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Original Article

Pentacyclic triterpenes and other constituents in propolis extract from *Melipona beecheii* collected in Yucatan, México

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ABSTRACT

Thirteen pentacyclic triterpenes, methyl 3-oxours-12-en-23-oate, marsformosanone, taraxerone, β-amyrenone, α-amyrenone, lupenone, 24-methylencycloartan-3-one, moretenol acetate, β-amyrin acetate, germanicol acetate, 24-methylencycloartanyl acetate, β-amyrin, and α-amyrin were identified in a chloroform-methanol propolis extract from *Melipona beecheii.* Additionally, were identified in this propolis, hexadecanoic acid, methyl ester, octadecanoic acid, methyl ester and 1-triacontanol. The purification of the propolis extract was carried out using different chromatographic techniques, including vacuum liquid chromatography, gravity column chromatography and gel filtration chromatography Sephadex LH-20. The identification of the metabolites was performed using mass spectrometry.

Introduction

The Meliponini tribe, the members of which are also known as stingless bees, belongs to the Apidae family, and Meliponinae subfamily. These are tropical bees, among which *Trigona* and *Melipona* are the most well-known (Heard, 1999; Ferreira et al., 2017a,b). For millennia, the inhabitants of the Southern region of Mexico and neighboring countries have maintained the tradition of breeding and raising melipona bees, from which various products have been obtained, such as honey for food and medicine and beeswax used as a sealant and an art tool. (National Research and Council, 2007).

The *Melipona beecheii* species has been of great importance and widely cultivated since the pre-Columbian era, in particular by Mesoamerican cultures, such as the Maya. *M. beecheii* has been practically the only bee species domesticated by the Mayan culture due to its nest size, the excellent flavor, therapeutic properties attractive golden appearance of its honey, also the tameness of the bees (Quezada-Euán et al., 2001).

Besides the honey and wax, *M. beecheii* produces propolis, an apiarian product with a bitter flavor and a resinous aspect, which presents a color variance from greenish-yellow to reddish-brown. Propolis consists basically of a mix of wax and resin exudates from different plants which the bee gathers to utilize as auxiliary material for hive protection (Bracho Pérez et al., 2009). Propolis is a product with commercial interest due to the practice of meliponiculture and it is appreciated for its qualities in the treatment of respiratory illnesses, inflammatory diseases, fatigue, hemorrhoids and gastritis, plus, it can also be used as a base for the elaboration of remedies and food products in traditional indigenous medicine in Mexico. (Guzmán et al., 2011).

Various studies carried out on different propolis have demonstrated that its chemical composition and biological activity depend on the vegetal species, resin sources and balm that the pollinizing bees collect (Bankova, 2005). With this in mind, diverse secondary metabolites have been identified, such as flavonoids, caffeic acid esters, diterpenes, benzophenones and volatile elements including sesquiterpenes (Demestre et al., 2009; Bankova et al., 2000; Tomás-Barberán et al., 1993).

The chemical composition of *M. beecheii* propolis, as with all other melipone bees, is highly variable and, despite the availability of studies such as those of Pino et al. (2006), Torres-González et al. (2016), and Fonte-Carballo et al. (2016) in which volatile metabolites were identified; as well as the main families of metabolites

