Abstract: The detailed metabolite profiling of *Laguncularia racemosa* was accomplished by high-performance countercurrent chromatography (HPCCC) using the three-phase system *n*-hexane–tert-butyl methyl ether–acetonitrile–water 2:3:3:2 (*v*/*v*/*v*/*v*) in step-gradient elution mode. The gradient elution was adjusted to the chemical complexity of the *L. racemosa* ethyl acetate partition and strongly improved the polarity range of chromatography. The three-phase solvent system was chosen for the gradient to avoid equilibrium problems when changing mobile phase compositions encountered between the gradient steps. The tentative recognition of metabolites including the identification of novel ones was possible due to the off-line injection of fractions to electrospray ionization mass spectrometry (ESI-MS/MS) in the sequence of recovery. The off-line hyphenation profiling experiment of HPCCC and ESI-MS projected the preparative elution by selected single ion traces in the negative ionization mode. Co-elution effects were monitored and MS/MS fragmentation data of more than 100 substances were used for structural characterization and identification. The metabolite profile in the *L. racemosa* extract comprised flavonoids, hydrolysable tannins, condensed tannins and low molecular weight polyphenols.

Keywords: high-performance countercurrent chromatography; off-line MS/MS detection; three-phase solvent system; step-gradient; *Laguncularia racemosa*

1. Introduction

Countercurrent chromatography (CCC) is an all-liquid method, with no solid support, in which the stationary liquid phase is retained in the apparatus using centrifugal force only [1]. The principle behind this technique underlies the partitioning of a sample in a biphasic liquid solvent system [2]. Among many advantages, the technique is highly versatile; has high loading capacity; is easy to scale up; and eliminates sample loss by chemical degradation and irreversible adsorption [1,3,4]. CCC is a powerful tool in the phytochemical working field as it enables plant extract fractionation with existing major compounds and also isolation or fortification of minor compounds [5,6]. This characteristic is even more noticeable when semi-preparative and preparative scales are employed [7,8]. Standard CCC separation experiments do not provide the full flexibility, solely operating with a two-phase solvent system, and the isocratic elution–extrusion mode [9,10].

To improve the polarity range in the CCC operation field, three-phase solvent systems were developed/applied, due to the great differences in polarities between the upper,
middle and lower phases. Recently, some applications have been reported but only half of them actually were used as three phases in the separation process [11–15]. Tri-phasic systems are built from a two-phase system normally composed of n-hexane, acetonitrile and water in combination with a fourth solvent such as methyl acetate, ethyl acetate, methyl tert-butyl-methyl-ether or dichloromethane to create the third phase. Very few tri-phasic systems are described due to limited solvent combinations that form these three stable phases in a convenient volume percentage [11].

In analogy to solid phase chromatography, gradient elution in CCC intends to shorten the duration of a separation process and may also improve resolution. A common way to perform gradient elution is to change the mobile phase polarity over time [16], although gradient elution mode in CCC is less frequently used as the biphasic liquid system is in the equilibrium state and the change of composition in one phase corresponds directly to a change in the respective other liquid phase [17]. However, if a three-phase solvent system is used for gradient elution purposes, all phases involved for the experiment were previously in contact. Disturbance of the equilibrium and collapse of phase layers are omitted during the separation process while maintaining the broad polarity range for the recovery process.

In this work, a three-phase solvent system in step-gradient elution mode high-performance countercurrent chromatography (HPCCC) with off-line ESI-MS/MS detection was used for metabolite profiling of the ethyl acetate partition of the leaves from the mangrove plant Laguncularia racemosa. A similar approach was used on Anogeissus leiocarpus for compound identification but using centrifugal partition extraction (CPE), and off-line NMR detection [15]. L. racemosa (Combretaceae), popularly known as white mangrove, is the only occurring specie in the genus [18], and is considered as a strict mangrove [19], characteristic for growing in brackish coastal environments [20], and with excellent function for stabilizing shorelines against erosion [21]. From aspects of ethno-medicinal use, the plant is applied as astringent and tonic for dysentery and fever [22]. To date, there are only a few studies on the production of secondary metabolites [23–27], probably due to its high complexity of polar natural products.

2. Results and Discussion

The composition of L. racemosa ethyl acetate solvent partition (EtOAcPart) was initially investigated by TLC and LC-ESI/TOFMS analysis (Supplementary Figure S1). The liquid mass-spectrometry profile showed a high chemical complexity containing metabolites in a larger polarity range, making this mixture an ideal case study for the application of three-phase gradient elution. Some multiple-solvent biphasic systems have been proposed to great extend the polarity in CCC [28]. However, as the phases are in steady mixing contact during the complete separation process, a change of the mobile phase composition during gradient elution directly influences the stationary phase composition as well, and as consequence, disturbs the equilibrium and could lower or even lead to low chromatographic resolution [29,30]. To circumvent the equilibrium obstacle during the gradient elution procedure, a three-phase solvent system was used instead of two (or more) different biphasic systems. In this approach all phases involved in the separation were previously saturated with each other. The tri-phasic system n-hexane–tert-butyl-methyl ether–acetonitrile–water 2:3:3:2 (v/v/v/v) was used in the semi-preparative purification of L. racemosa EtOAcPart.

2.1. HPCCC of L. racemosa Metabolites by Off-Line ESI-MS/MS Profile Detection in the Sequential Order of Recovery

The L. racemosa EtOAcPart was separated by semi-preparative HPCCC chromatography, and the off-line injection profiling by injections of recovered fractions to ESI-MS/MS distinguished 17 principal phenolic constituents (Figure 1). However, as a result of the highly concentrated injections of respective HPCCC fractions, the selected single ion-based projection of the HPCCC experiment revealed more than 100 different metabolites (1–109) (Partially shown in Figure 1 and Supplementary Figure S2). Preliminary LC-ESI/TOF MS
analysis was not capable of detecting all minor compounds due to concentration levels below the detection limits.

