Selection of Three-Phase Solvent System for Countercurrent Chromatography – A Practical Guide Using Syzygium malaccense Leaves Extract as an Example

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Countercurrent chromatography (CCC) is a liquid-liquid partition technique with no use of a solid support. The choice of solvent system for CCC separations is the most important and time-consuming step of the fractionation process. Recently, three-phase solvent systems (TPSS) have emerged for separation of mixtures exhibiting a large variation of compounds polarity. This study proposed a strategy to select rationally a TPSS to be used for fractionation of Syzygium malaccense leaves extract by high-speed countercurrent chromatography. Solvent system tests were based on volume phase ratio, sample solubility, compounds partition and stationary phase retention, in this order. A good compound distribution along the separation process was obtained using n-hexane - ethyl acetate - acetonitrile - water (2:1:1:1, v/v) in stepwise gradient elution mode. Twelve compounds could be identified by NMR analysis. Flavonoid, phenylpropanoid, benzoic acid, hydrolysable tannin, fatty acid and carboxylic acid are some of the classes encountered in Syzygium malaccense leaves extract evidencing its complexity and broad hydrophobicity range.

Keywords: Three-phase solvents system; countercurrent chromatography; Syzygium malaccense; gradient elution.

1. Introduction

Countercurrent chromatography (CCC) is a liquid-liquid partition technique without the use of a solid support. The separation mechanism is based on solutes distribution between two liquid phases. The separation process involves the use of a biphasic liquid system that will work as stationary and mobile phases. The chromatographic columns are capable of maintaining the static liquid stationary phase using centrifugal force field, while the liquid mobile phase is pushed through it, resulting of static partition and dynamic elution.

The choice of solvent system for CCC separations is the most important step of fractionation process. The search for a suitable solvent system for separation of compounds is, usually, a challenge and time-consuming due to large number of possible solvent combinations that forms, at least, a two-phase solvent system. Therefore, it is convenient to have some strategies to guide this choice properly.

Two-phase solvent systems having different and defined polarity ranges are well known and successfully applied in CCC fractionations. Biphasic systems are limited to separate mixture of compounds with a narrow polarity range, when used in isocratic elution mode. However, plant extracts commonly contain a complex mixture of compounds with a broad range of polarity, making very difficult the separation process by ‘traditional’ CCC. In some cases, it is necessary to develop separation methods to amplify solvent system polarity spectrum.

In this context, triphasic systems have emerged with an advantage: the separation of mixtures exhibiting a large variation of compounds polarity due to difference in polarity between the upper phase (UP) and the lower phase (LP) interposing the intermediate phases (IP). Unfortunately, only a few CCC studies obtained solvent combinations that provide a stable three-phase solvent systems (TPSS) and an appropriate distribution pattern of solutes between the immiscible phases.

Three-phase systems are built by an organic-aqueous two-phase system normally composed of n-hexane, acetonitrile and water in combination with a fourth solvent such as methyl acetate, ethyl acetate, methyl t-butyl ether, diethyl ether or dichloromethane to create the third

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A TPSS composed by five solvents was described: in addition to the two-phase $n$-hexane, acetonitrile and water, dichloromethane and ethyl acetate were added. Three-phase solvent system comprising triethylamine solution instead of water also have been reported. Another TPSS composed by $n$-hexane-acetonitrile-ammonium sulfate was firstly reported very recently. As well as in the two-phase solvent system comprising triethylamine solution, the compositions of TPSSs can be optimized according to their physical parameters such as volume ratio, viscosity and specific gravity of phases.

Elution of mobile phase in TPSSs can be done in a variety of ways. In the pioneering study of Shibusawa et al., using TPSS in CCC, the separation was performed using IP and LP only. After that, other studies used two phases of the TPSS in the separation.

The stepwise gradient elution process was employed in several papers. It was observed that the stepwise elution involving the three phases is useful for the separation of compounds with a broad range of hydrophobicity in a single operation. In some examples, two-phases were used as stationary phase: the separation is initiated by filling the column with a mixture of IP and LP. Then, UP was the first mobile phase, switched to IP and finally eluting the column with LP. Another case applied a mixture of UP and IP as stationary phase, then LP was first mobile phase, switched to IP and finally eluting the column with UP. When two phases of a TPSS are retained in a rotating column as the stationary phase it can be considered a combination of columns.