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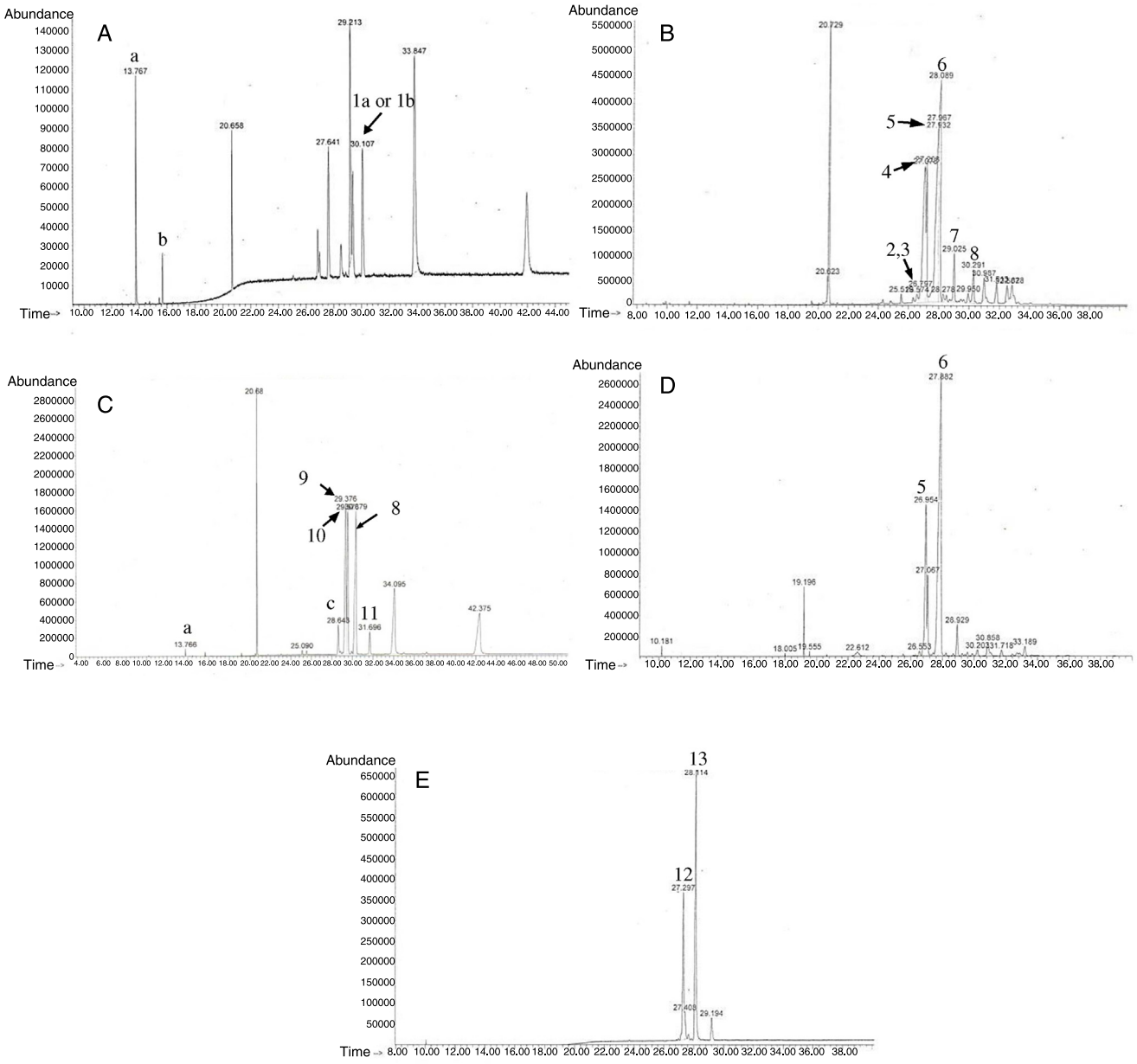


Fig. 1. GC profile of the fractions I–V of propolis from *Melipona beecheii*. A. Fraction I; B. Fraction II; C. Fraction III; D. Fraction IV; and E. Fraction V.

present in its methanol and ethanol extracts, current data is still scarce, especially in comparison with chemical component studies of *Apis mellifera* propolis.

Based on the above, and with the purpose of contributing to the available information on *M. beecheii* chemical composition, the main objective of this research was to identify the secondary metabolites present in the propolis of this species, starting with GC-MS and IR analysis, with which a pentacyclic triterpenes predominance was found, as well as other components including two fatty acids, aliphatic esters and a long chain alcohol.

Material and methods

General experimental procedures

Vacuum Liquid Chromatography (VLC) and column chromatography purifications were performed using E.M. Merck TLC-grade silica gel 60_{GF} and E.M. Merck silica gel (70–230 mesh), respectively. Gel permeation column chromatography purifications were carried out using Sephadex LH-20 (Sigma, size 25–100). Analytical TLC experiments were carried out using aluminum-backed silica gel ($60F_{254}$) plates (E.M. Merck, 0.2 mm thickness); the various components in the chromatograms were visualized by dipping the plates in a solution of phosphomolybdic acid (20 g) and ceric sulfate (2.5 g) in 500 ml of sulfuric acid (5%), followed by drying and gentle heating. GC-MS analyses were run on a Hewlett Packard 5890 gas chromatograph connected to a mass selective detector (MSD) (model 5975) [GC conditions: Split injection of 1 ml of sample; Ultra 1 column (25 m × 0.2 mm i.d.), flow rate 1.0 ml/min (Nitrogen); oven temperature program T_1 = 100 °C (3 min), T_2 = 280 °C (30 min), gradient 10 °C/min, injector 300° and detector (FID) 300 °C]. The components of each fraction were identified by comparing the MS spectra with those previously reported, by matching fragmentation patterns with those in the NIST05 library.

