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Comparative LC–MS-based metabolite profiling of the ancient tropical rainforest tree *Symphonia globulifera*

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ABSTRACT

In the last few years, several phytochemical studies have been undertaken on the tropical tree *Symphonia globulifera* leading to the isolation and characterisation of several compounds exhibiting antiparasitic activities against *Plasmodium falciparum*, *Trypanosoma brucei* and *Leishmania donovani*. The comparative LC–MS based metabolite profiling study conducted on the tree led to the identification of compounds originating from specific tissues. The results showed that renewable organs/tissues can be used as the starting material for the production of polycyclic poly-prenylated-acylphloroglucinols, therefore reducing impacts on biodiversity. This study also underlined the lack of knowledge on the secondary metabolites produced by *S. globulifera* since only a small number of the total detected features were putatively identified using the database of known compounds for the species.

1. Introduction

During the last few years, an increasing number of phytochemical studies have been undertaken on the tropical tree *Symphonia globulifera*, illustrating the growing interest of the scientific community for this species (Lenta et al., 2007; Marti et al., 2010; Ngouela et al., 2006). *S. globulifera* L. f. (Clusiaceae) is usually found within humid mixed species forests and freshwater swamp forests of America and Africa (Budde et al., 2013). *S. globulifera* are typically large canopy trees and are morphologically distinctive with large aerial roots and a bright yellow latex. Of the 17 accepted species of Symphonia, it is the only one that is recognised in its genus and is found outside of Madagascar (Dick et al., 2003; Newman and Cragg, 2012). It is considered of pharmaceutical interest as it produces several secondary metabolites exhibiting multiple antiparasitic activities (Lenta et al., 2007; Ngouela et al., 2005). Since the first study by Locksley et al. in 1966, ca. 40 compounds have been isolated from *S. globulifera* (Locksley et al., 1966a). Although these compounds predominantly fall within two important families (polycyclic poly-prenylated-acylphloroglucinols (PPAPs) and xanthones), some biflavonoids have also been described (Li et al., 2002; Mkounga et al., 2009).

Considering the importance and size of the tree, only few organs have been previously studied. Locksley reported the isolation of xanthones from the heartwood (Locksley et al., 1966a,b), and although PPAPs are present in all of the different tissues, they were first isolated from the roots bark (Gustafson et al., 1992; Marti et al., 2010; Nkengfack et al., 2002). The seeds have also been studied unravelling the presence of guttiferone A (**1**) together with gaboxanthone (**2**), globuliferin (**3**) and symphonin (**4**) (Ngouela et al., 2005). Finally, morelloflavone (5), GB2 (**6**) and manniflavanone GB3 (**7**) were isolated from leaves and twigs (Li et al., 2002; Mkounga et al., 2009). Despite all of these previously conducted studies, there is still a considerable gap in the knowledge as some of the renewable organs/tissues secretions (e.g. latex, pericarps or flowers), have not yet been studied. The broad spectrum of activities of PPAPs has also been highlighted in the recent article describing the total synthesis of guttiferone A (**1**) (Horeischi et al., 2014), however this elegant synthesis involves a total of 13 steps, precluding any further chemical transformations that are required to improve the products biological activities (both sensitivity and specificity).

