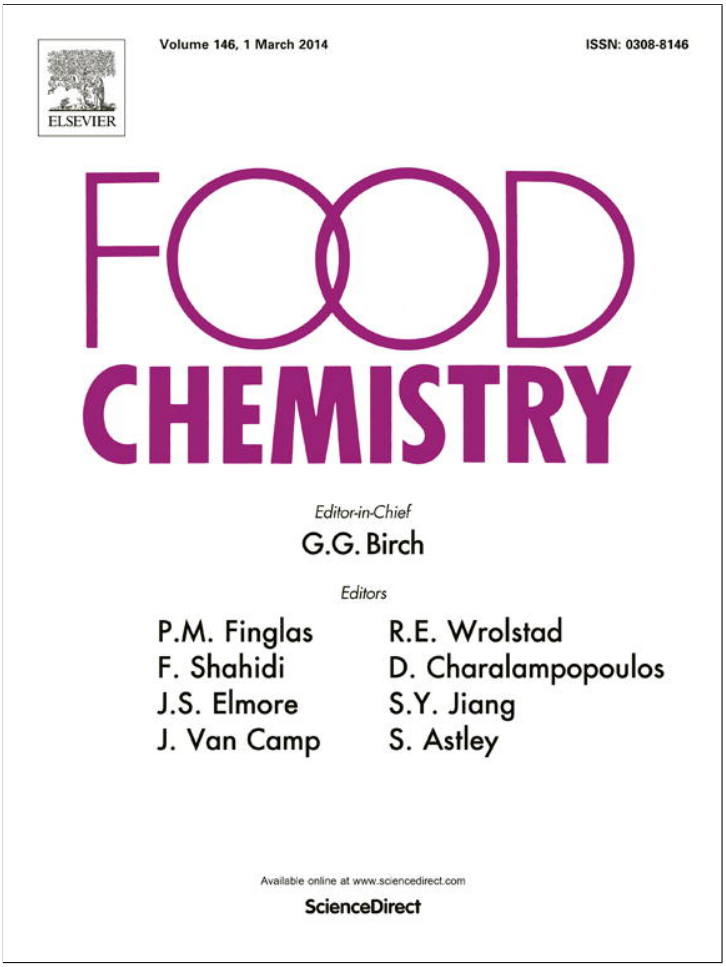

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Phytochemical markers of different types of red propolis

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ABSTRACT

Propolis is a resin that bees collect from different plant sources and use in the defense of the bee community. The intricate composition of propolis varies depending on plant sources from different geographic regions and many types have been reported. Red coloured propolis found in several states in Brazil and in other countries has known antimicrobial and antioxidant activity. Different analytical methods have been applied to studies regarding the chemical composition and plant origins of red propolis. In this study samples of red propolis from different regions have been characterised using direct infusion electrospray ionisation mass spectrometry (ESI(-)-MS) fingerprinting. Data from the fingerprints was extracted and analysed by multivariate analysis to group the samples according to their composition and marker compounds. Despite similar colour, the red coloured propolis samples were divided into three groups due to contrasting chemical composition, confirming the need to properly characterise the chemical composition of propolis.

1. Introduction

Propolis is a resin that bees collect from different plant sources and uses in the defense of the bee community by coating and strengthening the inside walls of the hive. It is also used to cover holes and cracks and to repair combs (Bankova, de Castro, & Marcucci, 2000), avoid insect invasions (Gonnet, 1968) and to reduce the microbial growth on the walls of the hive, preventing wind and water from entering (Simone-Finstrom & Spivak, 2010). Brazil is well known for its green propolis, produced by *Apis mellifera* (honeybees), which derives its resins mainly from the native shrub *Baccharis dracunculifolia* (Bankova et al., 1999; Banskota et al., 1998). However, due to the great biodiversity of Brazil, propolis from different geographic regions can vary profoundly in composition and several types of propolis are found in Brazil, including the less common red propolis. (Marcucci, Sawaya, Custódio, Paulino, & Eberlin, 2008; Sawaya et al., 2004).