Considering all the information collected about composition and elution modes involved in TPSSs, this study proposed a strategy to rationally select a three-phase solvent system to be used for fast fractionation of Syzygium malaccense leaves extract by high-speed counter-current chromatography.

2. Material and Methods

2.1. Chemical reagents and solvents

Organic solvents for the preparation of crude extract and thin-layer chromatography (TLC) analyses were analytical grade (Dinâmica Química Contemporânea, Indaiatuba, Brazil). Aqueous solutions used pure water produced by Milli-Q® system (18.2 MΩ) (Merck® Millipore, Direct-Q 5, Germany). Solvents used in HPLC analyses, solvent system tests and HSCCC separations were HPLC grade (Tedia Brazil, Rio de Janeiro, Brazil).

2.2. Plant material

Syzygium malaccense leaves (604.38 g) were collected in Cacoal, Rondônia, in the Brazilian Amazon region. The leaves were dried (168.66 g) and submitted to maceration with ethanol-water 7:3 (v/v) for 72 hours. Solvent was renewed every 24h. The extract was filtered, solvent was evaporated, and the crude extract (29.04 g) was obtained after freeze-drying (Liotop, model L101, São Carlos, Brazil).

2.3. HPLC–DAD analysis of S. malaccense crude extract and TPSS phases

HPLC analysis of S. malaccense crude extract and TPSS phases were performed on an Agilent 1260 Infinity Quaternary LC (Agilent Technologies, Germany), equipped with a Quat Pump VL pump (model G1316) and diode-array detector (DAD) 1260 (model G4212B). This instrument was equipped with an Agilent Poroshell HPH C18 column (2.1x 100 mm, 2.7 μm) (Agilent Technologies, EUA). Acidified water (0.1% formic acid, v/v) and acidified acetonitrile (0.1% formic acid, v/v) were used as mobile phases A and B, respectively. For S. malaccense crude extract analysis, the gradient was programmed as follows: 0 min, 5% B; 30 min, 50% B; 33 min, 100% B; 37 min, 100% B; 40 min, 5% B; and 45 min 5% B. The flow rate was 0.3 mL/min, injection volume was 5 μL and sample concentration was 2 mg/mL in methanol. For TPSSs phase analysis, the gradient parameters were optimized: 0 min, 0% B; 5 min, 0% B; 45 min, 100% B; 50 min, 100% B; 52 min, 0% B; and 57 min 0% B. The flow rate was 0.3 mL/min, injection volume was 10μL and sample concentration was 2.5 mg/mL in methanol (upper and intermediate phases) and in methanol-$\text{H}_2\text{O}$ 1:1 (v/v) (lower phase).

2.4. TLC analysis of crude extract and solvent system phases

TLC (silica gel plates 60 F254, Merck Art. 05554, Darmstadt, Germany) was developed with ethyl acetate – acetone – water 25:15:10 (v/v). Results were visualized by UV lamp - lightweight UV cabinet (Solab, SL-204, Brazil) in short- and long-waves ($\lambda=254$ and 365 nm, respectively) and then using spray-reagent H$_2$SO$_4$ 10% in ethanol and vanillin 10% in ethanol before heating in a hot plate (Novatecnicia®, NT 103, Brazil).

2.5. Three-phase solvent system tests

TPSSs (Table 1) were tested according to previous publications. For evaluation, 10 mg of the extract was dissolved in a flask containing 8 mL of TPSS fully equilibrated. The flask was shaken, and the compounds were allowed to partition between the three phases. Each phase was analysed by TLC and the most promising systems were analysed by HPLC.

2.6. High-speed countercurrent chromatography equipment

HSCCC fractionations were performed on the 98 mL column of a Quattro HT Prep countercurrent chromatograph (AECS®, Bridgend, United Kingdom) equipped with two bobbins containing two polytetrafluoroethylene...
A 10 mL sample loop (Model 5020, Rheodyne®) was used to inject the sample. Separations were performed at room temperature. The rotation speed is adjustable up to 865 rpm. The HSCCC system was connected to a constant flow pump by quaternary gradient HPLC pump with integrated vacuum degasser (Model PU-2089 plus, Jasco, Japan) and a fraction collector (Model 2110, Bio-rad, USA).