Figure 1. Selected electrospay ionization mass spectrometry electrospray ionization mass spectrometry ions traces (negative mode) of phenolics of L. racemosa EtOAc Part detected in the off-line injected high-performance countercurrent chromatography (HPCCC) fractions. HPCCC separation using \( n \)-hexane-\( \text{tert-} \)butyl-methyl ether-acetonitrile-water 2:3:3:2 (\( v/v/v/v \)) as triphasic solvent system.

Large advantage of the off-line injection profiling methodology of preparative HPCCC-fractions by an ESI-MS detector in the sequence of recovery is the ‘on-the-fly’ delivery of the respective molecular weight- and MS/MS-fragmentation data of all ionizeable compounds in one step. This is a very fast process to get the required data for immediate compound identifications in the respective HPCCC-fractions. A full mass-spectrometry guided metabolite profile with more than ten automatically selected precuresur ions for MS/MS on a larger lab-scale preparative HPCCC fractionation can be achieved in a 60 to 100 min experimental mass-spectrometry time frame. This mass-spectrometry approach is roughly by a factor of hundred faster than the single investigation of resepective HPCCC fractions by LC-ESI-MS/MS analysis. The results displayed in a single data file are ready
to use and not mutiple analysis sets need to be compared for guiding the decisions in fractionation process. This powerful approach was previously applied by Costa et al. [8] on the complex metabolite mixture extracted from the Brazilian plant *Salicornia gauchichiana*.

In case of the investigated *L. racemosa* ethylacetate solvent partition, the elution ranges of a large selection of higher and lower concentrated target molecules (Table 1) were visualized in the recovered HPCCC-fractions by selected single ion traces for performing the accurate fractionation, recovery and preventing unintentional mixing of already separated compounds. Additionally, the existing compound co-elution effects, and the sequential elution orders of separated isobars/isomers were clearly detected and visualized (Figure 1).

**Table 1.** Detected compounds in the HPCCC off-line ESI-MS/MS phenolic profile of *Laguncularia racemosa* EtOAcPart.

| Cpd | CCC-Fraction | MS [M – H]⁻ (m/z) | MS/MS [M – H]⁻ (m/z) | LC-RT(min) | ESI/TOF MS Formula (Error in ppm) | Identification |
|-----|--------------|--------------------|----------------------|------------|-----------------------------------|----------------|
| 1   | 11–15        | 255                | 237, 226, 209, 156   | n.d.       | -                                 | Dihydrocrysin  |
| 2   | 21           | 269                | 13.7                 | 269.04593  | *C₁₅H₁₀O₅ (1.4)                   | Apigenin       |
| 3   | 19–21        | 271                | 1.9                  | 271.04885  | *C₁₅H₁₁O₅ (45.6)                  | Naringenin     |
| 4   | 23           | 273                | 29.1                 | 273.08108  | *C₁₅H₁₃O₅ (15.5)                  | Afzelechin     |
| 5   | 19           | 285                | 40.8                 | 285.04413  | *C₁₅H₁₁O₅ (12.6)                  | Kaempferol     |
| 6   | 31–41        | 287                | 12.1                 | 287.05883  | *C₁₅H₁₁O₆ (9.5)                   | Dihydrokaempferol |
| 7   | 97–115       | 289                | 6.2                  | 289.07438  | *C₁₅H₁₁O₆ (9.0)                   | (Epi)-catechin |
| 8   | 29–33        | 301                | 35.4                 | 301.03937  | *C₁₅H₁₀O₇ (13.3)                  | Quercetin      |
| 9   | 17–19        | 305                | 2.4                  | 305.0706   | *C₁₅H₁₂O₇ (12.8)                  | (Epi)-gallocatechin |
| 10  | 21–23        | 315                | 42.8                 | 315.0466   | *C₁₅H₁₁O₇ (14.1)                  | Isorhamnetin   |
| 11  | 25–27        | 317                | 28.9                 | 317.03536  | *C₁₅H₁₀O₇ (16.0)                  | Myricetin      |
| 12  | 81–93        | 319                | 9.1                  | 319.04883  | *C₁₅H₁₁O₆ (9.0)                   | Dihydromyricetin |
| 13  | 33–41        | 329                | 21.7                 | 329.05816  | *C₁₇H₁₃O₇ (25.9)                  | Tricin         |
| 14  | 133–149      | 393                | -                    | -          | -                                 | Myricetin derivative |
| 15  | 29–33        | 415                | -                    | -          | -                                 | Quercetin alkyl derivative |
| 16  | 127–131      | 419                | -                    | -          | -                                 | (Epi)-gallocatechin alkyl derivative |
| 17  | 55–57        | 431                | -                    | -          | -                                 | Myricetin alkyl derivative |
| 18  | 97–105       | 433                | 27.5                 | 433.08215  | *C₂₀H₁₇O₁₁ (10.4)                 | Quercetin pentoside |
| Cpd | CCC-Fraction | MS [M – H]− (m/z) | MS/MS [M – H]− (m/z) | LC-RT(min) | ESI/TOF MS Formula (Error in ppm) | Identification |
|-----|--------------|------------------|---------------------|-----------|--------------------------------|----------------|
| 19  | 89–91        | 433, 319, 193    | -                   | -         | -                              | Dihydromyricetin alkyl derivative |
| 20  | 91–95        | 441, 289         | 19.3                | -         | 441.08208 C22H18O10 (1.5)    | (Epi)-catechin gallate           |
| 21  | 105–119      | 447, 301         | 29.3                | -         | 447.09851 C21H19O11 (11.7)   | Quercetin desoxyhexoside        |
| 22  | 115–133      | 449, 317, 316    | 22.4                | -         | 449.07395 C20H17O12 (3.1)    | Myricetin pentoside             |
| 23  | 113–127      | 457, 331, 305, 169 | 12.1              | -         | 457.07859 C22H18O11 (2.1)    | (Epi)-gallocatechin gallate     |
| 24  | 21–23        | 461              | -                   | -         | -                              | Quercetin derivative            |
| 25  | 123–131      | 463, 317, 316    | 24.6                | -         | 463.09083 C21H19O12 (5.7)    | Myricetin desoxyhexoside        |
| 26  | 141–145      | 463, 301         | 25.9                | -         | 463.09187 C21H19O12 (7.9)    | Quercetin hexoside              |
| 27  | 153–165      | 467              | -                   | -         | -                              | Quercetin derivative            |
| 28  | 67–79        | 469, 317         | n.d.                | -         | -                              | Myricetin galatte               |
| 29  | 85–91        | 471, 319, 301, 193 | n.d.              | -         | -                              | Dihydromyricetin alkyl derivative |
| 30  | 115–131      | 477, 301, 179    | 15.2                | -         | 477.06812 C21H17O13 (1.4)    | Quercetin glucuronide           |
| 31  | 133–163      | 479, 317, 316    | 22.1                | -         | 479.08428 C21H19O14 (2.4)    | Myricetin glucuronide           |
| 32  | 89–97        | 585, 433, 301    | 32.7                | -         | 585.09204 C27H21O15 (5.9)    | Quercetin pentoside gallate     |
| 33  | 85–95        | 599, 447, 301    | 26.8                | -         | 599.10714 C28H23O15 (4.8)    | Quercetin desoxyhexoside gallate |
| 34  | 95–113       | 601, 449, 317    | 28.6                | -         | 601.08787 C27H21O16 (7.3)    | Myricetin pentoside gallate     |
| 35  | 29–33        | 603, 301         | n.d.                | -         | -                              | Quercetin [2M – H]−              |
| 36  | 125–131      | 611, 305         | -                   | -         | -                              | (Epi)-gallocatechin [2M – H]−    |
| 37  | 91–115       | 615, 463, 317, 179 | 23.4              | -         | 615.1014 C28H23O16 (3.6)    | Myricetin desoxyhexoside gallate |
| 38  | 137–153      | 615, 463, 301    | 31.6                | -         | 615.10412 C28H23O16 (8.1)    | Quercetin hexoside gallate      |
| 39  | 113–127      | 629, 477, 317, 289 | 21.5              | -         | 629.07893 C28H21O17 (0.8)    | Quercetin glucuronide gallate   |
| 40  | 133–139      | 631, 479, 317    | 28.9                | -         | 631.09859 C28H23O17 (7.2)    | Myricetin hexoside gallate      |
| 41  | 45–57        | 635, 317         | n.d.                | -         | -                              | Myricetin [2M – H]−              |
Table 1. Cont.