Propolis from Melipona beecheii

The propolis samples from *Melipona beecheii* (352.38 g) were collected in the meliponary "Flor de Mayo" in Maní, Yucatán, Mexico (20.3931° N, 89.3918° W) by the scraping technique of the upper parts of the beehive.

Extraction and isolation

The ground propolis (335.4 g) was extracted twice with a mixture of $CHCl_3$:MeOH 1:1 at room temperature (24 h); the solution was filtered and evaporated to produce 2.12 g (0.63%) of crude extract, which was subjected to VLC purification, eluting with increasing amounts of ethyl acetate in hexane to produce seven main fractions (A–G). Fraction A was purified using a combination of Sephadex LH-20 ($CHCl_3$:MeOH 1:1) and gravity column chromatography (hexane-acetone 9:1), with which it was possible to obtain two semi-pure fractions, I (2.4 mg) and II (6.7 mg). On the other hand, fraction B was purified using gravity column chromatography (hexane/ethyl acetate 9:1), which resulted in the isolation of the fractions III (1 mg), IV (1.9 mg) and V (9.8 mg) in a semi-pure form.

Results

The yield of crude *M. beecheii* propolis extract was 0.54%. Successive chromatographic purifications of the $CHCl_3$:MeOH 1:1 crude extract of *M. beecheii* propolis, using a combination of VLC, gel filtration on Sephadex LH-20 and gravity column chromatography, resulted in the isolation of five semi-pure fractions, I–V. The GC-MS analysis of the five fractions allowed the detection of thirteen pentacyclic triterpenes and other constituents. The identification of the different kinds of metabolites was obtained based on the molecular weight and its fragmentation pattern in mass spectrometry.

The chromatographic profile in the CG-MS analysis (Fig. 1A) of the fraction I showed the presence of one pentacyclic triterpenes identified as methyl 3-oxours-12-en-23-oate (**1**, t_R 30.11 min). The mass spectrum of metabolite **1** showed a parent ion peak at *m/z* 468 suggesting the molecular formula $C_{30}H_{48}O_3$. It is not possible to distinguish by mass spectrometry in the case of the isomeric forms (**1a** and **1b**) for metabolite **1**. Additionally in the fraction I were found two metabolites with a parent ion peak at *m/z* 270 ($C_{17}H_{34}O_2$) and *m/z* 298 ($C_{19}H_{38}O_2$), identified as hexadecanoic acid, methyl ester (**a**, t_R 13.77 min) and octadecanoic acid, methyl ester (**b**, t_R 15.67 min) respectively.

On the other hand, the GC-MS analysis of the fraction II (Fig. 1B) allowed the identification of seven pentacyclic triterpenes (2–8). The signal by GC at t_R 26.56 min showed a mass spectrum, which indicated a mixture of two metabolites, observing two parent ion peak at *m/z* 422 and *m/z* 424, suggesting a molecular formula of $C_{30}H_{46}O$ and $C_{30}H_{48}O$, identified as marsformosanone (**2**) and taraxerone (**3**), respectively (Paul et al., 1974; Oladoye et al., 2015). The metabolites **4** (t_R 27.03 min), **5** (t_R 24.93 min) and **6** (t_R 28.08) presented the same parent ion peak at *m/z* 424 ($C_{30}H_{48}O$). The metabolites **4** and **5** presented a base ion peak at *m/z* 218 and the fragment ions at *m/z* 203 and *m/z* 189 which allowed identified as β-amyrenone (**4**) and α-amyrenone (**5**) (Mathe et al., 2004; Cely-Veloza et al., 2014; Ferreira et al., 2017a,b). The metabolite **6** presented a base ion peak at *m/z* 205 and an ion peak at 189 characteristic of a lupane triterpen skeleton, which allowed to identify it as lupenone (**6**) (Prashant and Krupadanam, 1993). The metabolites **7** (t_R 29.02 min) and **8** (t_R 30.29 min) presented parent ion peak at *m/z* 438 ($C_{31}H_{50}O$) and *m/z* 468 ($C_{32}H_{52}O_2$) respectively and according to their fragmentation patterns were identified as 24-methylencycloartan-3-one (**7**) (Alsaadi and Al-Maliki, 2015) and moretenol acetate (**8**) (Abdel El-Fattah et al., 1992).