The existence of a red coloured propolis has been reported in several countries: the northeastern coast of Brazil (states of Alagoas, Bahia, Paraiba, Sergipe and Pernambuco) (Daugsch, Moraes, Fort, & Park, 2008); Cuba (Pinar Del Rio) (Piccinelli et al., 2011); Venezuela (Trusheva et al., 2004); México (Champoton) (Lotti, Campo Fernandez, Piccinelli, & Marquez Hernandez, 2010) and China (Izuta et al., 2009). Studies regarding the plant sources of red propolis are very recent, dating back to 2008 and only for red propolis from Northeastern states of Brazil,(Daugsch et al., 2008; Silva et al., 2008) followed in 2011 by studies of Cuban red propolis (Piccinelli et al., 2011). Determining the botanical origin of propolis is not a simple task and two approaches have been applied: the comparative chemical composition and palynological analysis (López & Sawaya, 2012). The widespread geographical origin of red propolis indicates that the botanical origin of red propolis in diverse countries is different since weather and flora are specific for each region. Four species have been suggested to be the plant origin of red propolis: *Dalbergia ecastophyllum* for Brazilian red propolis and *Clusia scrobiculata*, *Clusia minor*, *Clusia major* for red propolis from Venezuela (Tomás-Barberán, García-Viguera, Vit-Olivier, Ferreres, & Tomás-Lorente, 1993; Trusheva et al., 2004, 2006), and *Clusia rosea* for propolis from Cuba (Cuesta-Rubio, Frontana-Uribe, Ramírez-Apan, & Cárdenas, 2002) .

Red propolis is biologically active, having antimicrobial and antioxidant activity (Cabral et al., 2009), among others. Due to these properties, propolis can be used as a natural food preservative (Tosi, Ré, Ortega, & Cazzoli, 2007). Furthermore, its use as a food additive was evaluated by the oral administration of propolis to ewes, resulting in an improvement of their biochemical parameters (Morsy et al., 2013). The first studies about red propolis date back to 1996 relating to the anti-psoriatic, anti-inflammatory and

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analgesic effects of Cuban red propolis (Ledón et al., 1996; Rodriguez et al., 1997). However it wasn't until 2004 that the chemical composition of red propolis began to be investigated, in samples from Venezuela (Trusheva et al., 2004). Since 2007 further studies of red propolis show that the determination of its chemical composition is a necessary complement to studies of its varied biological activity, and to boost its promising potential in the food industry (Alencar et al., 2007; Awale et al., 2008; Barth & Pinto da Luz, 2009; Cabral et al., 2009; Cuesta-Rubio et al., 2007; Daugsch et al., 2008; Fernandez et al., 2008; Li, Awale, Tezuka, & Kadota, 2008; Oldoni et al., 2011; Piccinelli et al., 2011; Righi et al., 2011; Silva et al., 2008). Most studies have analysed samples from one specific geographical origin, and these studies used diverse analytical methods, so qualitative and quantitative variations in composition have been observed even between samples from the same region (López & Sawaya, 2012). It is difficult to ascertain if these differences are the result of the varied analytical methods used, or truly represent differences in the composition of the samples.

Direct infusion ESI(-)-MS fingerprinting has been successfully and widely applied to the characterisation of propolis. For instance, it has been applied to characterise *A. mellifera* propolis samples from different countries (Sawaya et al., 2004) and to native stingless bee propolis from different regions in Brazil (Sawaya et al., 2007). It has also been used to compare plant resins with propolis samples to confirm their plant origins (Marcucci et al., 2008; Sawaya, Cunha, Marcucci, de Oliveira Rodrigues, & Eberlin, 2006). Therefore in this study, samples of red propolis from different regions have been compared and characterised using direct infusion negative ion mode electrospray ionisation mass spectrometry (ESI(-)-MS) fingerprinting and data was treated by multivariate analysis. MS data were acquired using a high resolution and accuracy Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR–MS) in order to obtain the molecular formulas of the marker compounds. Further identification was performed via ultra-high-efficiency liquid chromatography (UPLC) coupled to with mass spectrometry detection and fragmentation (MS/MS).

2. Materials and methods

2.1. Red propolis samples

Red coloured propolis was collected in hives by different beekeepers in the following Brazilian states: Sergipe (samples 1, 2, 5 and 6); Alagoas (samples 3 and 8); Paraíba (samples 4 and 7) and Roraima (samples 10–14). The sample of *Dalbergia eccastophyllum* (sample D) was collected in João Pessoa, state of Paraiba, and a sample of red propolis from Cuba (sample 9) was kindly donated by Dr. Osmany Cuesta-Rubio.

2.2. Extraction of propolis

Red propolis samples were extracted following the procedure described by Sawaya et al. (2004). The samples were dissolved in absolute ethanol, were shaken for 7 days under controlled speed and temperature and then filtered. The samples were kept in the freezer overnight (temperature of −16 °C) and then filtered again to remove wax. Solvent was then evaporated on a water bath at a temperature of 50 °C under a flow of nitrogen to obtain dry extracts of propolis.