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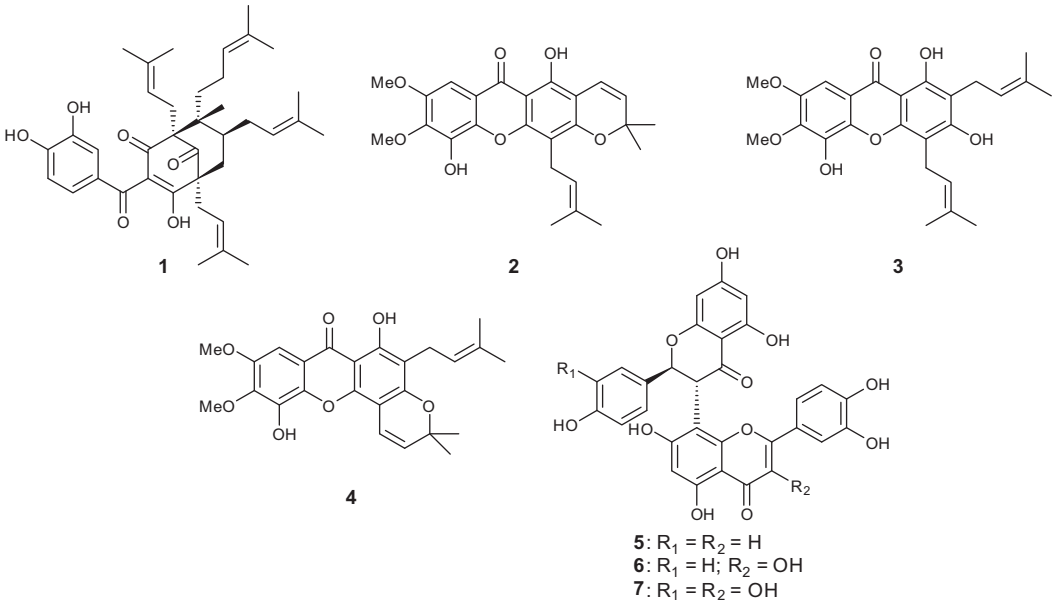

The growth of interest in *S. globulifera* secondary metabolites lead us to take advantage of the analytical techniques developed over the last decade in order to explore the metabolome of the different tissues of the tree. The recent progress in plant metabolomics have made possible the detection of thousands of compounds (Bertrand et al., 2014; Kim and Verpoorte, 2010; Wolfender et al., 2013). The large range of metabolite features that are detected by these analyses enables the comparison of samples for similarities and differences in composition. Ultra performance liquid chromatography coupled to high resolution mass spectrometer (UPLC-HRMS) has become a method of choice with regard to its sensitivity, accuracy and rapid separation of metabolites (Eugster et al., 2011; Tierney et al., 2013).

In the present study we applied a complete metabolomics work-flow on *S. globulifera* with the aim of highlighting the differences and/or similarities in the metabolite composition of methanolic extracts taken from the leaves, roots, latex, various barks, seeds, pericarps and flowers, in order to identify which tissues can be used as eco-compatible sources for the production of PPAPs derivatives.

2. Material and methods

2.1. Plant material

The leaves, roots, latex, bark, seeds, pericarps and flowers of *S. globulifera* L. f. were sampled in French Guiana (Macouria, 4.91874,-52.366624) and formally identified by Dr. G. Odonne. All of the samples were collected on an individual day of November 2012. A voucher (No. ODONNE 770) was deposited at the French Guiana herbarium (IRD).

2.2. Samples preparation

Freshly harvested plant tissues have been dried at room temperature and crushed. All extracts were prepared following the same protocol: 10 g of biological material was crushed into small parts and extracted (2×100 mL) with freshly distilled methanol (Carlo erba) for 48 h at room temperature. After rotary evaporation under reduced pressure, the crude extracts were fractionated using a bi-phasic system composed of freshly distilled ethyl acetate and water. The organic phases were dried over magnesium sulphate before being evaporated. All biological samples were reconstituted in methanol (MeOH) to obtain a final concentration of 0.5 mg mL^{-1}. Quality controls samples (QC's) were constituted by pooling 50 µL of each of the prepared samples. After preparation, the samples were stored at 4 °C until analysis.

