Article

Dereplication and Scale-up Countercurrent Chromatography in the Phytochemical Analysis of *Avicennia schaueriana* Stapf & Leechm. ex Moldenke

*Desreplicação e Aumento de Escala em Cromatografia Contracorrente na Análise Fitoquímica de **Avicennia schaueriana** Stapf & Leechm. ex Moldenke*

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Mangroves are transitional ecological communities between marine and terrestrial ecosystems, are an important renewable natural resource, and have high biological productivity. *Avicennia schaueriana* (Acanthaceae) is one of three plant species found in Brazilian mangroves. The goal of this study is to contribute to the knowledge of its chemical composition. This paper involves the gathering of a database with previously isolated compounds from the genus *Avicennia*, analyses of representative samples of *A. schaueriana* using liquid phase chromatography coupled to high resolution mass spectrometry (LC-HRMS), gas phase chromatography coupled to mass spectrometry (GC-MS), and the semi-preparative scale fractionation using countercurrent chromatography (CCC). LC-HRMS preliminary analysis, when compared to the database generated, enables the prediction of the chemical composition of the CHCl$_3$ extract of *A. schaueriana* branches and leaves through the dereplication of the results, and along with GC-MS preliminary analysis, give the possibility to show its complexity. CCC was able to fractionate and scale up efficiently. *p*-Methylbenzoic acid, veratric acid, vanillic acid, syringic acid, cinnamic acid, *p*-hydroxybenzaldehyde, syringaldehyde, and ferulic acid were purified by CCC and identified by NMR. These compounds were firstly described in *A. schaueriana*.

Keywords: *Avicennia schaueriana*, Acanthaceae; LC-HRMS; countercurrent chromatography; phenolic compounds.

1. Introduction

Mangroves are coastal transitional ecological communities between the marine and terrestrial ecosystems and have important renewable natural resources, providing high biological productivity. The vegetation occurs over muddy soil containing high salt concentration and low oxygen content, and it is rich in organic matter.[1-3] Brazil owns 25.000 km^2 of mangrove area along its coast between the states of Amapá and Santa Catarina, but interestingly it has only three plant species.[4] Brazilian mangroves' flora has been considered endangered since 1996[5] due to oil spillage,[2] urban and industrial invasions, and overfishing.[3] Nevertheless, traditional use for its medicinal properties against a varied number of diseases[6] highlights the urge for the characterization of phytochemicals presented in this Brazilian mangroves' flora.

Avicennia schaueriana is known as black mangrove. Locally it is called *siriúba*, *sereíba*, or *canoé* and it is one of the plant species occurring in all Brazilian mangroves' extensions.[7] The plant is adapted to survive in this characteristic environment, with salt glands[2] and pneumatophores coming up from the roots.[3] This study was encouraged by the lack of phytochemical data on it in the literature. *A. schaueriana* is chemically characterized by glycosylated iridoids,[8] quinones, steroids, terpenes,[9, 10] fatty acids, hydrocarbons, phenylpropanoids, and oxygenated sesquiterpenes.[11] For several years, the genus *Avicennia* belonged to the Avicenniaceae family, having been relocated in Verbenaceae,[12] Santalaceae, Dipterocarpaceae, and Celastraceae.[13] More recent studies of phylogenetic relationships have definitely placed it in the Acanthaceae family.[14, 15]

Countercurrent chromatography (CCC) is a liquid-liquid partition technique introduced in 1970[16] where the stationary phase is retained inside the equipment's coil, without the aid of solid support, using centrifugal force.[17] Hydrodynamic balance equipment uses two rotation axes that mimic planetary motion, promoting a variable centrifugal force field.[18-20] The separation fundamental principle involves the partition of the solute between two immiscible liquid phases.[21] The technique presents unique advantages: versatility in terms of sample polarity and variety of elution modes; low solvent consumption; high sample loading; total sample recovery; and easy scale-up.[17, 21, 22]

In this study, CCC was used as a preparative technique to purify phenolic acids, phenylpropanoids, and phenolic aldehydes from *A. schaueriana* branches and leaves chloroform extracts. Scale-up CCC made purification possible to further NMR analysis and, possibly, to contribute to future works on biological activities.

2. Experimental

2.1. Reagents

Analytical grade solvents (Tedia Brazil) were used for extraction and thin layer chromatography (TLC), CCC, liquid phase chromatography coupled to high resolution mass spectrometry (LC-HRMS), and gas phase chromatography coupled to mass (GC-MS) analyses. Aqueous solutions were prepared with pure water produced by an ultrapure water system (Milli-Q, 18.2 MΩ). Deuterated methanol solvent (methanol-*d*4) with TMS at 0.05% (Cambridge Isotope Laboratories, Inc., MA, USA) was used for NMR analysis.