2.3. High resolution and accuracy ESI(-)-FT-ICR–MS fingerprints

Mass spectra fingerprinting and MS/MS spectra were acquired using a 7.2T LTQ FT Ultra mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with a chip-based direct infusion nanoelectrospray ionisation source (Advion BioSciences, Ithaca, NY, USA) operating in the negative ion mode at the follow conditions: chip voltage 1.5 kV,gas pressure of 0.3 psi, tube lens −160 V, temperature 270 °C and fragmentation energy 15–40 eV. Data acquisition was performed along the 150–800 *m*/*z* range by the Xcalibur 2.0 software.

2.4. UPLC-ESI(-)-MS/MS

The chromatographic analyses of the ethanolic solutions of the dried propolis extracts (1 mg/mL) were performed on a UPLC Acquity chromatographer coupled with a TQD Acquity mass spectrometer (Micromass-Waters Manchester, England), with an ESI source. A C18 BEH Waters Acquity column (2.1 mm × 50 mm × 1.7 μm particle size) was used. Solvent A was mili-Q purified water with 0.1% formic acid and solvent B was methanol. The flow rate was 0.2 mL/min and 5 μL of samples were injected; with a linear gradient starting at 40% methanol and increasing to up 100% methanol in 9 min, held until 11 min and then returning to the initial conditions, followed by column re-equilibration. ESI ionisation in the negative ion mode was used under the following conditions: Capillary −3.00 kV, Cone −30 V, Source Temperature 150 °C, Desolvation Temperature 350 °C and Collision Energy 30 V, acquiring data between 100 and 800 *m*/*z*.

2.5. Multivariate data analysis

The analysis of the ESI(-)-MS fingerprints was done using the The Unscrambler 10.2 software. Principal Component Analysis (PCA) was performed on previously normalised and mean centred data. All ions with more than 10 % intensity in the fingerprints were included.

3. Results and discussion

As already mentioned, samples of red propolis from Brazil, Cuba, México and Venezuela have been studied and various classes of substances have been identified in their composition, such as: isoflavonoids, pterocarpans, chalcones, flavonoids, prenylated benzophenones and terpenes (Alencar et al., 2007; Awale et al., 2008; Cabral et al., 2009; Cuesta-Rubio et al., 2007; Daugsch et al., 2008; Fernandez et al., 2008; Lotti et al., 2010; Nunes et al., 2009; Oldoni et al., 2011; Piccinelli et al., 2011; Righi et al., 2011; Silva et al., 2008; Trusheva et al., 2004, 2006) among others.

Based on this composition, ethanolic extracts of the 14 samples of red coloured propolis from different regions of Brazil and Cuba were analysed by high resolution and accuracy ESI(-)-FT-ICR–MS in the negative ion mode. The data regarding the *m*/*z* values and abundances for the ions detected in the MS fingerprints were compared using a classical multivariate analysis method, Principal Component Analysis (PCA), and classified in groups based on the common ions in the fingerprints (Fig. 1a). PC1 × PC2 was able to represent 92% of the variation. Note that all the samples from Roraima were clearly placed in the same group since their composition was very unique and clearly different from the other samples of red propolis. The major, very abundant marker ion for the Roraima group was that of *m*/*z* 501.30, which was absent in the fingerprints of all other Brazilian propolis samples (Fig. 2a). Propolis from Roraima was not as intensely red as the other propolis samples. Therefore, Roraima samples were excluded from the following steps of this study, since they were considered not truly samples of red propolis, but represent a new type of Brazilian propolis. The remaining samples were divided into 2 groups. Group A included samples **3, 4, 7** and **8** from the states of Alagoas and Paraiba, whose