The GC-MS analysis of fraction III (Fig. 1C) resulted in the identification of three pentacyclic triterpenes (**9**–**11**). The mass spectrum of **9** and **10** showed that these metabolites have parent ion peak at *m/z* 468 and a molecular formula $C_{32}H_{52}O_2$ for both. These metabolites were identified based on their fragmentation pattern and by comparison in the literature as β-amyrin acetate (**9**) and germanicol acetate (**10**) (da Silva et al., 2018). The metabolite **11** (*m/z* 482, $C_{33}H_{54}O_2$) was identified as 24-methylencycloartanyl acetate (De Pascual et al., 1987). In addition, 1-triacontanol (**c**, t_R 28.64 min) was identified in the fraction III.

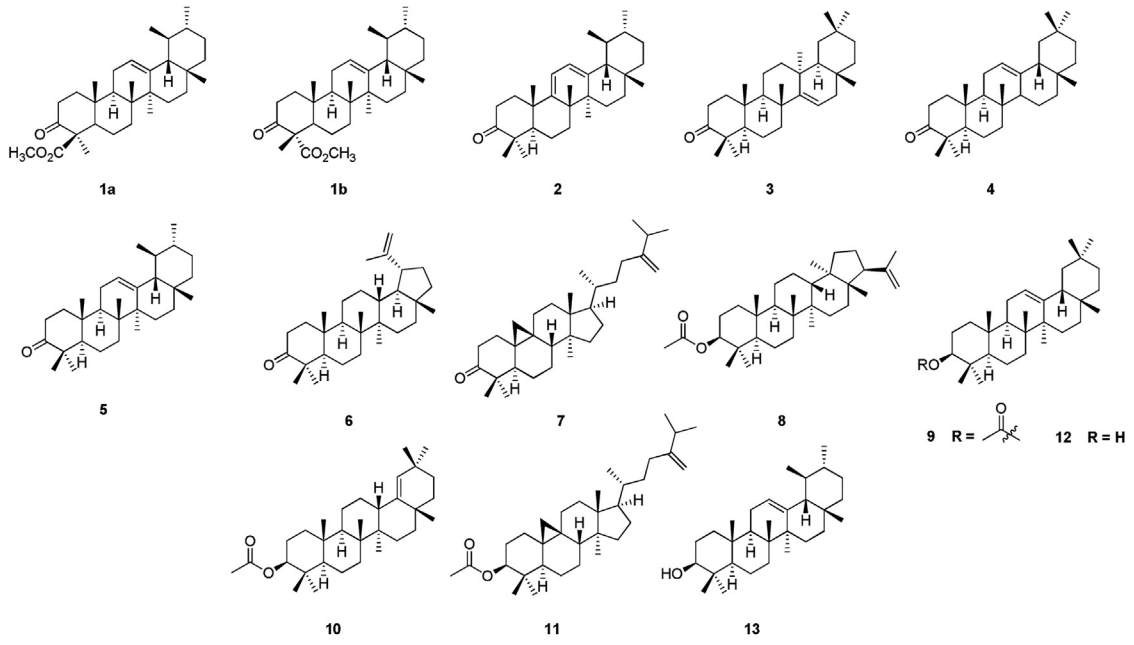
In the GC-MS analysis of fraction IV (Fig. 1D), were found the metabolites **5** and **6**. Finally, in the GC-MS analysis of fraction V (Fig. 1E), a triterpenes mixture was found, whose mass spectra showed the same molecular ion value at *m/z* 426 for both cases, suggesting a molecular formula of $C_{30}H_{50}O$ and based on its fragmentation pattern, these triterpenes were identified as β-amyrin (**12**) at t_R 27.29 min and α-amyrin (**13**) at t_R 28.11 min (Scheme 1) (Estrada-Vielma, 2003; Furukawa et al., 2002). All identified pentacyclic triterpenes are shown in Table 1.

