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Original Article

Diterpenes and a new benzaldehyde from the mangrove plant *Rhizophora mangle*

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ABSTRACT

This work describes the isolation, by high-speed counter-current chromatography, of the diterpenes manool, jhanol and steviol and the benzaldehyde *p*-oxy-2-ethylhexyl benzaldehyde from the stilt roots hexane extract of the mangrove plant *Rhizophora mangle* L., Rhizophoraceae. For this, a non-aqueous biphasic solvent system composed of hexane–acetonitrile–methanol 1:1:0.5 (v/v/v) was applied. As far as we know, only steviol was previously isolated in Rhizophoraceae and this is the first time that *p*-oxy-2-ethylhexyl benzaldehyde is reported.

Introduction

Mangroves are ecosystems with unusual variety of plants adapted to conditions of high salinity, frequent floods and muddy anaerobic soil. Brazil has the second largest mangrove area in the world but has only three genera of Angiosperms (Wu et al., 2008). Among them, *Rhizophora* is the most frequent and abundant genus. *Rhizophora mangle* L., Rhizophoraceae, popularly known as 'red mangrove', is a native Brazilian widespread mangrove tree (Tomlinson, 1986), occurring along all the coast, from the State of Pará until the State of Santa Catarina (Schaeffer-Novelli et al., 2000).

Several secondary metabolites have been isolated/identified/detected in four of the ten existing *Rhizophora* species (Wu et al., 2008; Nebula et al., 2013): ditepenoids and triterpenoids in *R. mucronata* (Misra et al., 1984; Ghosh et al., 1985; Anjaneyulu and Rao, 2001; Anjaneyulu et al., 2000, 2002) and in *R. apiculata* (Kokpol et al., 1990; Gao et al., 2011); phenylpropanoids and other volatiles, triterpenes, flavonoids and a flavoglycan polymer in *R. stylosa* (Neilson et al., 1986; Azuma et al., 2002; Li et al., 2007); flavonoids, condensed tannins and triterpenes in *R. mangle* (Williams, 1999; Koch et al., 2003; Kandil et al., 2004; Zhang et al., 2010; Costa et al., 2014).

In this study, high-speed counter-current chromatography (HSCCC) was used to isolate three diterpenes and a new benzaldehyde from the hexane extract of *R. mangle* stilt roots. Counter-current chromatography, CCC, is a liquid–liquid partition technique in which the liquid stationary phase is retained in the apparatus using centrifugal force only (Conway, 1990). High-speed counter-current chromatography, HSCCC, uses two rotation axes in a planetary motion, generating a variable centrifugal force field (Conway, 1990). The separation is based on partitioning of analytes between two immiscible liquids (generally a solvent mixture) forming a biphasic system (Marston and Hostettmann, 2006). The use of an all-liquid technique leads to many advantages over conventional chromatography (Berthod, 2002), being the recovery of labile compounds without chemical modifications on its structure crucial to the success of this work.

Materials and methods

Extract preparation

Rhizophora mangle L., Rhizophoraceae (Supplementary data), was collected at Reserva Biológica e Antropológica de Guaratiba, Rio de Janeiro, Brazil in October 2010. Plant was identified on campus by Dr Gustavo Duque Estrada. As this plant is the only *Rhizophora* species occurring in Brazilian mangrove areas being its morphology totally different from any other existing mangrove plant, there was no need of a voucher specimen. Dried and grounded stilt roots

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were submitted to maceration with ethanol–water (9:1, v/v). 55 g of the crude dried extract was dissolved in methanol–water (50:350, v/v) and partitioned between hexane, dichloromethane and ethyl acetate, in this order, affording four different extracts.

Selection and preparation of the biphasic solvent system and sample solution

Solvent system selection was made by dissolving a small amount of the sample in test tube containing the equilibrated biphasic system. The test tubes were shaken and the compounds allowed to partition between the two phases. Equal aliquots of each phase were spotted beside each other separately on silica gel TLC plates (Merck Art. 05554, Darmstadt, Germany), developed with the solvent system hexane–ethyl acetate 9:1. The results were visualized under UV light (254 nm). The results were visualized after spraying the plate with H_2SO_4 (10%) and vanillin (5%).