| Cpd | CCC-Fraction | MS [M – H]⁻ (m/z) | MS/MS [M – H]⁻ (m/z) | LC-RT(min) | ESI/TOF MS Formula (Error in ppm) | Identification |
|-----|--------------|-------------------|---------------------|-----------|----------------------------------|----------------|
| 42  | 87–91        | 639               | 319, 301            | 11.2      | 639.05562 C₂₀H₁₉O₁₇ (11.2)       | HHDP Dihydromyricetin |
| 43  | 25           | 657               | 317                 | -         | -                                | Myricetin derivative |
| 44  | 89           | 697               | 599                 | -         | -                                | Quercetin desoxyhexoside gallate derivative |
| 45  | 89–93        | 737               | 585, 301            | n.d.      | -                                | Quercetin pentoside digalloyl |
| 46  | 93–103       | 753               | 601, 449, 317       | 32.5      | 753.09740 C₃₄H₂₅O₂₀ (3.9)        | Myricetin pentoside digalloyl |
| 47  | 85–89        | 773               | 471, 301            | -         | -                                | Quercetin derivative |
| 48  | 97–103       | 867               | 433, 301            | n.d.      | -                                | Quercetin pentoside [2M – H]⁻ |
| 49  | 117–127      | 883               | 449, 317            | -         | -                                | Myricetin pentoside derivative |
| 50  | 115–123      | 892               | 457, 433            | -         | -                                | (Epi)-gallocatechin gallate derivative |
| 51  | 117–127      | 899               | 463, 449, 317       | -         | -                                | Myricetin pentoside derivative |
| 52  | 87–93        | 901               | 599, 301            | -         | -                                | Quercetin desoxyhexoside gallate derivative |
| 53  | 111–121      | 905               | 469, 457, 447, 425, 301 | - | - | Quercetin desoxyhexoside derivative |
| 54  | 113–125      | 907               | 449, 317            | -         | -                                | Myricetin pentoside derivative |
| 55  | 113–123      | 915               | 457                 | -         | -                                | (Epi)-gallocatechin gallate [2M – H]⁻ |
| 56  | 129–131      | 927               | 463, 317            | n.d.      | -                                | Myricetin desoxyhexoside [2M – H]⁻ |