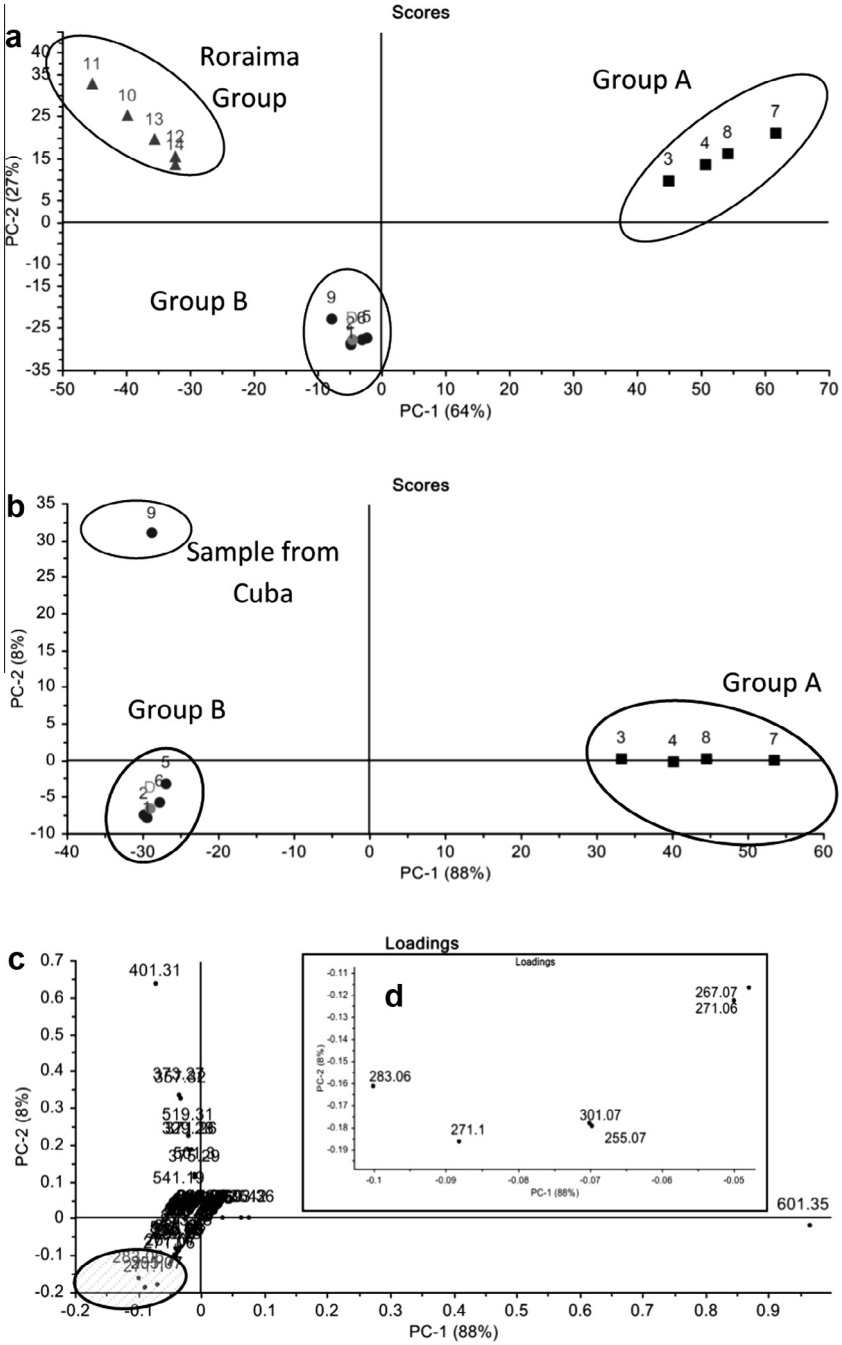

Fig. 1. (a) PCA scores including all red propolis samples and *D. ecastophyllum*; (b) PCA scores (samples); (c) PCA loadings (ions) after excluding the samples from Roraima and (d) Zoom of the markers of Group B, highlighted in Fig. 1c.

marker ion of *m*/*z* 601.35 (C38H49O6) was also very abundant. Group B contained Brazilian red propolis samples **1, 2, 5, and 6,** the Cuban sample (**9**) and the extract of *D. ecastophyllum* (sample **D**), considering that this plant contributes with resin for Group B. The spectra of Group B was much more diverse but also characteristic and its marker ions will be discussed further on.

After removing the Roraima samples from the study, only slight changes were observed in the disposition of the groups (Fig. 1b) and PC1 × PC2 was now able to represent as much as 96% of the variation. Group A contained the same samples, whereas the Cuban sample (**9**) was set apart from B due mainly to the abundant marker ion of *m*/*z* 401.31 (Fig. 1c). This separation indicates that Cuban red propolis must contain, besides *D. ecastophyllum* resins, other plant resins in its composition. The rest of the propolis samples of Group B clustered with the *D. ecastophyllum* sample (**D**), confirming the similarity in their composition. Although a large number of ions are common in the fingerprints of Group B (Fig. 1d), the main marker ions for this group are those of: *m*/*z* 255.06 ($C_{15}H_{11}O_4$), 267.06 ($C_{16}H_{11}O_4$), 271.06 ($C_{15}H_{11}O_5$), 271.10 ($C_{16}H_{15}O_4$), 283.06 ($C_{16}H_{11}O_5$), and 301.07 ($C_{16}H_{13}O_6$). Fig. 2 displays typical ESI(-)-FT-ICR MS fingerprints for each group of samples.