The selected solvent system was equilibrated in a separatory funnel at room temperature and phases were separated just before use. The upper layer was used as stationary phase while the lower layer as mobile phase, in head to tail direction. Sample solution was prepared by dissolving the sample in the solvent system used for separation (1:1, v/v).

CCC equipment and separation procedure

Semi-preparative HSCCC was performed on a Quattro HT-Prep counter-current chromatograph (AECS, Bridgend, United Kingdom) equipped with two bobbins containing two polytetrafluoroethylene multi-layer coils each (26 ml, 1 mm i.d. + 234 ml, 3.2 mm i.d. and 95 ml, 2 mm i.d. + 98 ml, 2 mm i.d.). The rotation speed is adjustable from 0 to 865 rpm. A 5 ml sample loop was used to inject the sample.

In the separation process, the 95 ml coil was first entirely filled with the upper stationary phase, and then the apparatus was rotated at 865 rpm, while the lower mobile phase was pumped into the column. The flow rate used was 2 ml/min. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, the sample solution (300 mg in 5 ml) was injected into the column through the injection valve. Fractions of 4 ml were collected: 50 during elution and 30 during extrusion.

Extract and fraction analyses and compound identification

Crude extract and each fraction from CCC were analysed by TLC (Merck Art. 05554, Darmstadt, Germany), developed with hexane–ethyl acetate 9:1 and/or 7:3, according to its polarity. The results were visualized after spraying the plate with H_2SO_4 (10%) and vanillin (5%).

^1H and ^{13}C NMR data measurements for the isolated compounds were recorded on a Varian VNMRS500 (California, USA) at 25 °C, operating at 500 MHz for ^1H and 125 for ^{13}C, using deuterated chloroform and TMS as internal standard.

Results and discussion

R. mangle is the only species occurring along all Brazilian coast in mangrove areas with different climatic and environmental conditions (Schaeffer-Novelli et al., 2000) and very little is known about its chemical profile, especially the one concerning non-polar compounds. Following our previous work on phenolic profile of the EtOAc extract of *R. mangle* leaves (Costa et al., 2014) where several compounds were described for the first time on the species, the stilt roots hexane extract was investigated.

Preliminary analysis

The hexane extract of *R. mangle* stilt roots was analysed by TLC and the plate (*not shown*) showed a complex mixture, with compounds of low polarity, possibly terpenoids, due to the purple colour developed after spraying the plate with sulfuric vanillin. This first information on complexity and polarity was important to guide solvent system testing.

Solvent system selection

The selection of the biphasic solvent system is the most important step in CCC, as it means the choice of both stationary and mobile phases at the same time (Yto, 2005) and, without consulting literature, this search can be very time-consuming.

More than 150 solvent systems were formerly used to isolate terpenoids by CCC (Skalicka-Woźniak and Garrard, 2014). From them, non-aqueous solvent systems have been used for the isolation of low-polarity compounds mainly from essential oils. The most commonly used are hexane:acetonitrile and hexane:methanol modified by chlorinated solvents (Leitão et al., 2012).

After testing hexane:acetonitrile (1:1, v/v), hexane:methanol (1:1, v/v), solvents were combined and a further system was evaluated: hexane:acetonitrile:methanol (1:1:0.5, v/v/v). When using only acetonitrile as the polar solvent, compounds were slightly more concentrated in the upper layer (composed mainly by hexane). On the other hand, when using only methanol as the