**Hydrolisable tannins and derivatives**

| Cpd | CCC-Fraction | MS [M – H]⁻ (m/z) | MS/MS [M – H]⁻ (m/z) | LC-RT(min) | ESI/TOF MS Formula (Error in ppm) | Identification |
|-----|--------------|-------------------|---------------------|-----------|----------------------------------|----------------|
| 57  | 93–115       | 169               | 125                 | 12.1      | 169.01664 C₇H₅O₅ (14.2)          | Gallic acid |
| 58  | 61–81        | 183               | 124                 | 5.9       | 183.01418 C₈H₇O₅ (12.2)          | Methyl gallate |
| 59  | 43–57        | 197               | 169, 125            | 14.3      | 197.04741 C₉H₅O₅ (9.5)           | Ethyl gallate |
| 60  | 81–93        | 301               | 283, 257, 229, 163  | 18.5      | 300.99939 C₁₄H₂₅O₈ (1.3)         | Ellagic acid |
| 61  | 19–21        | 315               | 300                 | 6.1       | 315.01809 C₁₅H₂₅O₈ (10.9)        | Ellagic acid methyl ether |
| 62  | 97–103       | 321               | 169                 | 3.9       | 321.03300 C₁₄H₂₀O₇ (24.3) *      | Galloyl gallate |
| 63  | 133–151      | 325               | 169                 | 3.3       | 325.06016 C₁₄H₁₅O₈ (11.3)        | Galloyl shikimate |
| 64  | 33–39        | 329               | 314                 | 44.2      | 329.02154 C₁₆H₂₅O₈ (8.9)         | Ellagic acid dimethyl ether |
| Cpd | CCC-Fraction | MS [M − H]⁻ (m/z) | MS/MS [M − H]⁻ (m/z) | LC-RT (min) | ESI/TOF MS Formula (Error in ppm) | Identification |
|-----|--------------|--------------------|-----------------------|-------------|----------------------------------|----------------|
| 65  | 163          | 331 271, 169, 125 |                       | 11.5        | 331.06888 C₁₂H₁₅O₁₀ (5.5)        | Galloyl hexoside |
| 66  | 81–87        | 335 183            |                       | 9.2         | 335.02817 C₁₅H₁₁O₆ (37.9) *      | Galloyl methyl gallate |
| 67  | 21–23        | 343 328            |                       | 44.1        | 343.04787 C₁₇H₁₅O₆ (5.6)          | Ellagic acid trimethyl ether |
| 68  | 59–79        | 349 197            |                       | 13.9        | 349.0416 C₁₆H₁₅O₉ (42.7) *       | Galloyl ethyl gallate |
| 69  | 105–119      | 425 301            |                       | 15.8        | 425.01469 C₂₀H₁₁O₁₁ (0.8)        | Ellagic acid pyrogallol ether |
| 70  | 103–119      | 469 425            |                       | 15.7        | 469.0039 C₂₁H₁₁O₁₃ (2.1)         | Valoneic acid dilactone |
| 71  | 161–165      | 481 439, 331, 301, 169 |                   | 1.2         | 481.06556 C₂₀H₁₇O₁₄ (6.6)        | HHDP hexoside |
| 72  | 155–165      | 483 439, 331, 313, 169 |                   | 11.5        | 483.07806 C₂₀H₁₉O₁₄ (0.1)        | Digalloyl hexoside |
| 73  | 85–89        | 497 301            |                       | 23.4        | 497.03631 C₂₃H₁₅O₁₃ (0.3)        | Valoneic acid dilactone ethyl ether |
| 74  | 83–91        | 625 471, 301       |                       | 28.6        | 625.07438 C₂₆H₂₅O₁₈ (28.1) *     | Ellagic acid dihexoside |
| 75  | 147–155      | 631 479, 301       |                       | 19.4        | 631.09323 C₂₇H₁₅O₁₈ (26.3) *     | NHDP hexoside |
| 76  | 155–165      | 633 479, 301       |                       | 7.8         | 633.07511 C₂₇H₂₁O₁₈ (2.8)         | HHDP galloyl hexoside |
| 77  | 133–151      | 635 483, 465, 313  |                       | 15.6        | 635.08832 C₂₇H₂₃O₁₈ (1.0)        | Trigalloyl hexoside |
| 78  | 135–139      | 733 635            |                       | n.d.        | -                                 | Trigalloyl hexoside derivative |
| 79  | 149          | 781 631, 301       |                       | 3.7         | 781.06132 C₃₄H₂₁O₂₂ (10.7)       | Punicalin |
| 80  | 155–167      | 783 481, 301       |                       | 2.7         | 783.07063 C₃₄H₂₃O₂₂ (2.5)        | DiHHDP hexoside |
| 81  | 133–165      | 785 633, 481, 301, 275 |                 | 9.6         | 785.08378 C₃₄H₂₅O₂₂ (0.7)        | HHDP digalloyl hexoside |
| 82  | 133–155      | 787 635, 617, 483, 465, 301 |    | 21.1         | 787.09741 C₃₄H₂₇O₂₂ (3.2)        | Tetragalloyl hexoside |
| 83  | 145–167      | 935 917, 633, 571, 365, 329, 299, 275 |     | 6.0         | 935.07728 C₄₁H₂₇O₂₆ (2.5)        | Galloyl diHHDP hexoside |
| 84  | 131–169      | 937 785, 769, 633, 617, 301 |            | 6.0         | 937.28345 C₄₁H₃₀O₂₆ (12.6) *     | HHDP trigalloyl hexoside |
| 85  | 155–167      | 939 787, 769, 617, 465 |                       | 26.2        | 939.11228 C₄₁H₃₁O₂₆ (1.5)        | Pentagalloyl hexoside |
| 86  | 155–169      | 951 907, 783, 605   |                       | -           | -                                 | DiHHDP hexoside derivative |
### Table 1. Cont.