2.2. Sample collection and extraction

Branches and leaves of different individuals identified as *A. schaueriana* were randomly collected across the region of *Reserva Biológica Estadual de Guaratiba* (Rio de Janeiro, RJ, Brazil) in November 2010 to ensure representativity. The specialized research group named *Núcleo de Estudo em Manguezais* (*NEMA/UERJ*), headed by Professor Dr. Gustavo Duque Estrada, gave support to identify the correct species. Scientific Research Authorization was given by Inea (*Instituto Estadual do Ambiente*), under number 301/2010. Dried and grounded branches and leaves (721.1 g and 806.8 g, respectively) were submitted to exhaustive maceration with ethanol-water 9:1 (v/v). Branches (AsB-crude) and leaves (AsL-crude) crude extracts were resuspended in methanol-water 1:7 (v/v) and partitioned between organic solvents with increasing polarity: hexane (Hex), chloroform ($CHCl_3$), and ethyl acetate (EtOAc) to produce four extracts for branches and leaves: Hex (AsB-Hex and AsL-Hex), $CHCl_3$ (AsB-$CHCl_3$ and AsL-$CHCl_3$), EtOAc (AsB-EtOAc and AsL-EtOAc), and H_2O (AsB-H_2O and AsL-H_2O).

2.3. Bibliographic research and database creation

Bibliographic research consisted of listing the previously isolated chemical compounds of eight species of the genus *Avicennia* with the highest confidence index of the base The Plant List:[23] *A. balanophora* Stapf & Moldenke, *A. bicolor* Standl., *A. germinans* (L.) L., *A. integra* N.C.Duke, *A. marina* (Forssk.) Vierh., *A. officinalis* L., *A. schaueriana*, and *A. tonduzii* Moldenke, excluding synonyms according to The Royal Botanic Gardens, Kew and Missouri Botanical Garden. Additional information (molecular formulas and monoisotopic masses) produced a database of chemical compounds named *Avicennia* chemical database used in this study. For instance, the negative ionized mass value [M – H]⁻ was calculated by subtracting the monoisotopic mass of the compound by the monoisotopic mass of the hydrogen atom (1.007825 Da). The survey was held using the keywords: *Avicennia*, compound, isolated, and LC-MS, in the databases: Google Scholar, ScienceDirect, and SciFinder.

2.4. LC-HRMS equipment

Preliminary analyses by LC-HRMS were performed using a 1200 series chromatograph (Agilent, California, USA) equipped with quaternary pump module G1311C and diode array detector (DAD) G4212B coupled to a MicrOTOF-II Mass Spectrometer (Bruker Daltonics, Inc., MA, USA) with an electrospray ionization source (ESI). Samples were injected at a 0.3 mL.min⁻¹ flow rate, at 40 ºC. Source temperature was set to 180 ºC. Drying gas (nitrogen) flow rate was set to 4.0 L.min⁻¹, and nebulizer gas (nitrogen) pressure was set to 0.4 bar. Data were acquired in the negative mode in a mass range of *m/z* 100 to 1500. Chromatograms and spectra were processed using Bruker Daltonics ESI Compass Data Analysis Version 4.0 SP 1 (Bruker Daltonics, Inc., MA, USA) software.

The chromatographic analyses were performed on a Poroshell EC-C18 column (100 mm × 2.1 mm i.d. × 2.7 µm) in gradient mode using an increasing concentration of MeOH in H_2O, both acidified with formic acid 0.1%, with a linear gradient from 50% to 90% of MeOH in 32 minutes, and from 90% to 10% of MeOH in 6 minutes. The error between experimental mass and calculated mass was set to the maximum value of 20 ppm.

2.5. GC-MS equipment

GC-MS analyses were performed using a GC-2010 Plus (Shimadzu) coupled to an electron ionization (EI) quadrupole GCMS-QP2020 Mass Spectrometer (Shimadzu) at 70 eV. Samples were manually injected at a 1 mL.min⁻¹ flow rate. Source temperature was set to 200 ºC and pressure was set to 57.4 kPa. Data were acquired in a mass range of *m/z* 40 to 700. Chromatograms and spectra were processed using GCMS Postrun Analysis LabSolutions software (Shimadzu) and database search was done using a local mass spectrum library NIST11. The analyses were performed on a DB-5 MS column (30 m × 0.25 mm i.d. × 0.25 µm) with a linear gradient from 60 ºC to 300 ºC in 30 minutes. The minimum similarity index between the experimental spectrum and the library for the compound determination was set to 85%, but all annotation was hand validated according to their fragmentation profile.

2.6. CCC selection and preparation of the biphasic solvent system and sample solution

Solvent system selection was performed by dissolving a small amount of AsB-$CHCl_3$ and AsL-$CHCl_3$ in test

tubes for each extract containing the solvent system. The test tubes were shaken to check the solubilization of the sample and the compounds could partition between the two phases. Equal volumes of each phase were applied on silica gel TLC plates (Merck Art. 05554, Darmstadt, Germany) developed with dichloromethane-methanol 9.8:0.2 (v/v). The results were visualized under ultraviolet (UV) radiation (Spectroline CL-80 model) at $\lambda = 254$ and 365 nm, followed by spraying vanillin 10% in ethanol (m/v) and sulfuric acid 10% in ethanol (v/v) solutions, and subsequent heating on a hot plate. The range of proportions of the two phased tested solvent system Hex-EtOAc-MeOH-H_2O (HEMWat) ranged from 2:2:2:2 to 3:2:3:2 (v/v).