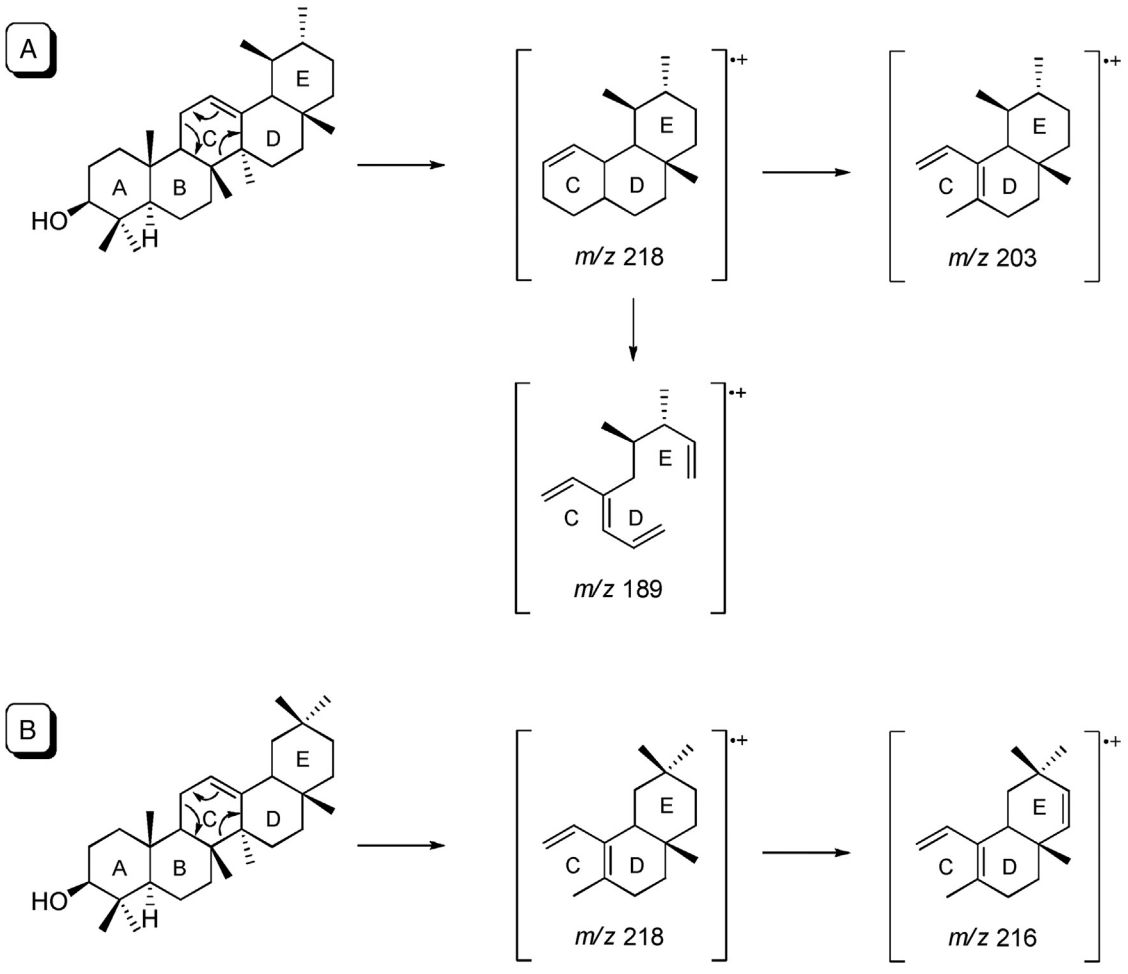
Discussion

Many of the metabolites identified in the propolis from *M. beecheii* have been reported in literature as possessing different biological activities, including antimicrobial, anti-inflammatory, cytotoxic, antioxidant, hepatoprotective and antiulcer, among others (Freire et al., 2002; Pinto et al., 2008; Abbas et al., 2009; Nikkon et al., 2010; Lucetti et al., 2010; Duan et al., 2011; Yoon et al., 2015). Among the majoritarian pentacyclic triterpenes present in the propolis, lupenone (**6**), α-amiryn (**13**) and β-amyrin (**12**) were identified (Fig. 1D and E). Aragao et al. (2008) reported that the mixture of α- and β-amyrin isomers, of ursane and oleanane

Table 1
Pentacyclic triterpenes identified in the propolis from *Melipona beecheii*.