| Cpd | CCC-Fraction | MS [M − H]− (m/z) | MS/MS [M − H]− (m/z) | LC-RT(min) | ESI/TOF MS Formula (Error in ppm) | Identification |
|-----|--------------|--------------------|------------------------|------------|-----------------------------------|----------------|
| Condensed tannins |
| 87  | 121–133      | 577 463, 425, 313, 289 | 3.9 | 577.13515 C_{30}H_{25}O_{12} (2.9) | (Epi)-catechin dimer |
| 88  | 123–125      | 593 575, 467, 441, 425, 305 | 2.6 | 593.15119 C_{30}H_{25}O_{13} (4.3) | (Epi)-catechin-(epi)-gallocatechin dimer |
| 89  | 107–119      | 609 457, 439, 321, 169 | 4.1 | 609.12858 C_{30}H_{25}O_{14} (5.9) | (Epi)-gallocatechin dimer |
| 90  | 125–129      | 897 745, 575, 463, 449, 423 | 13.0 | 897.14880 C_{44}H_{33}O_{21} (3.6) | (Epi)-catechin gallate-(epi)-gallocatechin gallate dimer |
| 91  | 123–131      | 913 463, 449, 317 | 8.0 | 913.14548 C_{44}H_{33}O_{22} (1.5) | (Epi)-gallocatechin gallate dimer |
| 92  | 137–155      | 913 761, 573, 449, 423 | 24.6 | 913.16762 C_{45}H_{37}O_{21} (17.1) | (Epi)-gallocatechin trimer |
| Others |
| 93  | 73–85        | 109 - | 3.0 | 109.02893 C_{6}H_{5}O_{2} (5.3) | Catechol |
| 94  | 67–81        | 124 - | n.d. | - | Amino catechol |
| 95  | 93–115       | 125 - | 12.1 | 125.02756 C_{6}H_{5}O_{3} (17.2) | Pyrrogallol |
| 96  | 77–83        | 153 109 | 9.1 | 153.02038 C_{7}H_{5}O_{4} (6.9) | Protocatechuic acid |
| 97  | 23–25        | 167 125 | 9.1 | 167.03454 C_{8}H_{5}O_{4} (2.6) | Vanillic acid |
| 98  | 67–79        | 168 124 | n.d. | - | Amino protocatechuic acid |
| 99  | 85–95        | 193 111 | 9.1 | 193.01665 C_{9}H_{5}O_{5} (12.5) | Trihydroxychromone |
| 100 | 15           | 209 187, 165, 125 | n.d. | - | Jasmonic acid |
| 101 | 59–69        | 217 155 | - | - | Unknown |
| 102 | 13–15        | 279 277, 243, 237 | 73.4 | 279.23401 C_{18}H_{31}O_{2} (3.8) | Linoleic acid |
| 103 | 11–15        | 281 277, 255 | 75.8 | 281.24987 C_{18}H_{33}O_{2} (4.5) | Oleic acid |
| 104 | 295          | 277, 275, 265, 251, 249, 185 | 70.2 | 295.2304 C_{18}H_{31}O_{3} (8.6) | Hydroxy linoleic acid |
| 105 | 305 221, 219, 179, 165, 125 | 12.1 | 305.06942 C_{12}H_{17}O_{5}S (2.1) | 5′-hydroxysulphonyloxyjasmonic acid |
| 106 | 11–17        | 383 337 | - | - | Unknown |
Table 1. Cont.

| Cpd | CCC-Fraction | MS [M−H]− (m/z) | MS/MS [M−H]− (m/z) | LC-RT(min) | ESI/TOF MS Formula (Error in ppm) | Identification |
|-----|--------------|----------------|------------------|------------|----------------------------------|----------------|
| 107 | 157–159      | 707            | 687, 671, 533, 359| n.d.       | -                                | Integracin D   |
| 108 | 97–99        | 875            | 441, 433, 289     | -          | -                                | Unknown        |
| 109 | 89–93        | 887            | 585, 301          | -          | -                                | Unknown        |

MS² numbers in bold indicate the most intense product ion. * indicate very minor compounds. HHTP = hexahydroxydiphenoyl ester; NHTP = nonahydroxytriphenoyl ester.

One of the special cases of isomer/isobar separation by HPCCC is the selected ion trace [M−H]− at m/z 615, as the HPCCC experiment separated flavonoid-glycosides with identical molecular weights as displayed in the low resolution ESI-MS injection profile (Figure 1). A set of two partly co-eluting positional isomers of myricetin-desoxy-hexoside-gallate (37) (fraction range 91–115) were absolutely separated from the later eluting isobar quercetin-hexoside-gallate (38) (fraction range 137–153).

The selected ion trace at m/z 635 displayed two strong HPCCC elution ranges with completely separated compound areas with 41 (range 45–57), and 77 (133–151) (Figure 1). However, the metabolite 41 with lower elution volume in the HPCCC run was identified by the ESI-MS/MS profile data as myricetin whereby the ESI-ion-source dimer [2M−H]− was generated in dominant intensity. This was confirmed by the exact identical position of m/z 317 ([M−H]−) in the HPCCC profile. Nevertheless, the late eluting metabolite 77 was identified as a [M−H]−-signal with a hexosid unit substituted by three galloyl-moieties indicated by MS/MS neutral loss cleavage (Δm/z 152) to m/z 483, and 331 of gallic acid releases. The tetra-galloyl-hexoside with [M−H]− at m/z 787 (82) (Table 1) co-eluted in this HPCCC run as seen in Figure 1, as well as the penta-galloyl-hexoside (85) (Table 1). A very large elution volume for recovery in the triphasic HPCCC experiment displayed the galloyl diHHDP hexoside (83) seen by [M−H]− at m/z 935 (range 145–167) (Figure 1 and Table 1). Although the constitution of certain compounds had been different, the polarity differences were not sufficient for a successful HPCCC separation as seen for the selected ion traces [M−H]− m/z 585 (quercetin pentoside gallate, 32), and m/z 599 (quercetin desoxyhexoside gallate, 33) (Figure 1).

Using literature to guide the identification process of the minor, and very minor concentrated derivatives, literature was verified and the few previously isolated compounds in *L. racemosa* were listed with molecular weights as a comparative database. Most of the unknown compounds were characterized by ESI-MS/MS fragmentation and indicative neutral loss pattern. High accuracy molecular weights acquired by LC-ESI/TOF MS were used to ratify and/or verify the proposed molecular formulas. Phytochemical investigations describing chemical compounds on other genus of Combretaceae helped to support the results based on chemotaxonomic knowledge. From the aspect of natural product classes, the chemical composition of the EtOAcPart was distinguished in four main groups as flavonoids, hydrolysable tannins, condensed tannins and other low molecular weight polyphenols (Supplementary Figure S2). The chemical structures and substitution patterns of fractionated and identified compounds are shown in Figure 2.
Figure 2. *Laguncularia racemosa* EtOAcPart general structures and tentative substitution patterns of some of the existing compounds. (a) Flavonoids, (b) hydrolysable tannins, (c) condensed tannins and (d) other low molecular weight polyphenols.
Fractions had been combined on the basis of TLC analysis and the electrospray mass-spectrometry profiling experiment. Supplementary Figure S3 displays the TLC-analysis on the combined fractions of the HPCCC experiment. Table 1 lists HPCCC chromatographic elution, and ESI-MS/MS informations; LC ESI-TOF-MS data (when present) and tentative identification. Although L. racemosa EtOAcPart showed quite complex constituents, most phenolic compounds were well separated.