The selected solvent system for both fractionations by CCC of AsB-$CHCl_3$ and AsL-$CHCl_3$ was prepared and equilibrated in a separatory funnel at room temperature and upper and lower phases were separated in different bottles and degassed in an ultrasound machine for 15 minutes. The lower/aqueous phase was used as the stationary phase while the upper/organic phase, as mobile phase, characterizing the normal elution mode, in tail to head direction. Sample solutions were prepared by dissolving each sample in the solvent system 1:1 (v/v).

2.7. CCC equipment and separation procedure

CCC was performed on a Quattro HT-Prep countercurrent chromatograph (AECS Ltd., Bridgend, United Kingdom) equipped with two bobbins containing two polytetrafluoroethylene multilayer coils each (26 mL, 1.0 mm i.d. + 234 mL, 3.2 mm i.d. and 95 ml, 2.0 mm i.d. + 98 ml, 2.0 mm i.d.). Rotation speed is adjustable from 0 to 865 revolutions per minute (rpm).

For the analytical scale fractionations of AsB-$CHCl_3$ and AsL-$CHCl_3$, the 26 mL coil was filled with the stationary phase and then, the apparatus was rotated at 860 rpm, while the mobile phase was pumped into the coil. The flow rate was set to 0.5 mL.min^{-1} for both. After the mobile phase front emerged and hydrodynamic equilibrium was established in the coil, the sample solutions were each injected into the coil through the injection valve. Fractions of 1 mL were collected: 50 during elution and 30 during extrusion when rotation was reduced to 200 rpm. The sample solution for branches fractionation was prepared as 70 mg.mL^{-1} using 70 mg of sample while the sample solution for leaves one was prepared as 100 mg.mL^{-1} using 150 mg of sample.

For the semi-preparative scale fractionation of AsL-$CHCl_3$, was used the 98 mL coil, following the same previous procedures with some adjustments: flow rate was set to 1.9 mL.min^{-1} and fractions of 3.8 mL were collected (50 during elution and 30 during extrusion). Sample solution in this fractionation was prepared as 100 mg.mL^{-1} using 500 mg of sample.

Partitioned fractions derived from fractionations by CCC were qualitatively analyzed by TLC with the same parameters previously described. Fractions were dried in a SpeedVac concentrator (Thermo Scientific Savant SPD131DDA), resuspended in equal volumes of MeOH, and applied to the plate using a volumetric capillary tube.

2.8. Fractions analysis and compound identification

^{1}H and HSQC NMR data measurements of purified compounds were recorded on an 800 MHz Bruker Avance III (Ettlingen, Germany) operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C at 25 ºC, methanol-$d4$ was used as the solvent for every sample and the TMS signal was used for chemical shift reference. Spectra processing was performed using MNova (Mestrelab Research S.L.).

3. Results and Discussion

3.1. Sample collection and extraction

Partitions of 60 g of AsB-crude and 70 g of AsL-crude between Hex, $CHCl_3$, and EtOAc gave four extracts for branches: AsB-Hex: 7.0 g; AsB-$CHCl_3$: 5.0 g; AsB-EtOAc: 4.5 g; AsB-H_2O: 15.0 g, and four extracts for leaves: AsL-Hex: 5.0 g; AsL-$CHCl_3$: 5.0 g; AsL-EtOAc: 6.0 g; AsL-H_2O: 20.0 g.

3.2. Bibliographic research and database creation

349 compounds were identified in 102 references from 1913 to January 2021 in four of eight species of the genus *Avicennia*: *A. germinans*, *A. marina*, *A. officinalis*, and *A. schaueriana*, generating the *Avicennia* chemical database. No publications were found for the other four species. The genus is chemically characterized by glycosylated iridoids, flavonoids, lignans, steroids, diterpenes, diterpenoids, triterpenes, triterpenoids, saponins, quinones, naphthalene derivatives, phenylpropanoids, glycosylated phenylpropanoids, fatty acids, phthalates, alcohols, and hydrocarbons (Table S1, Supplementary material).

3.3. Preliminary analyses

AsB-$CHCl_3$ and AsL-$CHCl_3$ were analyzed by TLC (Figure S1, Supplementary material). The blue spots revealed under UV radiation at 254 nm wavelength showed a complex mixture constituted mainly in phenylpropanoids in both extracts.[24]

AsB-$CHCl_3$ and AsL-$CHCl_3$ were then analyzed by LC-HRMS. The chromatograms (Figure S2, Supplementary material) provided additional information on chemical profiles of the branches and leaves extracts: they revealed qualitative similarity, sharing notable coincident peaks. LC-HRMS data from AsB-$CHCl_3$ and AsL-$CHCl_3$ was compared to the *Avicennia* chemical database (Table S1, Supplementary material) to enlighten their chemical composition through the dereplication of the results.