After the chemometric analysis of the direct insertion MS fingerprints grouped the samples and indicated their marker ions, the next step was to characterise them. As isomers with the same molecular formula cannot be separated even with high resolution

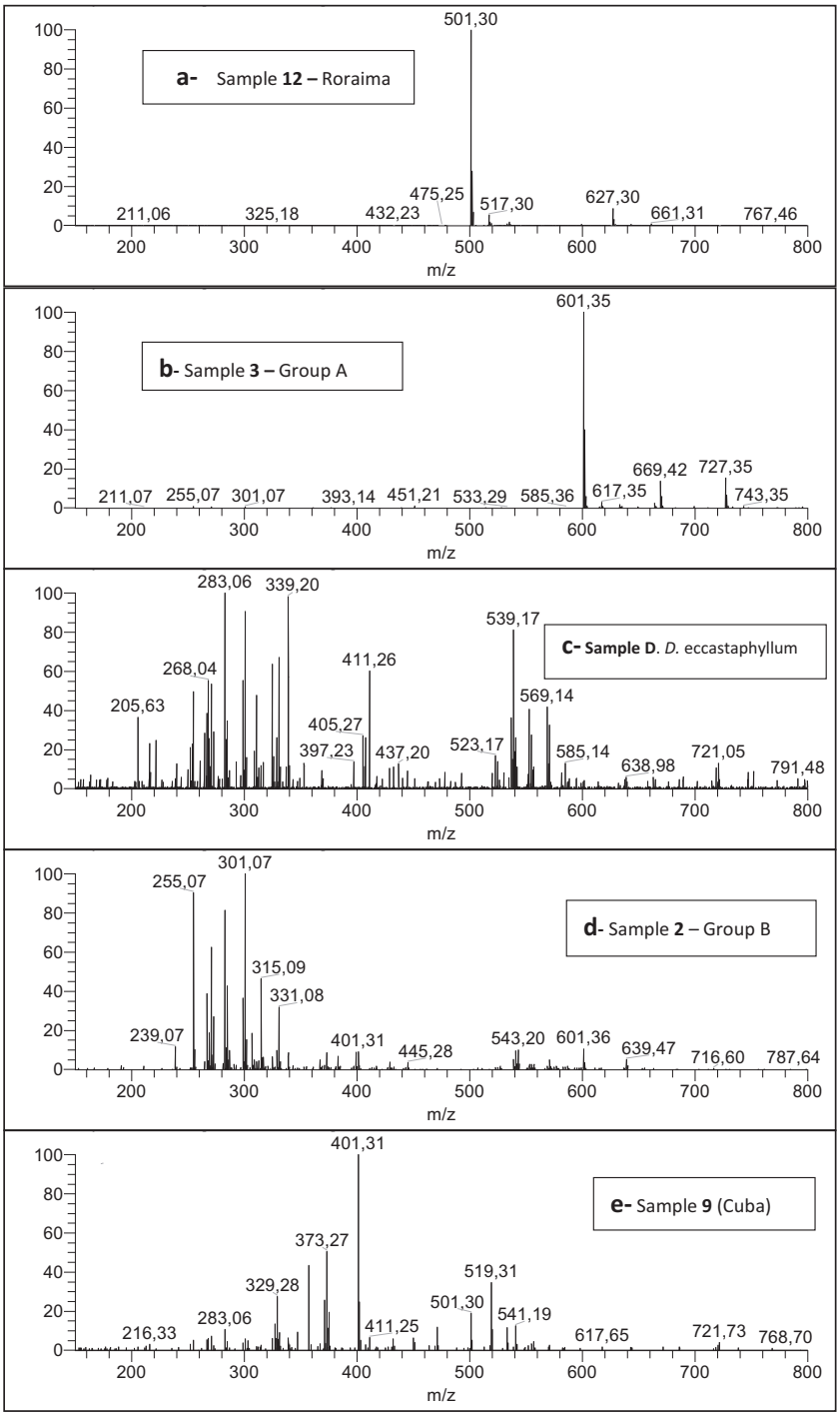

Fig. 2. ESI(-)-FT-ICR–MS fingerprints in the negative ion mode of: a- sample 12 (Roraima); b- sample 3 (Group A); c- sample of *D. ecastophyllum* resin; d- sample 2 (Group B) and e- sample 9 (Cuba).

