

**CHEMICAL COMPOSITIONS AND ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF PROPOLIS PRODUCED BY *Frieseomelitta longipes* AND *Apis mellifera* BEES**

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In this study, we investigated the chemical compositions and antioxidant and antimicrobial activities of propolis produced by the stingless bee *Frieseomelitta longipes* and the honeybee *Apis mellifera* collected from colonies in North Brazil. In terms of volatile composition, both mono- and sesquiterpenes were detected in the propolis of *F. longipes* while only sesquiterpenes were detected in that of *A. mellifera*. Out of 50 volatiles identified in all samples, 26 were found exclusively in *F. longipes* propolis and 8 were found exclusively in *A. mellifera* propolis. The chemical profiles of the propolis extracts were determined by atmospheric pressure chemical ionization-mass spectrometry and liquid chromatography-electrospray ionization-tandem mass spectrometry allowed to identify several prenylated benzophenones. *A. mellifera* extracts exhibited major antioxidant activity as assessed by the 2,2-diphenyl-1-picrylhydrazyl method and all extracts exhibited antioxidant activity as assessed by the  $\beta$ -carotene/linoleic acid method. The ethanolic extracts of the propolis showed promisor activity against all tested microorganisms.

Keywords: volatiles; phenolics; terpenes; stingless bee; honeybee.

## INTRODUCTION

Since ancient times, propolis has been used as an alternative medicine owing to its biological action. Besides its antimicrobial activity, it has several therapeutic properties, such as antitumor, antioxidative, and anti-inflammatory activities.<sup>1</sup> Propolis has become popular as an alternative medicine and is a constituent of several cosmetics and health products. Many studies have been conducted to elucidate the chemical compositions and biological activities of propolis from different regions of the world.<sup>2,3</sup>

Propolis is a resinous material found in colonies of many eusocial bee species. It is derived from plant resins collected by foragers and used to close gaps in hives, trapping the bodies of small invaders. It works as a physical barrier against natural enemies and also as a biochemical weapon against pathogenic or opportunistic microbes. Recent findings have also revealed its important role in colony immunity.<sup>4</sup>

The propolis used worldwide is harvested from the same bee species, *Apis mellifera*, also known as honeybees. Although from the same bee, the product is not standard because its composition can differ depending on the plants of the region where, and the season when, it is produced. In Brazil, for example, there are two well-known types of propolis: green propolis, produced in Southeast and Central Brazil from *Baccharis dracunculifolia*, a plant of the Asteraceae family, and red propolis, produced in the littoral regions of Northeast Brazil from *Dalbergia ecastaphyllum*, a plant of the Leguminosae family.<sup>2,5,6</sup>

The chemical composition of propolis can also differ depending on the bee species. Several other eusocial bees can be managed in hives, such as those of the Meliponini tribe (also known as stingless bees), which produce pure propolis with great potential for commercial use.<sup>7</sup> Stingless bees occur in all tropical and subtropical areas of the globe and

consist of more than 500 different species. Unlike honeybees, stingless bees mix propolis with wax to produce a building material, known as cerumen, which is used to construct all the structures in their nests, such as brood combs, entrances, and food pots. Recent studies have found that propolis from stingless bees has interesting characteristics and sometimes stronger biological activities than propolis from honeybees.<sup>8-11</sup> However, its commercial use remains limited because little is known about its composition or whether its properties make propolis production from these species economically viable.

Propolis contains volatile and fixed compounds. The volatile fraction corresponds to what are also called essential oils and contains mono- and sesquiterpenes, as well as alkanes and various aromatic compounds.<sup>9,12,13</sup> The fixed compounds in propolis comprise triterpenoids, flavonoids, lignans, phenolic esters of caffeic and coumaric acids, diterpenes, and prenylated benzophenones, among others.<sup>2,3</sup>

In the present study, we aimed to investigate whether the propolis produced by different bee species in the same area and at the same time are different.

We hypothesized that each bee species would use different resin sources and have specific processing methods, therefore producing unique propolis. To test this hypothesis, we compared the chemical compositions of propolis produced by two bee species, *Apis mellifera* (Apidae: Apini) and *Frieseomelitta longipes* (Hymenoptera: Apidae: Meliponini), located in the same area of the Amazon region of Brazil over the same time period.

## MATERIALS AND METHODS

### Propolis collection

Propolis samples were collected from colonies in the apiary of

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Embrapa Eastern Amazon, in the city of Belém, Brazil. Three colonies of *F. longipes* and one colony of *A. mellifera* were used. The colonies of the stingless bees and *A. mellifera* were separated by 50 meters. The surrounding area is predominantly composed of native secondary forest of the Amazonian ecosystem, but there are also urban areas and experimental Embrapa fields close to the apiary. To stimulate the production of propolis, two 2-cm-wide louvers were made in the side of the hives between the cover and the colony nest and sealed with adhesive transparent tape. Propolis was collected 15 days after the introduction of the louvers. Three samples of propolis collected from *F. longipes* colonies (FL-1, FL-2, and FL-3) and one collected from the *A. mellifera* colony (AM) were sent to the Laboratory of Biotechnology and Fine Chemistry at the Federal University of Roraima for analysis.

### Volatiles extraction

The propolis samples were chilled and powdered using a pestle and mortar. Portions of 15 to 20 g were subjected to hydrodistillation for 3 h in a modified Clevenger apparatus and the essential oil collected was dried over anhydrous sodium sulfate. The yield from the extraction was determined by the proportional relationship between the weight of the propolis samples and the weight of the obtained oil.