2.3. LC–MS data acquisition

Metabolomics analysis was performed using an Acquity UPLC system coupled to a Synapt G2-HDMS mass spectrometer system (Waters Corp., MA, USA). For the chromatographic separation a HSS C18 reversed-phase column (1.7 µm, 2.1 mm 100 mm; Waters Corp., MA, USA) was used with a gradient of water/methanol/formic acid (from 95:5:0.1 to 0:100:0.1 in 6 min). The mass spectral data were acquired in electrospray in both positive and negative mode. The capillary voltage, source cone voltage, and extraction cone voltage were maintained at 3.25 kV, 30 V, and 4 V, respectively for positive mode and at −2.5 kV, −40 V, and 4 V, respectively for negative mode. Nitrogen was applied as the desolvation gas at a flow rate of 900 L h^{-1}. The source and desolvation temperatures were maintained at 120 °C and 550 °C, respectively. Mass spectra were acquired over the m/z range of 100–1200 at a mass resolution of 22 000 FWHM (full width half at maximum). Leucine enkephalin (m/z 555.26930) was used as lock mass every 20 s during each run. Each sample was analysed in triplicate and one QC sample was injected every three biological samples. Three QC's were injected before and after all samples.

2.4. Data processing

LC–MS chromatograms were aligned using the R package XCMS with the following parameters: a signal-to-noise ratio threshold of 3:1 for peak selection, a step size of 0.2 min and a minimum difference in m/z for peaks with overlapping retention times of 0.05 Thomsons (Th). Annotation of the peak list generated by XCMS (Benton et al., 2010; Tautenhahn et al., 2008) was performed using the R package CAMERA (Kuhl et al., 2011). After annotation a matrix of peak intensities for each detected feature was created

and imported into Matlab 2013a Mathworks for the next processing steps. Sample filtering requiring a peak to be present in at least 80% of the samples to be considered as an informative peak (Kirwan et al., 2013). Missing values imputation was realised using KNN algorithm (Hrydziuszko and Viant, 2012). The spectral signal intensities were next normalised to a reference profile, in order to reduce variance arising from differing dilutions of the biological extracts, the probabilistic quotient normalisation (PQN) method was applied (Dieterle et al., 2006).

2.5. Statistical analysis

The normalised data were mean centred and principal component analysis (PCA) was conducted to search for similarities between samples (PLS-Toolbox, version 6.7, Eigenvector Research). Differences between the groupings observed within PCA scores plots were further investigated using one-way ANOVA and Tukey's post hoc test. Multiple fold change were calculated using each of the seven tissues as control groups alternatively. After calculation of the mean fold change, the top 200 features of each group ($p > 0.001$) were selected and putatively annotated using our database of compounds isolated from *S. globulifera*.

3. Results and discussion

To investigate the metabolites composition of the different organs and secretions of *S. globulifera*, we conducted an untargeted metabolomics study using LC–MS. Unsupervised multivariate statistics were performed to see if any differences in the metabolomes of the organs and secretions were observable. After conversion of the .raw Waters data files into .mzML files, chromatograms were aligned using XCMS (Benton et al., 2010; Smith et al., 2006; Tautenhahn et al., 2008), and a peak list was generated using the R package CAMERA (Kuhl et al., 2011). After data processing (detailed in materials and methods), the matrix containing peak intensities of each detected feature for each sample was analysed by principal component analysis (PCA). After verification that no important technical error was introduced during the data acquisition, QC's well clustered on the PCA (Supplementary information), QC's were excluded from the sample set for the statistical analyses. As shown in Fig. 1, all of the organs and secretions can be very well clustered in both ionisation modes although a better separation on PC1 is observed for the negative mode (Fig. 1b). Inspection of the PCA output illustrated that the largest differences were observed between leaf and flower extracts along PC1, whilst other extracts (barks, latex and roots) were grouped closely together. The Pericarp and seed extracts appeared to be fairly distinct from the other samples as a nice separation was observed on both PC1 and PC2. In both ionisation modes, the LC–MS profiles seemed to be very different for each of the extracted organs. To better understand the differences observed by PCA, analysis of the variance (ANOVA) on each detected feature was performed followed by Tukey–Kramer post hoc analysis and the Benjamini-Hochberg correction (applied at a false discovery rate of 5%).