The compounds identified on the database, with ions [M – H]⁻, were compared to the analyses in negative ionization mode using Data Analysis software. 96 compounds were identified and classified accordingly: glycosylated iridoids, fatty acids, quinones, flavonoids, phenylpropanoids, diterpenes, and lignans (Table S2, Supplementary material).

GC-MS analysis of AsB-CHCl₃ and AsL-CHCl₃ enabled the annotation of 24 compounds, 9 of which were present in both extracts (Table 1). Comparing experimental GC-MS data to the *Avicennia* chemical database (Table S1, Supplementary material), it was possible to suggest the presence of 9 compounds previously described in the genus *Avicennia*: lupenone, megastigmatrienone, ethyl palmitate, methyl palmitate, isovanillic acid, palmitic acid, 4-Hydroxy-3,5,5-trimethyl-4-(3-oxo-1-butenyl)-2-cyclohexen-1-one, dotriacontane and heneicosane.

3.4. CCC solvent system selection

AsB-CHCl₃ and AsL-CHCl₃ fractionations by CCC started with the biphasic solvent system selection. HEMWat was the selected system, which is more suitable for medium polarities samples.[25] It covers a high polarity range since it is composed by fully different polarities solvents.[26]

The solvent system was tested by changing the solvents volume ratio to achieve a satisfactory composition that provides a suitable partition coefficient (K). Due to the previous analyses, the extracts' polarities were already established, thus, two ratios of HEMWat were tested: 2:2:2:2 (v/v) (**system a**) and 3:2:3:2 (v/v) (**system b**). TLC analysis indicated a more suitable distribution of compounds presented in AsB-CHCl₃ and AsL-CHCl₃ in the system a, while in system b they were slightly more concentrated in the lower/aqueous phase, compared to the upper/organic one. Thus, system a was selected for fractionations by CCC (Figure 1).

3.5. CCC separation, fractions analyses, and scale-up

Analytical scale fractionation of AsB-CHCl₃ provided stationary phase retention of 73% and thirteen fractions combined by TLC profile similarity – F1 to F13 (Figure 2). Fractions F6, F7, F8-F9, and F10-F11 were analyzed by NMR and compounds (**1**), (**2**), (**3**), and (**4**) could be identified. GC-MS analysis of these fractions was performed for molecular formula and structure confirmation. All 4 compounds were detected in AsB-CHCl₃ preliminary analysis by LC-HRMS.

Analytical scale fractionation of AsL-CHCl₃ was performed following the same methodology as AsB-CHCl₃ and provided stationary phase retention of 78% (Figure S3, Supplementary material). Scale-up was held to obtain a greater amount of purified compounds to faster and accurate NMR analysis. It was executed with a semi-preparative coil (98 mL), having different i.d. and similar lengths. Coil length (L) is given by:[27]

$$L = \frac{V \text{ (volume)}}{A \text{ (area)}}, \text{ where } A = \frac{\pi \, d^2}{4}$$

- Analytical coil length (26 mL = 26.000 mm³):

$$A = \frac{3.14 \times 1^2 \text{ mm}}{4} = 0.785 \text{ mm}^2, \text{ therefore, } L = \frac{26.000 \text{ mm}^3}{0.785 \text{ mm}^2} \approx 33.12 \text{ m}$$

- Semi-preparative coil length (98 mL = 98.000 mm³):

$$A = \frac{3.14 \times 2^2 \text{ mm}}{4} = 3.140 \text{ mm}^2, \text{ therefore, } L = \frac{98.000 \text{ mm}^3}{3.140 \text{ mm}^2} \approx 31.21 \text{ m}$$

Therefore, the volumetric scale-up factor (SUF) was used.[28] Thus, it was possible to calculate it by the ratio between largest coil volume and minor coil volume:

$$SUF = \frac{98 \text{ mL}}{26 \text{ mL}} \approx 3.77.$$

This value was used to increase the flow rate and sample injection volume. Consequently, the volume of each fraction was increased proportionally according to the SUF. Rotation, fraction collector, and sample concentration values were not modified (Table 2).[28]

Semi-preparative scale fractionation provided stationary phase retention of 90%. It can be noted higher stationary phase retention in bigger coils since the internal diameter directly affects this parameter.[19, 28] The procedure generated sixteen fractions combined by TLC profile similarity – F1 to F16 (Figure 3). Fractions F2-F3, F8, F10, and F11-F12 were analyzed by NMR and compounds (**5**), (**6**), (**7**), and (**8**) could be identified. GC-MS analysis of these fractions was performed for molecular formula and structure confirmation. All 4 compounds were detected in the AsL-CHCl₃ preliminary analysis by LC-HRMS.

3.6. Compound identification

All compounds were identified by comparison to Simulate and Predict NMR Spectra database site[29] and manually validated with the interpretation of the resonances (Figure 4).

¹H and HSQC NMR, and GC-MS were used for structure elucidation. Data from the preliminary analysis by LC-HRMS of AsB-CHCl₃ and AsL-CHCl₃ (Table S3, Supplementary material) were used for confirmation of their accurate *m/z*. The spectra can be found in the supplementary material (Spectra S1-S26).