MS, the samples were subjected to ultra-high performance liquid chromatography with tandem mass spectrometry (UPLC–MS/MS) to verify if the retention time and fragmentation patterns of the markers were similar for all the samples included in each group. Furthermore, this information was compared with data from literature or with the results of standards analysed in the same manner. The identification of important markers of the red propolis samples will be discussed individually.

Red propolis from Alagoas, recently obtained the Geographical Indication (GI) by the Brazilian National Institute of Industrial Property (INPI), and is the only red propolis of certified origin in Brazil (SEBRAE, 2012). In this study, red propolis from Paraiba was placed together with the samples from Alagoas in Group A, due to their marker ion of *m*/*z* 601.35. The neutral molecular formula obtained for this compound is $C_{38}H_{50}O_6$, and three propolis components have been described for this formula: Guttiferone E (Piccinelli et al., 2011; Trusheva et al., 2006), Oblongifolin A (Piccinelli et al., 2011) and Xanthochymol (Piccinelli et al., 2011; Trusheva et al., 2006). These prenylated benzophenones are isomers that have been found in red propolis samples from Alagoas (Brazil) (Piccinelli et al., 2011; Trusheva et al., 2006). Although no standards of these benzophenones could be obtained, the MS/MS fragmentation

of the marker ion of *m*/*z* 601.35 was found compatible with these structures (Fig. 3) and with reported data (Ishida, Negri, Salatino, & Bandeira, 2011). The UPLC–MS/MS chromatogram of Group A samples contained 1 peak (retention time of 9.95 min) with the precursor ion of *m*/*z* 601.35 and the same fragment ions of *m*/*z* 465.3, 409.1 and 109.0. Xanthochymol (*m*/*z* 601.3) was isolated from a red propolis sample from Manaus and its ESI(-)-MS/MS presented the same fragment ions of *m*/*z* 465.3, 409.1 and 109.0 (Ishida et al., 2011), as observed herein. Other peaks with the same mass, similar retention times but different fragmentations were found in these samples, therefore the presence of the other isomers (Guttiferone E and Oblongifolin A) cannot be discarded, since their structures and possible fragmentation patterns (Fig. 3) are similar. Species of the Guttiferae family such as: *C. minor*, *C. major*, *C. scrobiculata* (in Venezuelan propolis) and *C. rosea* (in Cuban propolis); have been shown to produce prenylated benzophenones (Cuesta-Rubio et al., 2002; Tomás-Barberán et al., 1993; Trusheva et al., 2004, 2006). These results indicate that the main plant source of resins for Group A red propolis samples is a species of the Guttiferae family, though further studies will be necessary to identify the species. Other prenylated benzophenones (Scrobiculatones A and B) were found in propolis from Cuba and Venezuela (Cuesta-Rubio et al., 2007; Trusheva et al., 2004) and 18-ethyloxy-17-hydroxy-17,18-dihydroscrobiculatones A and B were only described in propolis from Venezuela (Trusheva et al., 2004). There is however no evidence of the presence of prenylated benzophenones in the sample of Cuban propolis analysed herein (Fig. 2e)

All the samples in Group B share compounds found in *D. ecastophyllum* resins and this species has been identified as the plant source of resins in red Brazilian and Cuban propolis (Piccinelli et al., 2011). Comparison of the ions found in the high resolution and accuracy ESI(-)-FT-ICRMS fingerprints of these samples with reported data suggested the presence of several flavonoids and isoflavones. The UPLC–MS/MS of all samples was therefore compared with standards of: chrysin, kaempferol, quercetin, naringenin, pinocembrin, formononetin, biochanin A and daidzein. The flavonoids: chrysin, kaempferol, quercetin and naringenin were not present in any of the samples. Daidzein (*m*/*z* 253.05, rt 2.9 min) was present in samples **4, 5, 8** and **9** from Paraiba, Sergipe, Alagoas and Cuba. Pinocembrin (*m*/*z* 255.06, rt = 4.9 min) as found to be present in all the propolis samples of Group B (Fig. 4) and in *D. ecastophyllum*. The isoflavones: formononetin (*m*/*z* 267.06, rt 4.5 min) and biochanin A (*m*/*z* 283.06, rt 5.3 min) were found in all the propolis samples analysed (except for those from Roraima) and in the resin of *D. ecastophyllum* (Fig. 4), and pinocembrin was found in all samples (except **3** and **4,** belonging to Group A). Previous reports of red propolis from Sergipe, presented formononecin and biochanin A as the major components (Zulueta, Esteve, & Frígola, 2009). The high resolution MS together with the UPLC–MS analysis (Fig. 4), allowed the identification of biochanin A, formononecin and pinocembrin as three marker ions of Group B and of *D. ecastophyllum* resins in red propolis.