Number	t_R (min.)	Metabolite	Molecular weight	Molecular formula
1	30.10	Methyl-3-oxours-12-en-23-oate	468	$C_{31}H_{48}O_3$
2	26.56	Marsformosanone	422	$C_{30}H_{46}O$
3	26.56	Taraxerone	424	$C_{30}H_{48}O$
4	27.06	Olean-12-en-3-one	424	$C_{30}H_{48}O$
5	27.93	α-Amyrenone or β-amyrenone	424	$C_{30}H_{48}O$
6	28.08	Lupenone	424	$C_{30}H_{48}O$
7	29.02	24-Methylencycloartan-3-one	438	$C_{31}H_{50}O$
8	30.29	Moretenol acetate	468	$C_{32}H_{52}O_2$
9	29.37	β-Amyrin acetate	468	$C_{32}H_{52}O_2$
10	29.57	Germanicol acetate	468	$C_{32}H_{52}O_2$
11	31.69	24-Methylencycloartanyl acetate	482	$C_{33}H_{54}O_2$
12	27.29	β-Amyrin	426	$C_{30}H_{50}O$
13	28.11	α-Amyrin	426	$C_{30}H_{50}O$





Scheme 1. Retro Diels–Alder fragmentation in GC-MS analysis in ursane and oleanane-type triterpenes. A. α-amyrin and B. β-amyrin.

skeleton, respectively, possess anti-inflammatory and analgesic activities which, together with the antiviral and anti-inflammatory properties of lupenone, could be linked with the mitigation of the symptoms of respiratory diseases experienced by people with these conditions, when consuming propolis (Yoon et al., 2015). In addition to the reported biological activity of the α- and β-amyrin isomers, gastroprotective activity in rats, exposed to the irritant effect of capsaicin, was found to reduce the damage to the gastric mucosa to insignificant effects at concentrations of 100 mg/kg of this pair of isomers, as has been reported by Navarrete et al. (2002). Other bioactive properties of these triterpenes are the hepatoprotective, antiulcer and insecticidal effects (Pinto et al., 2008; Abbas et al., 2009; Nikkon et al., 2010; Duan et al., 2011). In addition to β-amyrin (**12**), its antioxidant, cytotoxic and anti-inflammatory activity has been reported (Manrique and Santana, 2008).

Taking into account that the chemical composition of propolis varies according to the flora from which it is obtained (Manrique and Santana, 2008), these same metabolites have been found in the propolis of other bee species in the American continent (Bracho Pérez et al., 2009) as is the case of the β-amyrin acetate (**9**), which is known to have anti-inflammatory, antinociceptive, antioxidant and cytotoxic activity, among others, the presence of which has been reported in the propolis of bees from other parts of the American continent and now in the propolis from *M. beecheii*.

Among other important metabolites which were found in the propolis from *M. beecheii*, mention can be made of the germanicol acetate (**10**), and the 24-methylencicloartan-3-one (**7**) among those that have been reported with different biological activities, including antibacterial, anticancer and anti-inflammatory (Shimizu et al., 1996; Yasukawa et al., 2000; Ragasa et al., 2011).

In contrast, no information has been published on the biological activity of the methyl 3-oxours-12-en-23-oate (**1a** and **1b**); however, it is known to have similarities with β-boswellic acid, the properties of which have been studied along with those of its derivatives, among which are anti-inflammatory, anti-cancer, immunomodulatory and neuroprotective activities, among others (Mehta et al., 2014).

Conclusion

The investigation of the chemical composition of propolis from various species of bees is of utmost importance, given that, in different parts of the world, several studies have been conducted relating to the chemical composition of the substances with the biological properties they possess, an aspect that has great value and interest for science. Motivated by this, the analysis of the medium-low polarity fraction of propolis of *M. beecheii* was carried out, providing its chemical composition and the assumption of some of its biological properties. A total of thirteen pentacyclic triterpenes were identified by GC-MS in a $CHCl_3$-MeOH extract of propolis from *M. beecheii*, among which can be mentioned, α-amyrin, β-amyrin, lupenone, germanicol acetate and β-amyrin acetate, among others, of which a great variety of biological properties have been reported. Many of the pentacyclic triterpenes found in the propolis of *M. beecheii* have been reported in propolis from other areas of the world, illustrating that these components contribute to the properties of this propolis and to increasing the knowledge of the varieties of plants that are involved in the formation of the same.

Author's contribution

AYP (PosDoctorate) and ASF (BSc student) equally contributed in collecting propolis samples, running the laboratory work, analysis of the data and drafting the paper. PYN contributed in the collection of propolis and running the laboratory work. JRS contributed in analysis of data and drafted the paper. MCF contributed in chromatographic analysis. RBA contributed in chromatographic analysis and critical review of the manuscript. EOV designed the study, supervised the laboratory work and contributed in critical review of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjp.2019.01.006.

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