Compound (**1**) was identified as *p*-methylbenzoic acid (1.4 mg). HR-ESI-MS *m/z* 135.04644 [M – H]⁻, 8,6 min (error 9,6 ppm calculated for $C_8H_7O_2^-$). ¹H NMR (500 MHz, methanol-*d*4): δ 7.22 (*d*, 2H, H_3; H_5), δ 7.76 (*d*, 2H, H_2; H_6). HSQC (500 MHz, methanol-*d*4): δ 7.22 (*d*, 2H, H_3; H_5) – δ 128.52 (C_3; C_5), δ 7.76 (*d*, 2H, H_2; H_6) – δ 126.85 (C_2; C_6).

Compound (**2**) was identified as veratric acid (1.0 mg). HR-ESI-MS *m/z* 181.05186 [M – H]⁻, 38,4 min (error 6,8 ppm calculated for $C_9H_9O_4^-$). ¹H NMR (500 MHz, methanol-*d*4): δ 3.86 (*s*, 3H, H_8), δ 3.87 (*s*, 3H, H_9), δ 6.97 (*d*, 1H, H_5), δ 7.58 (*d*, 1H, H_2), δ 7.62 (*dd*, 1H, H_6).

Table 1. Compounds identified in AsB-CHCl₃ and AsL-CHCl₃ on the GC-MS analysis, with respective retention times, peak area, and similarity index with the mass spectra library NIST11. A minimum similarity value of 85% was considered.

BRANCHES

Peak	Identified compound	Retention time (min)	Peak area (%)	Similarity (%)
1	*o*-Guaiacol	6.11	1.07	95
2	4-Hydroxy-3-methylacetophenone	9.32	8.59	89
4	Syringol	9.78	6.44	93
6	Vanillin	10.48	0.92	94
7	Cinnamic acid	10.75	1.53	93
9	Isovanillic acid	12.45	4.52	94
12	Veratric acid	13.26	5.35	92
13	Syringaldehyde	13.64	1.56	94
14	Methoxyeugenol	14.14	2.25	88
15	4-Hydroxy-2-methoxycinnamaldehyde	14.55	1.04	91
19	4-Hydroxy-3,5,5-trimethyl-4-(3-oxo-1-butenyl)-2-cyclohexen-1-one	15.13	1.45	92
20	Syringic acid	15.275	2.54	93
21	Methyl palmitate	16.51	0.93	88
22	Palmitic acid	16.84	1.42	89
23	3,5-Dimethoxy-4-hydroxycinnamaldehyde	17.09	0.72	86
24	Ethyl palmitate	17.19	0.82	89
30	Dotriacontane	21.65	1.16	92
39	Stigmast-5-en-3-ol oleate	26.56	1.53	85
40	2,6-*Bis*(3,4- Methylenedioxaphenyl)-3,7-dioxabicycle(3.3.0)octane	27.30	1.52	91