The direct visual comparison of the ESI(-)-MS fingerprints of *D. ecastophyllum* and of the propolis sample in Group B (Fig. 2c and d) presents differences. Furthermore, several compounds reported as belonging to *D. ecastophyllum* resins or red propolis (López & Sawaya, 2012) were not found in either. This may be due to variation in the composition of *D. ecastophyllum* resins, which may change throughout the year. It may also be caused by differences in the stability of the compounds, as only the most stable would remain in the propolis samples which are exposed to the environment. Further studies will be necessary to elucidate these matters.

PCA analysis divided the Brazilian red propolis samples into two groups based on the predominance of marker ions. However the ion of *m*/*z* 601.35 (marker ion for Group A) was also present in low abundance in the fingerprints of Group B samples (Fig. 2d), but was absent in the fingerprints of *D. ecastophyllum*, samples from Roraima and the Cuban sample. The isoflavones such as formononetin (*m*/*z* 267.06, rt 4.5 min) and biochanin A (*m*/*z* 283.06, rt 5.3 min), which are markers for Group B, were also present in lower abundances in the fingerprints of all the other red Brazilian samples and in the fingerprints of *D. ecastophyllum.* These results indicate that two plant species (*D. ecastophyllum* and probably a species of Guttiferae) are the main sources of resins for red Brazilian propolis. The relative contribution of each species to the composition of propolis varies regionally and possibly seasonally, resulting in two different types of red propolis; one defined by its isoflavone content and the other by benzophenones. A new type of Brazilian propolis was found in Roraima, whose very abundant and unique ESI(-) marker ion of *m*/*z* 501.30 (Fig. 2a) was absent in all of the other red propolis samples from other regions.

Only isoflavones from *D. ecastophyllum* were identified in sample **9** from Cuba, which has also been reported (Cuesta-Rubio et al., 2007; Piccinelli et al., 2011). Prenylated benzophenones have been

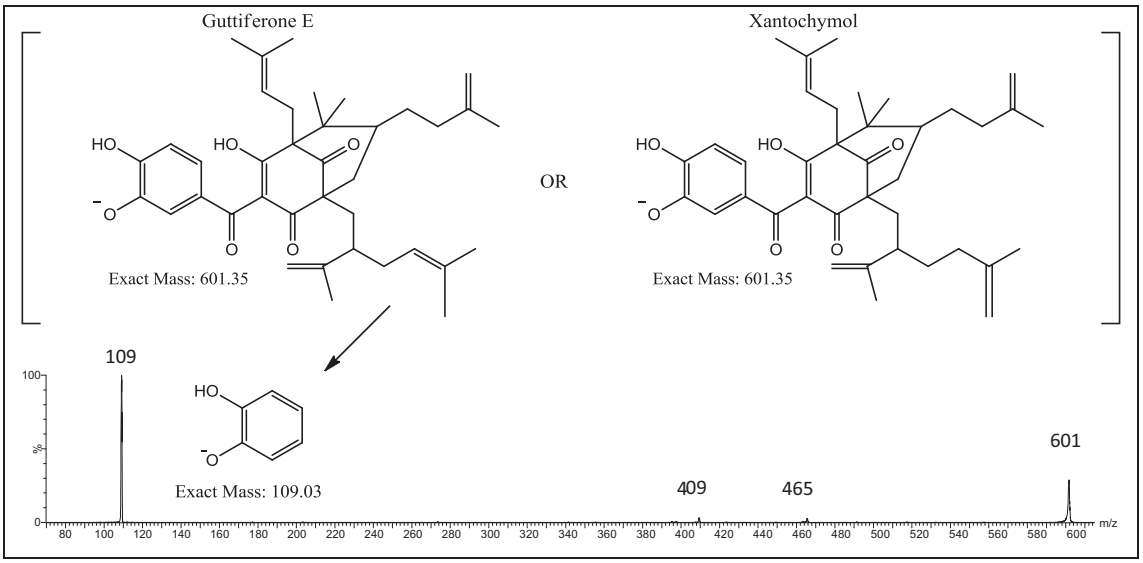

Fig. 3. Structures of deprotonated Guttiferone E and Xantochymol of *m*/*z* 601.35 and ESI(-)-MS/MS of this ion. Proposed structure for the main fragment ion *m*/*z* 109.