2.1.1. Flavonoids and Derivatives

Flavonoid derivatives were detected and identified in L. racemosa EtOAcPart by ESI-MS/MS as principal compounds in the recovered HPCCC fractions (Table 1, compounds 1-56). Flavonoids including flavonols, flavones, flavanols and flavanones were found in free form, linked to one sugar unit, as well as in the presence of galloyl substituents. The tentative identification of the flavonoid-aglyca (compounds 1–13) was done by comparison to specific fragmentation patterns, as the spectra of this flavonoid often displayed loss of small neutral fragments contributing to structure information [31–33]. The free flavonoid aglyca eluted during the first step of the gradient before the glycoside linked flavonoids, in accordance to mobile phase/compound polarity in the tail-to-head mode.

The flavonoid-O-substituted characteristically exhibited the neutral loss [34] attributed to a pentose unit [M − H − 132] −, hexose unit [M − H − 162] −, desoxy-hexose unit [M − H − 146] −, glucuronyl unit [M − H − 176] −, galloyl moiety [M − H − 152] − and combination of these substituents. A set of quercetin-O-pentoside, -O-desoxy-hexoside, -O-hexoside, -O-glucuronide and myricetin-O-pentoside, -O-desoxy-hexoside, -O-hexoside were detected in compounds 18, 21, 22, 25, 26, 30 and 31 [35–41]. The substituent gallate was found connected to (epi)-catechin (20), (epi)-gallocatechin (23) and myricetin (28) as well as in glycosylated forms of quercetin and ellagic acid. The sequence of compounds comprised ellagic acid itself and the -methyl, -dimethyl, ethyl gallate, respectively [37,38,42].

Aglycones apigenin, kaempferol, quercetin and tricin were previously reported in L. racemosa [8,26] in addition to the glycosylated derivatives quercetin-3-O-arabinoside and quercetin-3-O-rhamnoside [24]. Not fully identified derivatives could be distinguished by observed aglycone fragment ions in MS/MS.

2.1.2. Hydrolysable Tannins

Hydrolysable tannins, well-known in Combretaceae, were the second main class of natural compounds detected by the HPCCC and off-line injection ESI-MS/MS experiment (Table 1, compounds 57–86) [36–40,42,43]. It included derivatives of gallic acid, ellagic acid, gallotannins and ellagitannins. Some of the ellagic acid and its methyl-, dimethyl- and trimethyl ether derivatives were previously reported in L. racemosa [26]. Several studies describing the detection of hydrolysable tannins in species of Combretaceae can be found [44–46].

Common neutral loss cleavages observed in the MS/MS for simple gallic acid and its derivatives were related to the cleavage of carboxyl [M − H − 44] +, methyl [M − H − 15] +, ethyl [M − H − 29] + and galloyl [M − H − 152] −. They were found as ester or ether arrangements. Compounds 57–59, 62, 63, 66 and 68 were identified as gallic acid, methyl gallate, ethyl gallate, galloyl gallate, galloyl shikimate, galloyl methyl gallate and galloyl ethyl gallate, respectively [37,38,42].

Ellagic acid derivatives were characterized by the fragment ion m/z 301. At this point, LC ESI-TOF-MS was essential to distinguish derivatives from quercetin and ellagic acid. The sequence of compounds comprised ellagic acid itself and the -methyl, -dimethyl, -trimethyl, -pyrogallol and dihexoside ether forms (60, 61, 64, 67, 69, 74) [36,39,40,43]. Additionally, valoneic acid dilactone (70) and its ethyl ether derivative (73) were detected [36,40].

By comparison to literature [47], the molecular masses of compounds 65, 72, 77, 82 and 85 showed that they consist of a gallotannin series of molecules (mono-, di-, tri-,
tetra- and penta-galloyl hexosides) [37,38,42]. A similar series of monomeric ellagitannins (HHDP-, NHDP-, HHDP galloyl-, diHHDP-, HHDP digalloyl-, diHHDP galloyl- and HHDP trigalloyl-) were found in compounds 71, 75, 76, 80, 81, 83 and 84 [36,38,40]. The ellagic acid punicalin (79) was further detected at m/z 781.

2.1.3. Condensed Tannins

Condensed tannins (proanthocyanidins), formerly observed in L. racemosa wood and leaves [48–50], were recognized and characterized based on the detected flavanol-aglyca (4, 7, 9) and its gallate derivatives (20, 23). They were found as homo-dimers consisting of (epi)-catechin (87), (epi)-gallocatechin (89) and (epi)-gallocatechin gallate (91) [40]. Additionally, as hetero-dimers, existing as (epi)-catechin-(epi)-gallocatechin (88) and (epi)-catechin gallate-(epi)-gallocatechin gallate (90). The trimeric (epi)-gallocatechin (92) was also encountered. Compounds had fragmentation patterns related to the cleavage of flavanol units according to literature [51]: [M − H − 289]− for (epi)-catechin loss, [M − H − 305]− (epi)-gallocatechin loss, [M − H − 441]− (epi)-gallocatechin gallate loss and [M − H − 162]− for gallate loss.

Considering the elution order of compounds in respect to gradient polarity range, the flavonol-aglyca eluted before the gallate derivatives, both in the first step, while dimers and trimers stayed retained in the column until extrusion started.