### 2.7. Stationary phase retention volume calculation

The column of HSCCC was filled with the stationary phase, then, the first mobile phase was pumped from tail to head at 2.5 mL/min and the rotation speed was adjusted to 865 rpm. When the hydrodynamic mixing between the phases reached a steady state of equilibrium in the rotating column, the displaced volume of stationary phase was measured in a graduated cylinder. The stationary phase retention ratio ($S_f$) was calculated according to column volume ($V_c$) and the displaced stationary phase volume ($V_e$):

$$S_f = \left(\frac{V_c - V_e}{V_c}\right) \times 100$$

To simulate the sample injection, 5 mL of stationary phase was injected. After that, stationary phase volume was corrected by measuring the stationary phase stripping volume collected ($V_{str}$) and the corrected stationary phase retention ($S_f^*$) was obtained:

$$S_f^* = \left(\frac{V_c - (V_e + V_{str} + V_e^*)}{V_c}\right) \times 100$$

Final corrected stationary phase retention ($S_f^{**}$) was calculated in the second gradient step using the intermediate phase as mobile phase. In this case, a new displaced stationary phase volume ($V_{e^*}$) were measured after changing upper mobile phase to intermediary mobile phase:

$$S_f^{**} = \left(\frac{V_c - (V_e + V_{str} + V_{e^*})}{V_c}\right) \times 100$$

### 2.8. HSCCC fractionation procedure and sample preparation

The selected solvent system was prepared in a separatory funnel at room temperature. The three phases were separated and degassed by ultra-sonication for 5 min. The 98 mL column was completely filled with lower stationary phase. Then the rotation was set to 865 rpm and the upper mobile phase was pumped into the column at a flow rate of 2.5 mL/min. After reaching hydrodynamic equilibrium, the 10 mL sample solution was injected in the system. The sample was dissolved at fixed concentration (100 mg/mL) in the lower aqueous phase only. Upper phase was pumped during 80 min (corresponding to 40 fractions). Mobile phase was changed to middle phase and another 40 fractions were collected before extrusion using lower phase to push the column content out. Fractions were collected at 2 min intervals (Figure 1).

### 2.9. NMR identification of compounds

$^1$H, HSQC and/or HMBC data measurements for the selected fractions were recorded on 800 MHz Bruker Avance III (Ettingen, Germany) equipped with a 1.7 mm TCI cryoprobe at 25 °C. Dried samples were diluted in methanol-$d_4$ and transferred to 1.7 mm NMR tubes. All the NMR data acquired were processed accordingly using MNova (Mestrelab Research S.L.) and manually peak picking.
Mearnsitin (1): $^1$H NMR (800 MHz, CD$_3$OD): $\delta$ (ppm) 6.19 (C-6); 6.39 (C-8); 7.31 (C-2'); 7.31 (C-6'), 3.88 (–OCH$_3$). Mearnsitin-3-O-$\beta$-rhamnoside (2): $^1$H NMR (800 MHz, CD$_3$OD): $\delta$ (ppm) 6.22 (C-6); 6.38 (C-8); 6.89 (C-2', C-6'); 3.88 (–OCH$_3$); 5.31 (C-1''), 0.96 (C-6''). Quercetin (3): $^1$H NMR (800 MHz, CD$_3$OD): $\delta$ (ppm) 6.21 (C-6); 7.34 (C-2'); 6.92 (C-5'), 7.32 (C-6'). Quercetin-3-O-$\beta$-rhamnoside (4): $^1$H NMR (800 MHz, CD$_3$OD): $\delta$ (ppm) 6.21 (C-6); 7.34 (C-2'); 6.92 (C-5'); 7.32 (C-6'); 5.35 (C-1''), 0.94 (C-6''). Myricetin (5): $^1$H NMR (800 MHz, CD$_3$OD): $\delta$ (ppm) 6.18 (C-6); 6.38 (C-8), 7.34 (C-2', C-6'). Tartaric acid (6): $^1$H NMR (800 MHz, CD$_3$OD): $\delta$ (ppm) 4.56 (C-2). Dimethyl tartrate (7): $^1$H NMR (800 MHz, CD$_3$OD): $\delta$ (ppm) 4.56 (C-2), 3.78 (–OCH$_3$). Gallic acid (8): $^1$H NMR (800 MHz, CD$_3$OD): $\delta$ (ppm) 7.06 (C-2, C-6). Ethyl gallate (9): $^1$H NMR (800 MHz, CD$_3$OD): $\delta$ (ppm) 7.04 (C-2, C-6). Methyl ellagic acid (10): $^1$H NMR (800 MHz, CD$_3$OD): $\delta$ (ppm) 7.58 (C-2) e 3.65 (–OCH$_3$), p-coumaric acid (11): $^1$H NMR (800 MHz, CD$_3$OD): $\delta$ (ppm) 7.41 (C-2, C-6); 6.78 (C-3, C-5); 7.47 (C-7); 6.30 (C-8). Malyngic acid (12): $^1$H NMR (800 MHz, CD$_3$OD): $\delta$ (ppm) 3.74 (C-9); 5.37 (C-10); 5.36 (C-11); 3.76 (C-12); 3.71 (C-13); 5.34 (C-15), 5.32 (C-16).