A common approach to identifying metabolic features of importance within a dataset is to rank the significantly different peaks according to their fold change between sample groups. As fold change can be considered as the magnitude of change between two groups (Vinaixa et al., 2012), we calculated the fold change of all possible permutations using each of the seven sample groups as a control. We then calculated the mean of the fold change and ranked them. The features with the most important mean fold change (top 200) were maintained for further metabolite characterisation (Sumner et al., 2007).Compound annotation was

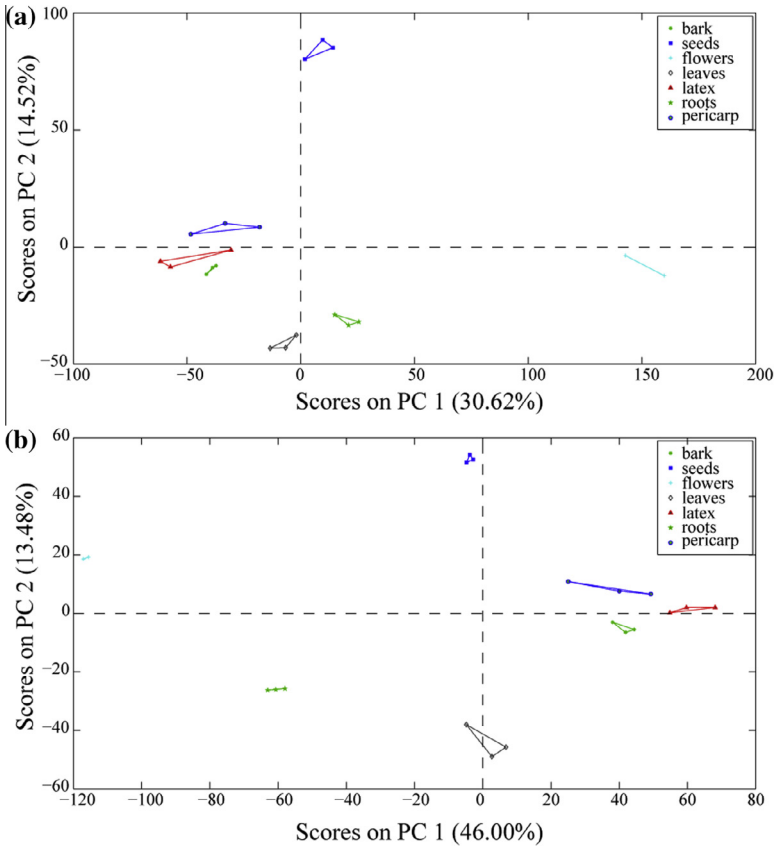

Fig. 1. Sample patterns of the extracts of organs and secretions of *S. globulifera* analysed by LC–MS. (a) PC1 occupies 31% and PC2 14% of total variance in positive mode. (b) PC1 occupies 46% and PC2 13% of total variance in negative mode.

Table 1
Annotated compounds in the dataset after comparison with a library of the isolated compounds from *S. globulifera* (putative identification of level 2 according to the Metabolomics Standard Initiative (Sumner et al., 2007)).