2.1.4. Low Molecular Weight Polyphenols

Other compounds were recognized and characterized based on precursors/derivatives of existing identified compounds in the off-line ESI-MS/MS profile or on the L. racemosa chemical database. Simple phenolic compounds included catechol (93) and pyrogallol (95), common occurring products in the hydrolysable tannins pathway [35]. Benzoic acid derivatives with frequent [M − H − 44]− corresponding to the neutral loss of CO2, comprised protocatechuic (96) and vanillic (97) acids [37]. The amino derivatives aminocatechol (94) and amino protocatechuic acid (98) were also detected [52]. The chromone detected at m/z 193, was identified as trihydroxy-chromone (99) and had its molecular formula confirmed by HRMS.

The jasmonic acid (100) and its sulphated derivative 5′-hydroxy-sulphonyloxy jasmonic acid (105), earlier isolated from the L. racemosa twigs and leaves [23], could be found at m/z 209 and 305, respectively. ESI/TOF MS data confirmed the proposed compounds. Ordinary oleic and linoleic fatty acids (102-104), jasmonic acid biosynthetic precursor, were further encountered. Another sulphated derivative isolated from L. racemosa leaves [26] was found at [M − H]− at m/z 707 and was identified as integracin D (107) [26]. Due to concentration limits, the compound could not be detected in the ESI/TOF MS analysis and structure was not fully confirmed.

3. Materials and Methods

3.1. Chemical Reagents and Solvents

Preparation of extracts was carried out with analytical grade solvents from Tedia Brazil (Rio de Janeiro, Brazil). LC-ESI/TOF-MS/MS analyses used HPLC grade solvents from Tedia Brazil (Rio de Janeiro, Brazil). HPCCC separations were performed with analytical grade solvents from Fisher Chemicals (Loughborough, UK). ESI-MS/MS analyses were done with HPLC grade solvents from VWR Chemicals (Radnor, PA, USA). NMR analyses used deuterated solvents from Cambridge Isotope Laboratories (Tweksbury, MA, USA) and TMS as internal standard. All aqueous solutions were prepared with pure water produced by Milli-Q water (18.2 MΩ) system (Thame, UK).

3.2. Preparation of the Extract

Laguncularia racemosa (3 kg) was collected at Guaratiba Biological and Anthropological Reserve (Rio de Janeiro, Brazil) in November 2010. Specialist researchers from the Nucleus of Mangrove Studies (University of the State of Rio de Janeiro) helped in the
localization, identification and collection of the plant. The leaves were dried and grounded in a laboratory mill (Laboratory Retsch mill, Haan, Germany) and 1800 g were submitted to maceration with ethanol–water 8:2 (v/v) in 10 cycles of 24 h. The solvent was evaporated under reduced pressure at 50 °C and the crude extract (255 g) was partitioned between water and organic solvents, affording different extracts: n-hexane (4 g), dichloromethane (8 g), ethyl acetate (15 g) and aqueous (215 g).

3.3. Thin Layer Chromatography

Preliminary analyses of EtOAcPart, solvent system evaluation tests and CCC fraction analyses were done by thin layer chromatography (TLC) on normal phase silica gel TLC plates (SiO$_2$-60, F254, Merck, Darmstadt, Germany, gel 60 RP-18, F254S) developed with EtOAc–acetone–H$_2$O 25:15:10 (v/v/v), and acetonitrile-H$_2$O 1:1 (v/v) for reversed phase C18-plates (RP18W, Macherey and Nagel, Düren, Germany). Results were visualized by using spray-reagent H$_2$SO$_4$ (10% m/v) in methanol with vanillin 5% in ethanol and flash heating on a hot plate 105 °C.

3.4. LC-ESI/TOF MS Preliminary Analysis

The EtOAcPart was also analysed by LC–ESI/TOF-MS with a 1200 Series LC-chromatograph (Agilent, Palo Alto, CA, USA) coupled with a MicrOTOF II time-of-flight mass spectrometer (Bruker Daltonics, Inc., Billerica, MA, USA). 5 µL injection was performed with an autosampler on a Poroshell EC-C18 column (100 × 2.1 mm; 2.7 µm, Agilent, Palo Alto, CA, USA). The source temperature was set at 200 °C, the drying gas (nitrogen) flow rate was 10.0 L/min and the nebulizer gas (nitrogen) pressure was 4 bar. Data were acquired in negative mode in the range of m/z 100–1500. The capillary voltage was 3.8 kV, the capillary exit voltage was −150 V, the skimmer 1 and 2 voltages were 50 V and 23 V, respectively, the hexapole 1 voltage was set to −23 V, the hexapole RF voltage was 120 Vpp, lens 1 transfer was 68 µs and lens 1 pre plus stage was 7 µs. Mass calibration was achieved by infusing ammonium formate in an isopropanol–water mixture (1:1, v/v) as an external standard. All data were analysed using Bruker Daltonics ESI Compass Data Analysis Version 4.0 SP 1 (Bruker Daltonics Inc., Billerica, MA, USA). The mobile phase consisted of spectroscopic grade methanol (B) and ultrapure water (A) containing 0.05% (v/v) formic acid. The linear gradient elution was set from 10% to 100% of B in 90 min at a flow rate of 0.3 mL/min.

3.5. High Performance Countercurrent Chromatography

3.5.1. Equipment

CCC separations were performed on a semi-preparative HPCCC system (model Spectrum, Dynamic Extractions Ltd., Gwent, UK) equipped with two counter-balanced bobbins with perfluoroalkoxy polymer (PFA) tubing (1.6 mm i.d.) wound in multi-layer coiled-columns, resulting in 143.5 mL total volume (V$_C$). The rotation speed was adjusted to the maximum velocity of 1600 rpm (240 g). Solvent phase systems were delivered by a constant flow pump (Agilent HP1200, Palo Alto, CA, USA) to the HPCCC system. A semi-preparative sample loop (7.15 mL) was used to inject the dissolved sample over a low-pressure valve (Upchurch Model V-450, with 1.6 mm i.d. fittings) to the chromatographic system. Fractions were collected by a fraction collector (Agilent HP1200, Palo Alto, CA, USA).