## 3. Results and Discussion

TLC and HPLC-DAD showing an extract with high chemical complexity and large polarity range made the *S. malaccense* preliminary chromatographic profile. The analysis also gave information on compound classes present: phenylpropanoids, flavonoids, phenolics and, possibly, its glycosides. The chemical characterization is in accordance with previous studies on the species.$^{23,24}$

The TLC plate (Figure S1) showed approximately nine major zones when visualized under UV light (UV-254 nm): phenylpropanoids with characteristic dark blue zones, chlorophylls with greenish spots and flavonoids with yellowish areas. In UV-365 nm, the orange-red zones characteristic of chlorophyll and the fluorescent blue color characteristic of phenolic acids are shown.$^{25}$ With the chemical detector: chlorophyll appeared greenish, flavonoids dark yellow and glycosylates reddish violet color.$^{24}$

The chromatographic profile by HPLC (Figure S2) of the extract showed the presence of five major peaks which UV spectra $\lambda = 365$ nm ($\lambda_{max} = \sim 252, 362; 262, 338; 245, 368$...
and 261, 364 nm) (FigureS3) were consistent with flavonoid derivatives.\textsuperscript{26-27} The UV data of the fifth peak was inconclusive.

Based on preliminary analyzes, medium to high polarity solvent systems were preferred, especially ones dealing with phenylpropanoids, flavonoids, phenolic acids.\textsuperscript{11-13} Table 1 describes a summary TPSSs tested combined as groups of solvents.

Among the tested TPSSs, systems A – C (Figure S4) belong to the same group of solvents (HEX - EtOAc - ACN - H\textsubscript{2}O). Systems B and C were not suitable for CCC use because they form phases with very different volumes proportion (22/67/11 and 66/17/17 of UP/IP/LP, respectively). In order to avoid wastage of solvent, systems with similar phase volume are recommended.\textsuperscript{28} When compared to A, the increase in EtOAc, ACN and H\textsubscript{2}O favored the formation of the intermediate phase in B, indicating high affinity of solvents by this phase in the given proportions. In C, the increase of HEX, lead to an increase in upper phase, indicating that there is low interaction of the solvent in the rest of the system.

In TPSSs D – H (Figure S4), belonging to the group of solvents HEX - MTBE - ACN - H\textsubscript{2}O, only the system H did not form three phases. Systems E, F and G, despite forming the three phases, had solubility problems when sample was added, probably due to the increase in HEX. Therefore, E, F and G were discarded. It is known that sample should be stable and soluble in the chosen solvent system for a successful CCC separation.\textsuperscript{1}

TPSS I (Figure S4) belongs to the group HEX - DCM - ACN - H\textsubscript{2}O. The system formed an emulsion – probably due to the presence of DCM – in the intermediate phase after adding the sample, being therefore discarded.

Figure 3. HPLC chromatogram profile of upper phases of TPSSs A, D and K. UV – \( \lambda = 280 \text{ nm} \). Gradient parameters: 0 min, 0% B; 5 min, 0% B; 45 min, 100% B; 50 min, 100% B; 52 min, 0% B; and 57 min 0% B

Figure 4. HPLC chromatogram profile of intermediate phases of TPSSs A, D and K. UV – \( \lambda = 280 \text{ nm} \). Gradient parameters: 0 min, 0% B; 5 min, 0% B; 45 min, 100% B; 50 min, 100% B; 52 min, 0% B; and 57 min 0% B
In TPSS J – L (Figure S4) group, being formed by HEX - MeOAc - ACN - H₂O, system J presented an emulsion formation when sample was added to the flask. System L formed phases with different volume ratios (12/75/13 of UP/IP/LP). Both were discarded.