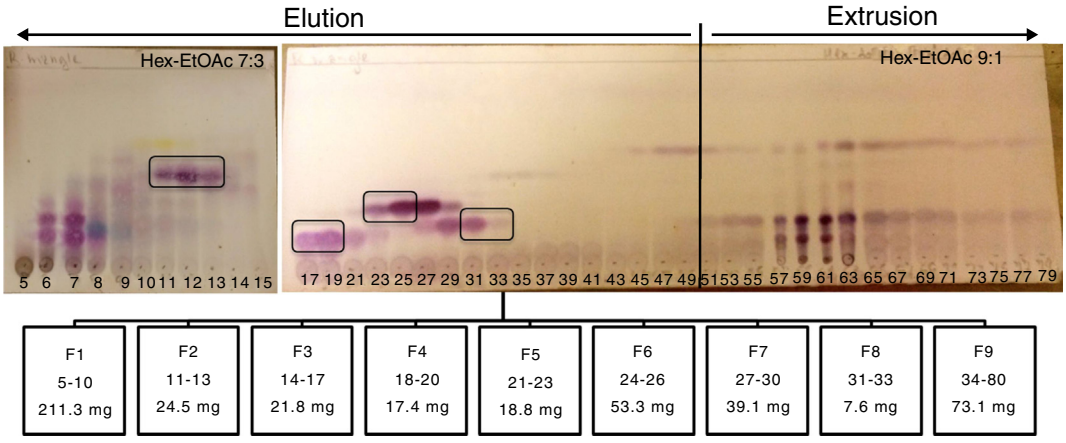

Fig. 1. Analyses of CCC fractions by TLC and combination by similarity. F2, F4, F6 and F8 are highlighted and correspond to compounds (**1**), (**2**), (**3**) and (**4**), respectively. *n*-Hexane–ethyl acetate was used as eluent (7:3) in fractions 5–15 and 9:1 in fractions 17–79. Compounds were visualized after spraying sulfuric vanillin.

polar solvent, compounds were heavily concentrated in the lower layer (composed mainly by methanol). Assuming that the suitable condition is achieved when having distribution coefficient approximately one, adding a small proportion of methanol to hexane:acetonitrile system gave satisfactory results.

CCC separation and fraction analyses

Hexane–acetonitrile–methanol at ratio 1:1:0.5 was employed for the semi-preparative fractionation of 300 mg of *R. mangle* stilt roots hexane extract affording nine main fractions combined by TLC similarity, F1 to F9 (Fig. 1). Elution process took place until fraction 50 being substituted by extrusion until fraction 80. Compounds **1**, **2**, **3** and **4** in F2, F4, F6 and F8, respectively, were isolated and were analysed by NMR. Compounds purity is not known.

Compound identification

Compound **1** was identified as the labdane diterpene manool by comparison with literature data (Ulubelen et al., 1991). It was obtained as a yellow amorphous solid and the molecular formula was determined as $C_{20}H_{34}O$ on the basis of HRESIMS ([M−18+H]$^+$ 273.2572). ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.68 (s, CH_3, H18), 0.87 (s, CH_3, H19), 0.80 (s, CH_3, H20), 1.27 (s, CH_3, H16), 1.00–2.50 (m, CH_2, H1; CH_2, H2; CH_2, H3; CH, H5; CH_2, H6; CH_2, H7; CH, H9; CH_2, H11; CH_2, H12), 4.51 (d, 1H, C17), 4.81 (d, 1H, C17), 5.06 (dd, 1H, C15), 5.22 (dd, 1H, C15), 5.90 and 5.94 (d, 1H, C14). APT NMR (125 MHz, $CDCl^3$): δ 14.43 (CH_3, C18), 17.70 (CH_2, C10), 19.38 (CH_2, C1), 21.71 (CH_3, C20), 24.42 (CH_2, C5), 27.63 (CH_3, C16), 33.56 (CH_3, C19), 33.61 (C, C3), 38.34 (CH_2, C6), 39.06 (CH_2, C2), 39.86 (C, C9), 42.19 (CH_2, C11), 55.57 (CH, C4), 57.31 (CH, C8), 73.58 (C, C12), 106.44 (CH_2, C17), 111.52 (CH_2, C15), 145.26 (CH_3, C13), 148.66 (C, C7).