Observed mass (uma)	Empirical formula	Exact mass (uma)	*m*/*z* diff (ppm)	Putative compound(s)
228.0514	$C_{13}H_8O_4$	228.04226	4.0	1,7-Dihydroxyxanthone
244.0478	$C_{13}H_8O_5$	244.03717	3.5	1,5,6-Trihydroxyxanthone; gentisein
260.0421	$C_{13}H_8O_6$	260.03209	3.8	1,3,5,6-Tetrahydroxyxanthone; norathyriol
262.0587	$C_{13}H_{10}O_6$	262.04774	4.2	maclurine
312.1109	$C_{18}H_{16}O_5$	312.09977	3.6	globuxanthone; mbarraxanthone
326.0938	$C_{18}H_{14}O_6$	326.07904	4.5	globulixanthone C
328.1100	$C_{18}H_{16}O_6$	328.09469	4.6	ugaxanthone; symphoxanthone
342.1127	$C_{19}H_{18}O_6$	342.11034	3.6	globulixanthone E
394.1583	$C_{23}H_{22}O_6$	394.14164	4.7	xanthone V1
556.1245	$C_{30}H_{20}O_{11}$	556.10056	4.3	morelloflavone
574.1393	$C_{30}H_{22}O_{12}$	574.11113	3.3	GB-2
586.3935	$C_{38}H_{50}O_5$	586.36582	4.7	14-Deoxy-7-*epi*-isogarcinol
600.3733	$C_{38}H_{48}O_6$	600.34509	4.7	symphonone H; symphonone I; symphonone A
602.3868	$C_{38}H_{50}O_6$	602.36074	4.6	guttiferone A; 7-*epi*-garcinol
618.1628	$C_{37}H_{30}O_9$	618.18898	4.3	globulixanthone F; symphonone C; 26-Hydroxy-isoxanthochymol
618.3827	$C_{38}H_{50}O_7$	618.3556	4.8	symphonone F; 36-Hydroxy-isoxanthochymol; 18-*epi*-symphonone F; symphonone G
636.3923	$C_{38}H_{52}O_8$	636.36622	4.1	35,36-Dihydroxy-isoxanthochymol;symphonone D; symphonone E
670.4538	$C_{43}H_{58}O_6$	670.42334	4.5	guttiferone B; guttiferone C; symphonone B; guttiferone D