With these preliminary observations on TPSSs, it was concluded that systems A, D and K were the ones fitting in basic CCC parameters, so will be further considered on compound partition test.

All three systems showed visually satisfactory compound partition between phases when analyzed by TLC (Figure 2). More accurate partition analyses were performed by HPLC. The chromatogram of the upper, intermediate and lower phases of the TPSSs indicated similar chromatographic profiles in the different systems (Figures 3–5). The use of three phases in separation process made very difficult the task of calculating partition values for major compounds in the mixture. Only visual results were examined. Systems A, D and K were considered able to fractionate the extract.

Among the many elution possibilities described for the TPSSs, we opt for the step-gradient. The decision was based not only on the extract broad polarity characteristics—ideal for the use of gradient—but also on extract partition results. Solvent system optimization for stepwise gradient elution mode in CCC is done by choosing systems that will provide extreme situations to create the gradient strengths. Compounds should be differently divided between the phases (Figure 6) such as the results shown in Figure 2.

Separation was then designed to use lower aqueous phase as stationary phase. Elution-extrusion of would comprise all phases: upper, intermediate and lower in a stepwise mode, (Figure 1). Because the volume of the stationary phase remaining in the column (Sf) is an essential parameter that directly influences the separation quality, the next step was the retention phase study in the CCC apparatus.

A ‘blank run’ was simulated to verify how TPSS behavior inside the equipment. It is important to examine if changes in the mobile phase would disturb the column hydrodynamic equilibrium in the gradient steps. The system should keep a high hydrodynamic stability during the entire fractionation process. The percentage of the stationary phase volume retained in the column relative to the total column capacity, is one of the most important parameters in CCC, as number of theoretical plates and chromatographic resolution depends on it.32

![Figure 5. HPLC chromatogram profile of lower phases of TPSSs A, D and K. UV – \(\lambda = 280\) nm. Gradient parameters: 0 min, 0% B; 5 min, 0% B; 45 min, 100% B; 50 min, 100% B; 52 min, 0% B; and 57 min 0% B](image)

![Figure 6. Scheme of compounds differently divided between the three phases to create the gradient strengths](image)
Retention results were analyzed in terms of $S_f$, $S_{f^*}$ and $S_{f^{**}}$ (meaning stationary phase retention, corrected stationary phase retention after sample injection and corrected stationary phase retention after changing mobile phase, respectively) (Table 2). All TPSSs tested provided differences between the three parameters confirming that the system equilibrium is sensitive to any variation on the liquid pumped into the column.

Systems A, D and K provided $S_f$ values above 80%, being acceptable to CCC separations. The higher $S_f$ value the better chance for compounds to be well separated. After blank sample was injected, $S_f$ reduced about 5% in the three cases. Result was expected and can arise from the nature, composition and/or mass of the loaded sample. The major difference encountered was when the mobile phase was changed from upper to middle phase in the second step of the gradient: while system A succeed in maintaining more than 70% of stationary phase inside the column, systems D and K dropped in half of the $S_{f^*}$ value, as shown in Table 2.

Based on the difference encountered on stationary phase volume calculation, TPSS A was chosen to fractionate $S$. *malaccense* leaves extract. A total of 120 fractions of 5 mL each were collected according to Figure 1. Resulting fractions were analyzed by TLC (Figure 7).

TPSS A showed a good distribution of the substances throughout the separation process in the three stages of the gradient. It is possible to check different polarities compounds distributed in the complete chromatographic run evidencing extract chemical complexity. The extrusion step could have been shortened, since compounds stopped eluting from column in fraction 105, in agreement to column volume.

Fractions 47, 49, 51, 81, 83, 87 and 89 were chosen to be analyzed by NMR and data was compared to literature (Table 3). It was possible to identify some compounds present on the $S$. *malaccense* leaves extract, evidencing the extract chemical complexity. Flavonoid, phenylpropanoid, benzoic acid, hydrolysable tannin, fatty acid and carboxylic acid are some of the classes found in these fractions (Figure 8).