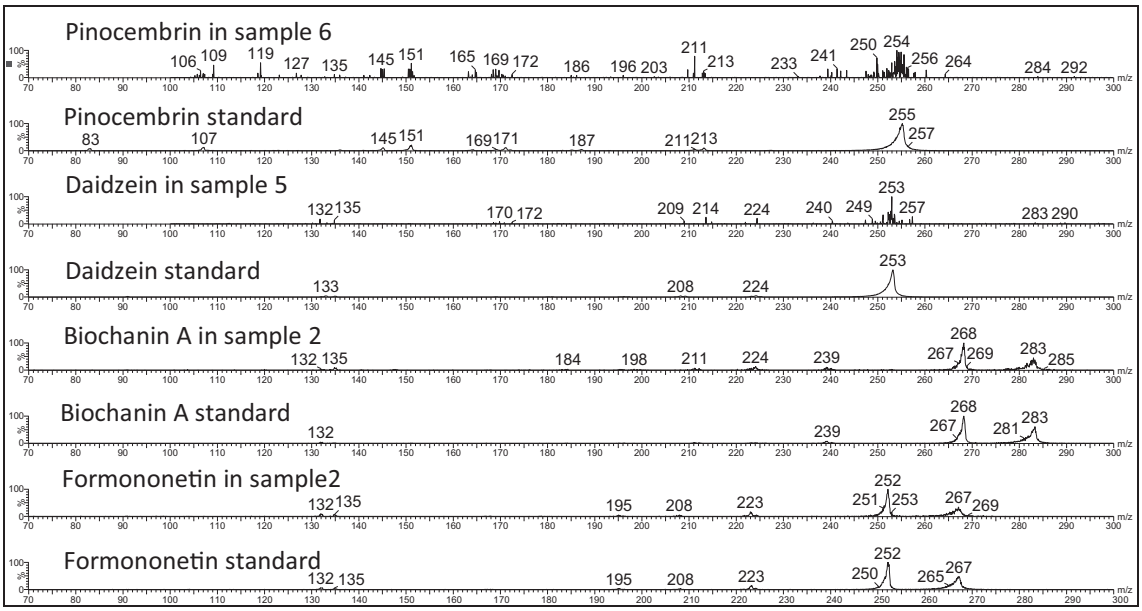

Fig. 4. UPLC-ESI(-)-MS/MS of pinocembrin, daidzein, biochanin A and formononetin standards and the identification of these compounds in propolis samples.

found in brown Cuban propolis (Cuesta-Rubio et al., 2007), Venezuelan propolis (Trusheva et al., 2004), and they are present in Brazilian red propolis, as observed in our samples (Piccinelli et al., 2011).

4. Conclusion

ESI(-)-MS in the negative ion mode of simple ethanol extracts provides characteristic profiles of chemical composition that work as fingerprints for different types of red propolis. Ethanolic extracts of 14 samples of Brazilian red coloured propolis were studied including a sample from Cuba and a sample of *D. ecastophyllum*. These samples were clearly classified into 3 main groups by PCA analysis and characteristic marker ions for each of the three groups could be identified. The samples from the state of Roraima, in Brazil, displayed a very abundant and unique marker ion of *m*/*z* 501.30 which seems to characterise these samples as a novel type of Brazilian propolis. Further studies are underway to characterise the marker ion and to identify plant sources. The other red samples (except that from Cuba) were divided into two groups according to the predominance of specific marker ions. In Group B, the marker ions were the same ones present in *D. ecastophyllum* (formononetin- *m*/*z* 267.06, biochanin A- *m*/*z* 283.06 and pinocembrin- *m*/*z* 255.06). For Group A the marker ion was that of *m*/*z* 601.35 (probably a benzophenone from a species of Guttiferae). These results indicate that at least two plant species are the main sources of resins for red Brazilian propolis and the relative contribution of each species to the composition of propolis varies regionally and possibly seasonally, resulting in two different types of Brazilian red propolis. Further studies evaluating the biological activity of these chemically characterised samples are underway as well as the search for the plant source of the prenylated benzophenones in Brazilian propolis.

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