LEAVES

| Peak	Identified compound	Retention time (min)	Peak area (%)	Similarity (%)
2	4-Hydroxy-3-methylacetophenone	9.32	19.87	89
3	Syringol	9.79	1.09	92
4	Isovanillin	10.48	4.25	95
6	Cinnamic acid	10.81	16.32	94
9	Heneicosane	12.98	1.16	89
11	Megastigmatrienone	13.36	2.49	91
12	Syringaldehyde	13.65	6.05	94
13	Methoxyeugenol	14.46	3.27	90
14	Syringic acid	14.47	1.37	93
15	4-Hydroxy-2-methoxycinnamaldehyde	14.57	5.30	92
16	4-Hydroxy-3,5,5-trimethyl-4-(3-oxo-1-butenyl)-2-cyclohexen-1-one	15.14	2.95	91
19	Phytol acetate	15.61	2.54	92
20	3,5-Dimethoxy-4-hydroxycinnamaldehyde	17.10	5.29	91
25	Lupenone	30.32	1.54	91
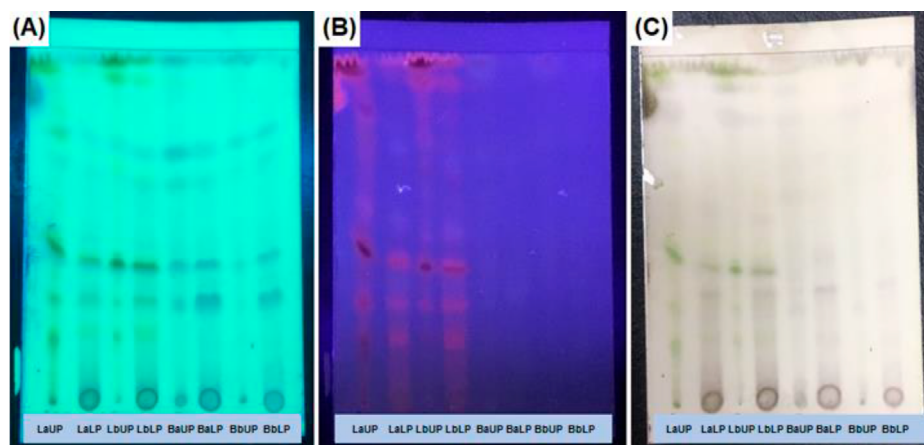
Figure 1. TLC analysis of solvent system selection for AsB-CHCl$_3$ and AsL-CHCl$_3$ using HEMWat solvent system. Mobile phase: CH$_2$Cl$_2$-MeOH 9.8:0.2 (v/v). Developers: (A) UV radiation at λ = 254 nm; (B) UV radiation at λ = 365 nm; (C) vanillin 10% in ethanol (m/v) and sulfuric acid 10% in ethanol (v/v) solution, and subsequent heating on hot plate. (L) Leaves; (B) Branches; (a) 2:2:2:2 (v/v) ratio; (b) 3:2:3:2 (v/v) ratio; (UP) Upper phase; (LP) Lower phase
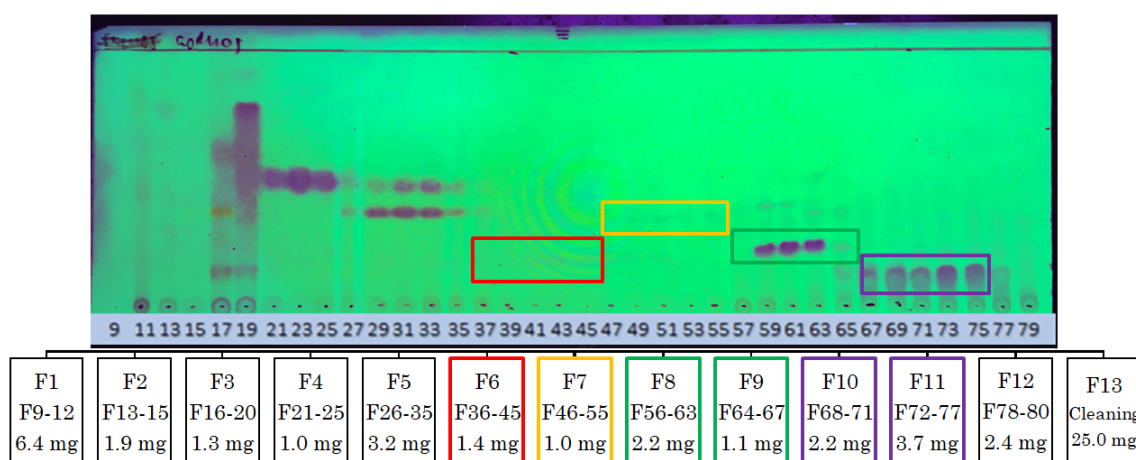

Figure 2. TLC analysis and flow diagram of AsB-CHCl$_3$ analytical scale fractionation by CCC. Fractions F6, F7, F8-F9, and F10-F11 are highlighted and correspond to compounds (**1**), (**2**), (**3**), and (**4**). The plate was eluted with mobile phase CH$_2$Cl$_2$-MeOH 9.8:0.2 (v/v). Compounds were visualized under UV radiation at 254 nm wavelength

Table 2. Conditions of AsL-CHCl$_3$ analytical and semi-preparative fractionations by CCC

Scale	Coil volume (mL)	Amount of injected sample (mg)	Sample concentration (mg.mL^{-1})	Flow rate (mL.min^{-1})	Injection volume (mL)	Fraction volume (mL)	Rotation (rpm)
Analytical	26	150	100	0.5	1.25	1.0	860
Semi-preparative	98	500	100	1.9	4.75	3.8	860

Compound (**3**) was identified as vanillic acid (3.3 mg). HR-ESI-MS *m/z* 167.03563 [M − H]$^-$, 33,7 min (error 3,9 ppm calculated for C$_8$H$_7$O$_4$$^-$). ^{1}H NMR (500 MHz, methanol-*d*4): δ 3.89 (*s*, 3H, H$_8$), δ 6.81 (*d*, 1H, H$_5$), δ 7.59 (*d*, 1H, H$_2$), δ 7.54 (*dd*, 1H, H$_6$); HSQC (500 MHz, methanol-*d*4): δ 3.89 (*s*, 3H, H$_8$) – δ 56.26 (C$_8$), δ 6.81 (*d*, 1H, H$_5$) – δ 115.50 (C$_5$), δ 7.59 (*d*, 1H, H$_2$) – δ 113.86 (C$_2$), δ 7.54 (*dd*, 1H, H$_6$) – δ 124.76 (C$_6$).

Compound (**4**) was identified as syringic acid (8.3 mg). HR-ESI-MS *m/z* 197.04718 [M − H]$^-$, 34,7 min (error 8,3 ppm calculated for C$_9$H$_9$O$_5$$^-$). ^{1}H NMR (500 MHz, methanol-*d*4): δ 3.88 (*s*, 6H, H$_8$; H$_9$), δ 7.33 (*s*, 2H, H$_2$; H$_6$); HSQC (500 MHz, methanol-*d*4): δ 3.88 (*s*, 6H, H$_8$; H$_9$) − δ 56.42 (C$_8$; C$_9$), δ 7.33 (*s*, 2H, H$_2$; H$_6$) − δ 108.26 (C$_2$; C$_6$).