3.5.2. Three-Phase Solvent System Test Evaluation

The three-phase solvent systems were composed of n-hexane–methyl acetate–acetonitrile–water and n-hexane–tert-butyl methyl ether–acetonitrile–water [11, 53, 54]. For the experiments for solvent system evaluation, 2 mg of the EtOAcPart were dissolved in a test tube containing 2 mL of each phase of the thoroughly equilibrated solvent systems. The test tubes were shaken vigorously for compound partition. After the phase layers had completely separated and distribution equilibrium was established, the resulting phase layers were analyzed by TLC (Supplementary Figure S4).
3.5.3. Solvent System and Sample Preparation

The selected solvent system n-hexane–tert-butyl methyl ether–acetonitrile–water (2:3:3:2, v/v/v/v) was thoroughly equilibrated in a separatory funnel at room temperature. The three phases were separated shortly before use and degassed by ultra-sonication for 5 min. The sample solution was prepared by dissolving the sample at fixed concentration (100 mg/mL) and coil-volume (5% V_C) in the lower aqueous phase only.

3.5.4. HPCCC Separation Procedure

Separation was performed in a normal step-gradient elution mode. The more aqueous lower phase was used as the stationary phase while organic upper and middle phases were used as mobile phases as shown in Figure 3. The system was completely filled with the lower aqueous stationary phase. Rotation was set to 1600 rpm. For the separation, the upper organic mobile phase was pumped at 4.0 mL/min. After reaching hydrodynamic equilibrium, the sample was injected to the HPCCC column. For the first elution step, 214.5 mL mobile phase (1.5 V_C) of upper phase was pumped through. For the second elution step, 2 V_C (286 mL) of the middle phase was pumped through the HPCCC system. Fractions were collected at 1 min intervals. For the extrusion step, rotation was reduced to 200 rpm and the column contents were pushed out of the system by lower phase at 8.0 mL/min and fractions were collected at 30 s intervals. The temperature control was maintained at 30 °C.

Figure 3. HPCCC three phase solvent system step-gradient procedure.

3.6. Metabolite Profiling by Offline Injections to ESI-MS/MS

The molecular weight profiles of the recovered HPCCC fractions were monitored by off-line ESI-MS/MS and were recorded in a single data file using the ion-trap mass-spectrometer HCT-Ultra ETD II (Bruker Daltonics, Bremen, Germany). Aliquots of 0.75 µL of odd numbered CCC fractions were directly filled to vials, dried and redissolved in 1.0 mL of methanol for conducting the ESI-MS analysis. Fractions were delivered to the ESI-MS/MS by a HPLC-pump (binary pump, G1312 A, 1100 Series, Agilent, Waldbronn, Germany) using the make-up solvent system with a flow rate of 0.5 mL/min composed of acetonitrile and water (1:1, v/v). ESI-MS/MS parameter settings were in the negative ionization mode, with scan-range between m/z 100–2000, where mostly deprotonated [M – H]^- ion signals were generated. An auto-MS/MS method fragmented the nine most intense peaks and to monitor and characterize co-eluting compounds. Drying gas was nitrogen (flow rate 10.0 L/min, 310 °C), and nebulizer pressure was set to 60 psi. Ionization voltage at HV capillary was 3500 V, HV end plate off set –500 V, trap drive 61.8, octupole RF amplitude 187.1 Vpp, lens 2 60.0 V, Cap Ex –115.0 V, max. accumulation time 200 ms, averages 5 spectra, trap drive level 120%, target mass range: m/z 500, compound stability 80%, Smart ICC target 70.000, ICC charge control ‘on’ and smart parameter setting ‘active’.
4. Conclusions

The combination of analysis of preparative HPCCC fractions with off-line injections to an ESI-MS/MS device was proven to be highly effective for a full metabolite chemical profile for polyphenols using the negative ionization mode. The use of a three-phase solvent system for HPCCC in a step-gradient elution mode was adequate to maintain equilibrium and chromatographic resolution while improving mobile phase strength. The ESI-MS/MS projection of the semi-preparative HPCCC experiment visualized over 100 compounds by selected single ion traces and was an adequate confirmation of the LC–ESI/TOF MS analysis. This study detected a variety of metabolites from different classes occurring in L. racemosa EtOAcPart and used chemotaxonomic data to guide the MS/MS putative structure elucidation.

Supplementary Materials: The following are available online, Figure S1: EtOAcPart preliminary analyses by LC-ESI-TOF-MS. The mobile phase was methanol (B) and water (A) containing 0.05% (v/v) formic acid. The linear gradient elution was set from 10% to 100% of B in 90 min. Figure S2: HPCCC off-line injection ESI-MS/MS profile of the the EtOAc Part by use of selected single ion traces for target compounds or classes. (a) Flavonoids, (b) hydrolysable tannins, (c) condensed tannins and (d) other compounds. Figure S3: TLC analysis of L. racemosa CCC combined fractions. On the left: reversed phase silica gel TLC plates developed with acetonitrile-H2O 1:1 (v/v). On the right: Normal phase silica gel TLC plates developed with EtOAc-acetone-H2O 25:15:10 (v/v/v). Visualization was done using 254 nm UV light and spray-reagent H2SO4 10% and vanillin 5%. F means a group of jointed fractions according to TLC similarity. Figure S4: Three-phase solvent system test by TLC. CCC solvent system: n-hexane–MTBE–ACN-H2O (1) 1-1-2-1, (2) 2-1-3-2, (3) 2-2-3-2, (4) 2-3-3-2 and (5) 2-3-3-2 (v/v/v); (U) upper, (M) middle and (L) lower phases. Normal phase silica gel TLC plates developed with EtOAc-acetone-H2O 25:15:10 (v/v/v), visualized using λ 254 nm UV light and spray-reagent H2SO4 10% and vanillin 5%.

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