Compound **2** was identified as the kaurane diterpene steviol by comparison with literature data (Subrahmanyam et al., 1999; Geuns et al., 2006). It was obtained as a white powder and the molecular formula was determined as $C_{20}H_{30}O_3$ on the basis of HRESIMS ([M−18+Na] 329.2461). ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.94 (s, CH_3, H20), 1.22 (s, CH_3, H18), 0.90–2.50 (m, CH_2, H1; CH_2, H2; CH_2, H3; CH, H5; CH_2, H6; CH_2, H7; CH, H9; CH_2, H11; CH_2, H12; CH_2, H14; CH_2, H15), 4.80 and 4.74 (d, 2H, H17). APT NMR (125 MHz, $CDCl^3$): δ 15.63 (CH_3, C18), 18.41 (CH_2, C11), 19.08 (CH_2, C2), 21.81 (CH_2, C6), 28.96 (CH_3, C20), 37.80 (CH_2, C3), 39.53 (C, C10), 40.69 (CH_2, C7), 41.26 (CH_2, C1), 42.39 (C, C8), 43.70 (CH_2, C12), 43.82 (C, C4), 44.20 (CH_2, C14), 48.94 (CH_2, C15), 55.08 (CH, C5), 57.02 (CH, C9), 81.54 (C, C13), 102.97 (CH_2, C17), 155.85 (C, C16), 183.79 (C, C19).

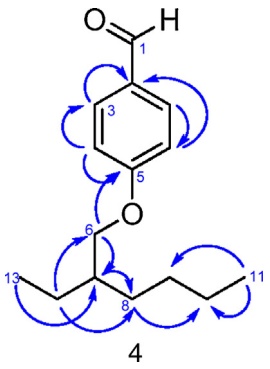

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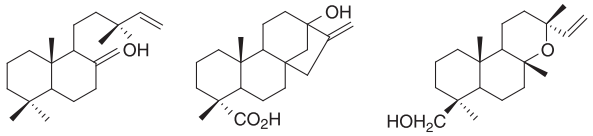
Fig. 2. Selected HMBC correlations of (**4**) *p*-oxy-2-ethylhexyl benzaldehyde.

Compound **3** was identified as the labdane diterpene jhanol by comparison with literature data (González et al., 1977; Stierle et al., 1988; Fraga et al., 1998). It was obtained as a yellow oil and the molecular formula was determined as $C_{20}H_{34}O_2$ on the basis of HRESIMS ([M−18+H] 289.2526). ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.75 (s, CH_3, H18), 0.83 (s, CH_3, H20), 1.27 (s, CH_3, H16), 1.30 (s, CH_3, H17), 0.85–1.80 (m, CH_2, H1; CH_2, H2; CH_2, H3; CH, H5; CH_2, H6; CH_2, H7; CH, H9; CH_2, H11; CH_2, H12), 3.13 and 3.40 (d, CH_2, H19), 5.86 and 5.89 (d, CH, H14), 4.93 and 5.15 (dd, CH_2, H15). APT NMR (125 MHz, $CDCl^3$): δ 15.28 (CH_2, C11), 15.77 (CH_3, C20), 17.15 (CH_3, C18), 17.83 (CH_2, C2), 19.68 (CH_2, C6), 25.47 (CH_3, C17), 28.50 (CH_3, C16), 35.27 (CH_2, C12), 35.63 (CH_2, C3), 36.89 (C, C4), 37.57 (C, C10), 38.49 (CH_2, C1), 42.88 (CH_2, C7), 49.64 (CH, C5), 55.57 (CH, C9), 72.05 (CH_2, C19), 73.27 (C, C8), 74.96 (C, C13), 110.32 (CH_2, C15), 147.81 (CH, C14).