Compound (**5**) was identified as cinnamic acid (10.5 mg). HR-ESI-MS *m/z* 147.04353 [M − H]$^-$, 29,3 min (error 11,0 ppm calculated for C$_9$H$_7$O$_2$$^-$). ^{1}H NMR (500 MHz, methanol-*d*4): δ 6.49 (*d*, 1H, H$_8$), δ 7.38–7.58 (*m*, 5H, H$_2$ – H$_6$), δ 7.63 (*d*, 1H, H$_9$); HSQC (500 MHz, methanol-*d*4): δ 6.49 (*d*, 1H, H$_8$) − δ 120.10 (C$_8$); δ 7.38–7.58 (*m*, 5H, H$_2$ – H$_6$) − δ 128.94–129.82 (C$_2$ – C$_6$); δ 7.63 (*d*, 1H, H$_9$) − δ 145.50 (C$_9$).

Compound (**6**) was identified as *p*-hydroxybenzaldehyde (3.1 mg). HR-ESI-MS *m/z* 121.02852 [M − H]$^-$, 7,1 min (error 8,1 ppm calculated for C$_7$H$_5$O$_2$$^-$). ^{1}H NMR (500 MHz,
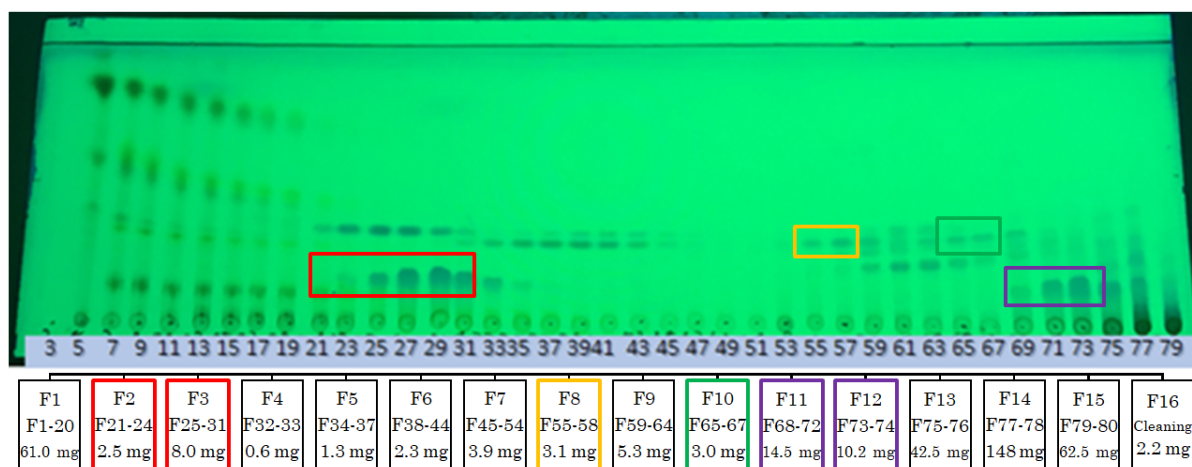
Figure 3. TLC analysis and flow diagram of AsL-CHCl$_3$ semi-preparative scale fractionation by CCC. Fractions F2-F3, F8, F10, and F11-F12 are highlighted and correspond to compounds (**5**), (**6**), (**7**), and e (**8**). The plate was eluted with mobile phase CH$_2$Cl$_2$-MeOH 9.8:0.2 (v/v). Compounds were visualized under UV radiation at 254 nm wavelength
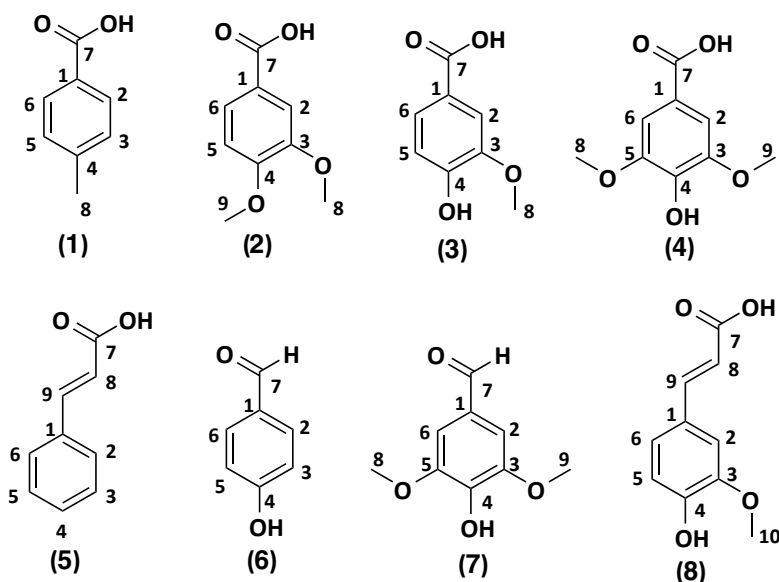