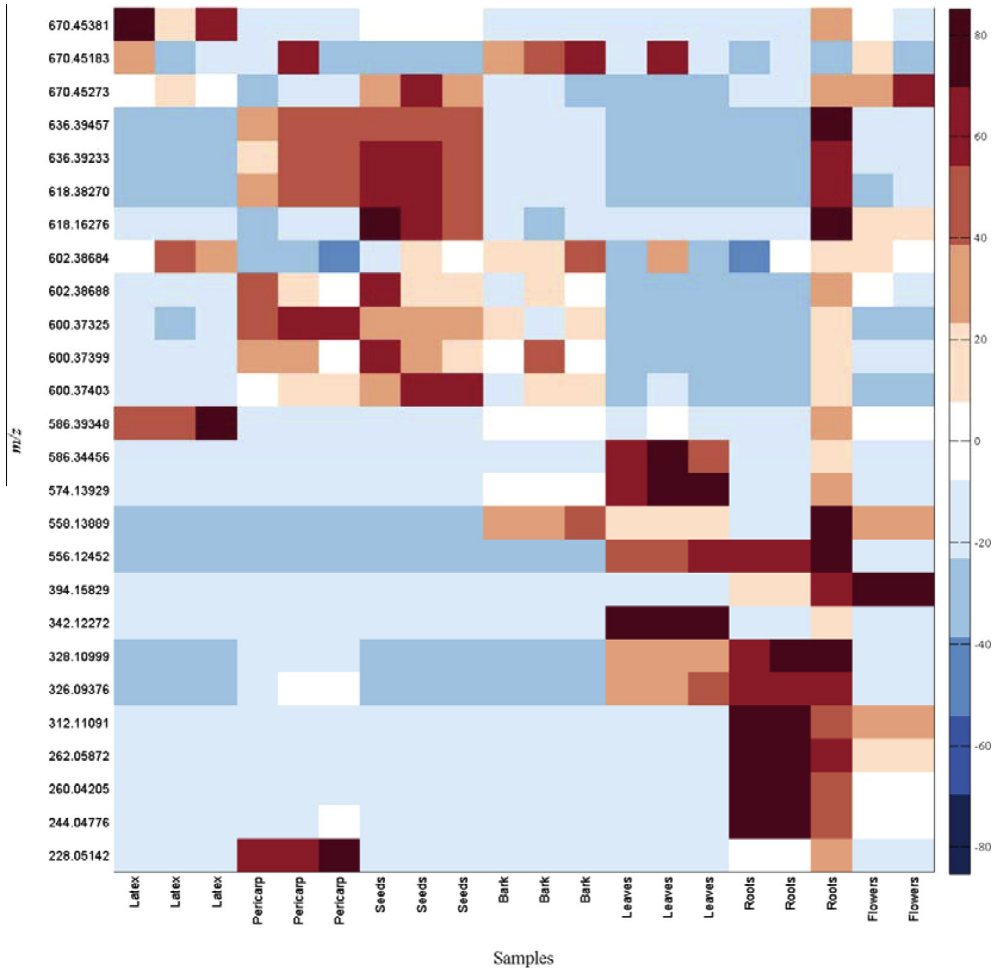

Fig. 2. Heatmap representation of the relative concentration of the compounds present in the extract (ESI-(−)).(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

performed by database searching focused on compounds that have only been isolated from *S. globulifera* (Gustafson et al., 1992; Ngouela et al., 2006; Ngouela et al., 2005). Metabolite searches were based upon accurate mass comparisons using a tolerance of 5 ppm, following removal of duplicates, 27 compounds belonging to three different families of chemicals: xanthones (with *m*/*z* ranging from 228 to 618), biflavonoids (with *m*/*z* 556–590) and PPAPs with *m*/*z* values above 586 where listed as putative chemomarkers (Table 1).

To have a better visualisation of the distribution of the annotated metabolites in the samples, a heatmap representation was used (relative quantification of each compound is available in the

Supporting information). As shown in Fig. 2, several compounds are present in very high concentration specifically in one or two organs or secretions (dark red squares), whilst others are not detected in specific tissues (dark blue squares).

As depicted in Fig. 2, the latex extract does not contain compounds with *m*/*z* values corresponding to those observed for xanthones (Table 1). On the other hand, three compounds seem to be present at high levels. After a search of their observed *m*/*z* at 586.3935, 602.3868 and 670.4538, only compounds belonging to the PPAPs family were annotated. Characterisation of the relative isotopic abundance (RIA) as proposed by Weber et al. (2011) for these *m*/*z* led to relatively high differences in the theoretical number of carbon atoms (C_{diff}) as we obtained a C_{diff} = 3.06, 3.67 and 8.86 for *m*/*z* 586.3935, 602.3868, 670.4538 respectively. These differences can be explained by the fact that RIA have been calculated based upon a single scan, whereas averaging of the intensities across multiple scans could improve the estimation. From these three compounds, one seems to be present in *t* high levels in the latex extracts compared to the other plant compartments. This compound had a *m*/*z* of 586.3935 (Δ 4.7 ppm), after a database search two isomers, 14-deoxy-7-*epi*-isoxanthochymol or the 14-deoxy-7-*epi*-garcinol (Gustafson et al., 1992), were proposed as putative identifications. Unexpectedly, guttiferone A was also identified as a major compound of the latex extract (*m*/*z* 602.3868, Δ 4.6 ppm). One last feature at *m*/*z* 670.4538 was also quite abundant in the latex extract. This compound was putatively identified as one of the guttiferone B (Gustafson et al., 1992) isomers (Δ 4.5 ppm), ie. guttiferone C, guttiferone D or symphonone B (Marti et al., 2010). Further observations of Fig. 2 focussing on the seed extracts indicated that none of the described xanthones (Table 1) are present as major components. Observing the higher masses, it was noticeable that several compounds were present in significant quantities. These compounds with a *m*/*z* range of 600–636 were first annotated as PPAPs. A close look at the mass defect (MD) of the different compounds led us to the conclusion that one of the listed compounds did not belong to the same PPAPs family (Sleno, 2012). The MD can be very useful as it should show a linear increase when adding multiples of the same chemical group or function (e.g. methylenes, hydroxyls, etc.). A difference in the MD for a given mass implies a difference in the elemental constitution and often a change in the chemical class of natural products (Wang et al., 2014). All the PPAPs have indeed a MD > 0.3 while this is not the case for the compound at *m*/*z* 618.1628 (Fig. 3).