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Compound **4** (Fig. 2) was identified as *p*-oxy-2-ethylhexyl benzaldehyde (Table 1). It was obtained as a colourless oil, $[\alpha]_D^{20} = -19.15$ ($c = 0.2$, MeOH), UV (MeOH): 203, 242 and 282 nm. The molecular formula was determined as $C_{15}H_{22}O_2$ on the basis

Table 1
^{1}H and ^{13}C NMR data of *p*-oxy-2-ethylhexyl benzaldehyde (**4**) (500 and 125 MHz; $CDCl_3$; δ in ppm; J in Hz).

	p-Oxy-2-ethylhexyl benzaldehyde (**4**)			
	δC	δH	HMBC	
			H ($^2J_{C-H}$)	H ($^3J_{C-H}$)
1	204.38, CH			
2	132.61, C		7.54 (H3)	7.71 (H4)
3/3′	131.02, CH	7.54 (dd; 3.3, 9.0)	7.71 (H4)	
4/4′	128.95, CH	7.71 (dd; 3.3, 9.0)	7.54 (H3)	
5	167.90, C		7.71 (H4)	4.22 (H6)
6	68.31, CH_2	4.22 (ddd; 5.7, 8.3)	1.68 (H7)	1.42/1.41 (H12)
7	38.89, CH	1.68 (ddd; 6.1, 12.2, 18.4)	4.22 (H6)	0.92 (H13)
			1.42/1.41 (12)	
8	30.52, CH_2	1.35 (m)	1.68 (H7)	4.22 (H6)
				1.42 (H12)
9	29.08, CH_2	1.32 (m)	1.35 (H8)	1.68 (H7)
				0.90 (H11)
10	23.14, CH_2	1.30 (m)	0.90 (H11)	1.35 (H8)
11	14.20, CH_3	0.90 (d; 7.5)		
12	23.91, CH_2	1.42 (m)	1.68 (H7)	4.22 (H6)
			0.92 (H13)	
13	11.12, CH_3	0.92 (d; 7.4)	1.42 (H12)	1.68 (H7)

of HRESIMS ([M+H]$^+$ 234.3355) indicating five degrees of instauration. The IR spectrum showed absorption at 3500–3250 and 1500–1400 cm^{-1} typical for aromatic signals, at 1700 cm^{-1} indicating the presence of a carbonyl group and at 1260 and 1100 cm^{-1} characteristic of an ether function. Four similar compounds differing in the number of carbons in the chain have been previously synthesized in studies on lipid bilayer intercalants (Cohen et al., 2008).

^{1}H, APT, HSQC and HMBC (Supplementary data) experiments were performed for the structure elucidation of compound **4**. The APT spectrum showed 13 carbon signals, allowing to distinguish carbon type: two methyls, five methylenes (including one connected to electronegative atom), four methines (including two aromatic signals and one carbonilic) and two quaternary aromatic carbons.

The presence of the carbonyl aldehyde was confirmed with the signal at 204.38 ppm. The aromatic ring could be seen in ^{1}H spectra at 7.71 and 7.54 chemical displacements. These signals have the AA′XX′ system, a standard to *para*-dissubstituted aromatic ring with 3.3 and 9.0 Hz typical coupling constant. The C3/C3′ and C4/C4′ have the same chemical shift due to symmetry, 131.02 and 128.95 ppm, respectively. The typical chemical shift for —CH_2O— group appears at 4.22 ppm in ^{1}H and at 68.31 ppm in APT experiment. Structure was confirmed by bidimensional HSQC and HMBC experiments (Table 1 and Fig. 2).

The non-aqueous solvent system used in this work showed to be effective on the isolation of the medium to low polarity diterpenes in *R. mangle* stilt roots hexane extract. The use of this solvent system, besides affording these labile compounds without chemical modification, additionally provided a new benzaldehyde derivative, which, as far as we know, is being described for the first time in nature. Concerning the diterpenes, literature reports the presence of kauranes, labdanes, pimaranes and beyeranes besides several aromatic compounds in Rhizophoraceae (Wu et al., 2008; Nebula et al., 2013) but only steviol was previously isolated in the family.

Authors' contributions

JNM (undergraduate student) did the compound isolation procedure. FSF (PhD student) contributed in compound identification by NMR and literature search. GRM (PhD student) did NMR experiments. GGL was FNC's PhD supervisor, where this work started, and contributed to critical reading the manuscript. FNC designed the study, supervised the laboratory work and wrote the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjp.2016.10.004.

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