Figure 4. Chemical structures of purified compounds from AsB-CHCl$_3$: (**1**) *p*-methylbenzoic acid, (**2**) veratric acid, (**3**) vanillic acid, and (**4**) syringic acid; and AsL-CHCl$_3$: (**5**) cinnamic acid, (**6**) *p*-hydroxybenzaldehyde, (**7**) syringaldehyde, and (**8**) ferulic acid.

methanol-*d*4): δ 6.92 (*d*, 2H, H$_3$; H$_5$), δ 7.78 (*d*, 2H, H$_2$; H$_6$), δ 9.77 (*s*, 1H, H$_7$); HSQC (500 MHz, methanol-*d*4): δ 6.92 (*d*, 2H, H$_3$; H$_5$) – δ 116.36 (C$_3$; C$_5$), δ 7.78 (*d*, 2H, H$_2$; H$_6$) – δ 132.93 (C$_2$; C$_6$).

Compound (**7**) was identified as syringaldehyde (3.0 mg). HR-ESI-MS *m/z* 181.04651 [M – H]$^-$, 29,0 min (error 20,8 ppm calculated for C$_9$H$_9$O$_4$$^-$). ^{1}H NMR (500 MHz, methanol-*d*4): δ 3.92 (*s*, 6H, H$_8$; H$_9$), δ 7.23 (*s*, 2H, H$_2$; H$_6$), δ 9.75 (*s*, 1H, H$_7$); HSQC (500 MHz, methanol-*d*4): δ 3.92 (*s*, 6H, H$_8$; H$_9$) – δ 56.71 (C$_8$; C$_9$), δ 7.23 (*s*, 2H, H$_2$; H$_6$) – δ 108.16 (C$_2$; C$_6$).

Compound (**8**) was identified as ferulic acid (24.7 mg). HR-ESI-MS *m/z* 193.04635 [M – H]$^-$, 46,2 min (error 22,2 ppm calculated for C$_{10}$H$_9$O$_4$$^-$). ^{1}H NMR (500 MHz, methanol-*d*4): δ 3.92 (*s*, 3H, H$_{10}$), δ 6.31 (*d*, 1H, H$_8$), δ 6.81 (*d*, 1H, H$_5$), δ 7.18 (*d*, 1H, H$_2$), δ 7.06 (*dd*, 1H, H$_6$), δ 7.59

(*d*, 1H, H$_9$); HSQC (500 MHz, methanol-*d*4): δ 3.92 (*s*, 3H, H$_{10}$) – δ 56.65 (C$_{10}$), δ 6.31 (*d*, 1H, H$_8$) – δ 115.91 (C$_8$), δ 6.81 (*d*, 1H, H$_5$) – δ 116.32 (C$_5$), δ 7.18 (*d*, 1H, H$_2$) – δ 111.51 (C$_2$), δ 7.06 (*dd*, 1H, H$_6$) – δ 123.79 (C$_6$), δ 7.59 (*d*, 1H, H$_9$) – δ 146.63 (C$_9$).

According to the *Avicennia* chemical database (Table S1, Supplementary material), all purified compounds are being firstly reported in the genus *Avicennia*, except for ferulic acid, which was described previously on *Avicennia marina*.[30] Seven of these compounds – veratric acid, vanillic acid,[31] syringic acid,[32] cinnamic acid,[33-35] *p*-hydroxybenzaldehyde, syringaldehyde,[36-39] and ferulic acid[40-43] – have been described in Acanthaceae. The existence of these compounds in *A. schaueriana* could be related to the response to environmental stress experienced by it on the mangrove where it was collected.[44]

Interestingly, it was verified that compound (**1**) may have originated from endophytic fungi presented in the *A. schaueriana* branches. This compound was previously described in the endophytic fungus *Cladosporium cladosporioides*,[45] also found in *A. schaueriana*.[46] Based on the structural similarity between the compound (**1**) and the others identified, the possibility that all of them may have originated from endophytic or exophytic fungal contamination is not excluded.

4. Conclusions

The comparison of the *Avicennia* chemical database to the obtained data by LC-HRMS was able to predict the chemical composition of AsB-CHCl$_3$ and AsL-CHCl$_3$. The preliminary analysis by LC-HRMS and GC-MS revealed the complexity of the chemical composition and settle that branches' chemical profile is similar to leaves' one. The fractionation by CCC allowed the purification of compounds from two different parts of the plant and the simple scale-up in an efficient way, providing perspective for future biological assays.

Eight compounds, including phenolic acids (*p*-methylbenzoic acid, veratric acid, vanillic acid, and syringic acid), phenylpropanoids (cinnamic acid and ferulic acid), and phenolic aldehydes (*p*-hydroxybenzaldehyde and syringaldehyde) were purified, highlighting the technique's advantages and its efficiency in the purification of plant compounds.

All chemical structures were elucidated by NMR. GC-MS analysis was used to structure confirmation. According to the *Avicennia* chemical database, the purified compounds are being firstly reported in *A. schaueriana*. It is important to consider the possibility of the compounds may have originated from endophytic or exophytic fungal contamination.

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