A database search using 5 ppm tolerance gave only one output, identifying the compound as a xanthone called globulixanthone F (Mkounga et al., 2009). This compound was of further interest since it was one of the most abundant peaks in the seed extract, whilst it was hardly detected in the other extracts (Fig. 3). Other compounds were also fairly abundant in the seed extract at *m*/*z* 618.3827and 636.3923. Looking at both accurate masses and mass defects, they were identified as a PPAPs compound having an empirical composition of $C_{38}H_{50}O_7$ (Δ 4.8 ppm). As detailed in Table 1, several compounds could be associated to this empirical formula, e.g. symphonone C, symphonone F or symphonone G (Marti et al., 2010). Another three compounds with a lower mass were observed at *m*/*z* 600.3733 and 600.3740. The three outputs given by the database query fitted very well with our expectations, and we could conclude that all the previously described isomers of symphonone A (Δ 4.7 ppm), ie. symphonone H and symphonone I were present in the seeds extract (Marti et al., 2010). The pericarp extracts had a very similar profile to those obtained from the seeds, as only one low mass compound and the absence of globulixanthone E aided the discrimination of both extracts (Fig. 2). The compound at *m*/*z* 228.0514 was putatively identified as 1,7-dihydroxyxanthone (Δ 4 ppm) (Locksley et al., 1966b). This compound was of further interest as it could not be found in the other extracts.

The study of the bark extracts revealed that none of the previously putatively identified compounds are present in a very high amount, only one isomer of guttiferone B at *m*/*z* 670.4518 (Δ 4 ppm) seemed to be relatively abundant. Looking at the distribution of the compounds relative amounts, the composition of the remaining extracts was significantly different, explaining the clear separation observed in the PCA plot along PC1. Looking at the roots extracts, three compounds at *m*/*z* 342.1227, 574.1393 and 586.3446 appeared to be quite abundant. The later accurate mass was already observed in the latex extracts and putatively identified as two isomers 14-deoxy-7-*epi*-isoxanthochymol or the 14-deoxy-7-*epi*-garcinol. As the matching ion observed in the latex extracts presented a different retention time, we could conclude that each of these two isomers were present in only one of the two extracts (latex or pericarp).

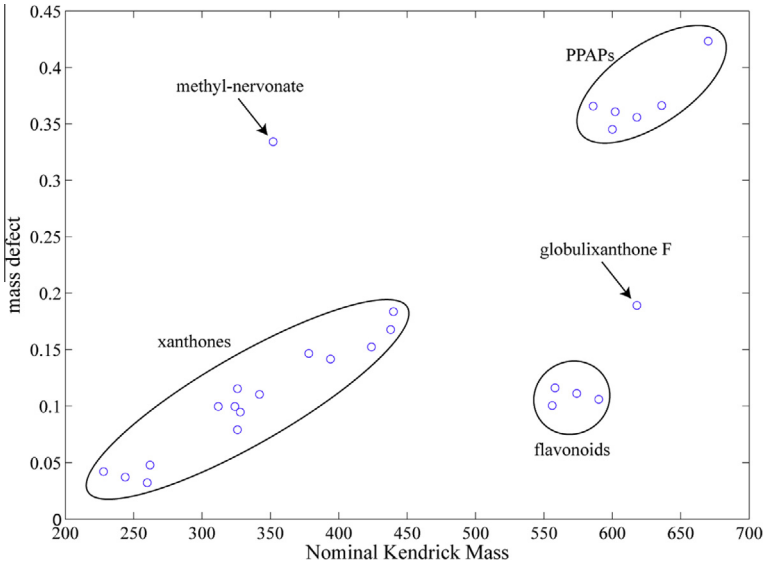

Fig. 3. Kendrick mass defect of all reported compounds from *S. globulifera*.