### Table 2

<table>
<thead>
<tr>
<th>TPSS</th>
<th>$S_f$ (%)</th>
<th>$S_{f^*}$ (%)</th>
<th>$S_{f^{**}}$ (%)</th>
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<tr>
<td>A</td>
<td>80.6</td>
<td>75.5</td>
<td>70.4</td>
</tr>
<tr>
<td>D</td>
<td>88.8</td>
<td>83.6</td>
<td>42.9</td>
</tr>
<tr>
<td>K</td>
<td>82.6</td>
<td>77.5</td>
<td>36.7</td>
</tr>
</tbody>
</table>

*a* Volume ratio of retention of the stationary phase using the first mobile phase = UP

*b* Volume ratio of retention of the stationary phase using the second mobile phase = IP

Figure 7. TLC plate corresponding to the fractionation of $S$. *malaccense* leaves extract by TPSS A. Eluted with the system: ethyl acetate – acetone – H$_2$O, 25:15:10 (v/v). Observed in short- (1) and long-waves (2) ($\lambda$=254 and 365 nm, respectively) and then using spray-reagent H$_2$SO$_4$ 10% in ethanol and vanillin 10% in ethanol before heating in a hot plate (3). Red arrows indicate the fractions analyzed by NMR.
Table 3. Summary of the compounds identified in the *S. malaccense* leaves extract obtained in HSCCC fractionation and indication of the experiments used in NMR

<table>
<thead>
<tr>
<th>Fractions</th>
<th>^H</th>
<th>HSQC</th>
<th>HMBC</th>
<th>Annotated compounds</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>47</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>Mearnsetin</td>
<td>34</td>
</tr>
<tr>
<td>49</td>
<td>X</td>
<td>X</td>
<td></td>
<td>Myricetin</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td><em>p</em>-coumaric acid</td>
<td>36</td>
</tr>
<tr>
<td>51</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>Myricetin</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td>Ethyl gallate</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>Methyl ellagic acid</td>
<td>38</td>
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<tr>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td>Malyngic acid</td>
<td>39</td>
</tr>
<tr>
<td>81</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>Mearnsetin-3-O-β-rhamnoside</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td>Quercetin</td>
<td>41</td>
</tr>
<tr>
<td>83</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>Mearnsetin-3-O-β-rhamnoside</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td>Gallic acid</td>
<td>35</td>
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<td></td>
<td>X</td>
<td></td>
<td></td>
<td>Quercetin 3-O-β-rhamnoside</td>
<td>42</td>
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<tr>
<td>87</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>Mearnsetin-3-O-β-rhamnoside</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>Gallic acid</td>
<td>-</td>
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<tr>
<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>Dimethyl tartrate</td>
<td>38</td>
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<tr>
<td>89</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>Gallic acid</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>Tartaric acid</td>
<td>43</td>
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<tr>
<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>Dimethyl tartrate</td>
<td>-</td>
</tr>
</tbody>
</table>

Among the twelve identified compounds, mearnsetin, mearnsetin-3-O-β-rhamnoside, myricetin, *p*-coumaric acid, quercetin, gallic acid, quercetin 3-O-β-rhamnoside were previously reported in *S. malaccense*. However, was the first time that ethyl gallate, ethyl ellagic acid, malyngic acid were described in the species.

### 3. Conclusion

The present study showed that the HSCCC is a powerful tool for the separation of compounds. The three-phase solvent system in stepwise elution mode approach was successful when dealing with complex mixtures. *S. malaccense* leaves extract was used as a practical example on how to choose the appropriate system. TPSSs were analyzed according to volume phase ratio, sample solubility in the system, compounds partition between the phases and stationary phase retention on CCC equipment. Using the TPSS *n*-HEX - EtOAc - ACN - H₂O at volume ratio of 2:1:1:1 (v/v) it was possible to fractionate *S. malaccense* leaves extract, having a good compound distribution along the separation. Twelve compounds could be concentrated, purified and identified by NMR analysis, evidencing its

Figure 8. Chemical structures of compounds 1-12
chemical complexity and large polarity range. From those, ethyl gallate, ethyl ellagic acid, malyngic acid were reported for the first time in *S. malaccense*.

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