As discussed above, the mass defect of the compound at *m/z* 574.1393 did not present the characteristics of the PPAPs family (Fig. 3) and querying the database, only one name could be attributed to this accurate mass: GB-2 (Δ 3.3 ppm) (Li et al., 2002; Mkounga et al., 2009). A compound with an accurate mass of 342.1227, that was only otherwise detected in the pericarp extract, fitted with only one database query: globulixanhtone E (Δ 3.6 ppm) (Nkengfack et al., 2002).

The root extracts were undoubtedly the richest in low molecular mass compounds. Six compounds with an accurate mass ranging from 244.0478 to 328.1100 were indeed fairly abundant in these extracts. Looking at both the compounds in the database with a tolerance of 5 ppm and the mass defect, we concluded that they belonged to the xanthones family. Only one putative identification could be proposed for each of the compounds at *m/z* 262.0588 and 326.0938, maclurine (Δ 4.2 ppm) and globulixanthone C (Δ 4.5 ppm) respectively (Nkengfack et al., 2002). The annotation became more complex for the other four compounds since multiple isomers were present in the database for each *m/z* value. Even for the lowest mass of *m/z* 244.0478, two isomers had been described, gentisein and its isomer 1,5,6-trihydroxyxanthone. The next compound had an accurate mass of 260.0421, making a difference of 16 *m/z* with the previous compounds. This difference could be attributed to the addition of a hydroxyl moiety on a trihydroxyxanthone already found in the plant. This hypothesis was confirmed by the database query, as the output consisted of two tetrahydroxyxanthones (Δ 3.8 ppm): norathyriol and 1,3,5,6-tetrahydroxyxanthone. The next accurate mass at *m/z* 312.1109 also gave two outputs after query of the database and the following xanthones (Δ 3.6 ppm) were proposed as putative structures: globuxanthone (Locksley et al., 1966a), mbarraxanthone (Locksley et al., 1966b). The last important feature observed in the root extracts had an accurate mass of 328.1100, only two compounds where obtained after querying the database with a tolerance of 5 ppm, namely: symphoxanthone (Locksley et al., 1966a) and ugaxanthone (Locksley et al., 1966b). Although we did not identify outliers in the PCA plots, the heatmap in Fig. 2 clearly illustrates that the third replicate extract of the root tissues differs in metabolite concentrations, especially for the higher masses. Despite these differences, the clustering was not affected as all three roots samples lie in the same area on the plots in Fig. 2.

The last section of the heatmap represents the relative concentrations of the flower extracts. Only two extracts are plotted as a problem occurred during the data acquisition. As illustrated in Fig. 2, only few compounds are present in significant concentrations in these extracts. The most significant, with at *m/z* 394.1583 is located in the xanthones region of Fig. 2. Query of the database gave only a single entry: xanthone V1 (Δ 4.7 ppm). A second compound present at a relatively high concentration had an accurate mass previously observed within the latex and bark extracts of 670.4527. As discussed above, this mass can be attributed to one of the three guttiferone isomer i.e. guttiferone B, C and D).

4. Conclusion

LC–MS has been demonstrated to be a method of choice for the study of methanolic extracts of the rainforest tree *S. globulifera*. The results obtained during this study demonstrated that the metabolome of the different organs, tissues, and secretions of the tree are rather different. PPAPs were mainly found in latex, pericarps, seeds and barks while xanthones and flavonoids were observed in the roots, leaves and flowers. The results showed that renewable organs/tissues can be used as starting material for the production of PPAPs, therefore reducing the impact of their agriculture on biodiversity. This study also underlined the lack of knowledge of the secondary metabolites produced by *S. globulifera* since only a small number of total features detected were putatively identified using the database of known compounds in the species.

This study also highlighted the applicability of the Kendrick mass defect in the field of natural products chemistry. It is indeed very easy to map the metabolites using this approach and it could potentially be a powerful tool for future chemotaxonomic studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2014.09.009.

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