### Volatile compound analysis by gas chromatography-mass spectrometry (GC-MS)

A Shimadzu gas chromatograph (model GC-2010) coupled to a mass spectrometer from the same manufacturer (model QP2010 Plus) was used for the analysis of volatile compounds. Separation was performed using a fused silica capillary column (RTX-5MS, 30 m × 0.25 mm × 0.25 μm). A 1 μL aliquot of a 15 mg mL<sup>-1</sup> ethyl acetate solution of the oil was injected into the chromatograph. The injector temperature was 220 °C, the interface temperature was 280 °C, and the column temperature was programmed to increase from 60 °C at 3 °C min<sup>-1</sup> to 280 °C. Helium was used as the carrier gas at a constant flow rate of 1.02 mL min<sup>-1</sup>. Mass spectra were acquired in the *m/z* range 40–600 using electron ionization with an ionization power of 70 eV and the ion source at 260 °C.

### Volatile constituent identification

The compositions of the essential oils were determined by comparing the values of their retention indexes with those obtained for a homologous series of *n*-alkanes (C<sub>7</sub>–C<sub>30</sub>) under the same conditions, according to the method of Van den Dool and Kratz.<sup>14</sup> Later, the experimental mass spectra were verified by comparison with those in the Wiley 8 and FFNSC 1.2 digital libraries and with data from existing literature.<sup>15</sup>

### Extract preparation

Powdered propolis samples (4.10–34.29 g) were subjected to extraction at 25°C three times using 250 mL absolute ethanol (99.5%) over a period of 24 h. The extracts were combined, concentrated on a rotary evaporator, and stored in a desiccator at reduced pressure.

### Chemical ionization at atmospheric pressure analysis

The mass spectra were acquired using an ion-trap type spectrometer (Thermo-LCQ fleet) equipped with an atmospheric pressure chemical ionization (APCI) source programmed to operate in positive and negative acquisition modes. The information was

recorded using acquisition continuum mode using LCQ Fleet Tune. The monitored track was *m/z* 100–1000. Stock solutions were diluted at 20 ppm in HPLC-grade methanol and applied by direct insertion from a 5-μL loop using a pump syringe as a transport vehicle. The operational conditions were: APCI(-): discharge voltage, 3 kV; vaporizer temp, 300 °C; IRS gas, 30 arbs; auxiliary gas, 10 arbs; sweep gas, 0 arb; capillary temp, 200 °C; capillary voltage, -40 V; tube lens, -115 V; syringe pump, 30 μL min<sup>-1</sup>.

### Liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) analysis

Propolis extracts were reconstituted in 500 μL of methanol, and 50 μL of this extract was diluted in 450 μL of the same solvent and analyzed using by Ultra High Performance Liquid Chromatography (Shimadzu, Nexera X2) coupled to a quadrupole time-of-flight mass spectrometer (Bruker, Impact II UHR-QTOF) controlled by the Otof Control and Hystar software packages (Bruker Daltonics) and equipped with an electrospray source operating in negative ionization mode. The parameters used for ESI(-) mode were: capillary voltage, 3.0 KV; nebulizer gas pressure, 4.0 Bar; dry heater temperature 220 °C. Full-scan MS spectra (*m/z* 100–1300) were acquired, and the ions of interest were selected by auto MS/MS scan fragmentation. Chromatographic separation was performed with a gradient mixture of solvents A (H<sub>2</sub>O/0.1 % formic acid, v/v) and B (methanol) using a C<sub>18</sub> column (Waters Acquity UPLC® CSH™ 1.7 μm, 2.1 × 100 mm). The column was maintained at 40 °C, the flow rate was 0.2 mL min<sup>-1</sup>, and the sample injection volume was 2 μL. The separations ran for 20 min using the program: 0–1 min: 40% B, 1–7 min: 90% B, 7–13 min: 97% B, followed by an isocratic elution in the period from 13 to 16 min at 97% B, and finally 16–18 min 40% B, keeping 40% until 20 min to re-equilibrate the column prior to another run. Sodium formiate solution was injected as calibrant at the start of each chromatographic run.

### 2,2-Diphenyl-1-picryl-hydrazyl (DPPH) radical-scavenging activity analysis

The analyses were performed according to the methodology described by Mensor *et al.*<sup>16</sup> with some modifications. For the extracts of *F. longipes* propolis, a 1 mg mL<sup>-1</sup> sample was used, and for *A. mellifera* propolis, a 0.1 mg mL<sup>-1</sup> sample was used. Different sized aliquots of these solutions were withdrawn (for *F. longipes*, 20, 30, 40, 50, 60, and 70 μL, and for *A. mellifera*, 25, 50, 75, 100, 125, and 150 μL), followed by addition of 1.5 mL DPPH (1 mmol L<sup>-1</sup>). A control solution consisting of 1.5 mL of a solution of DPPH and methanol was also prepared. The blank was formed by the same aliquots of samples and methanol. The determinations were performed using a UV-Vis spectrophotometer (Shimadzu UV-mini model 1240). The percentage uptake of the DPPH radicals was used to calculate percentage antioxidant activity (AA%). IC<sub>50</sub> values were determined using linear regression analysis.

### Antioxidant activity analysis using the β-carotene/linoleic acid system

For the oxidation of samples, we used the methodology described by Emmos *et al.*,<sup>17</sup> whereby 50 μL of a β-carotene solution (1 mg mL<sup>-1</sup>), 40 μL of linoleic acid, and 265 μL of Tween 80 were mixed and solubilized in 1 mL of chloroform. The chloroform was removed with nitrogen (N<sub>2</sub>) before the residue was then dissolved in aerated water (150 mL). A 5 mL aliquot of the emulsion was added to 100 μL of the propolis extracts (1 mg mL<sup>-1</sup>), after which

the mixture was a water bath at 40 °C. Oxidation was monitored in a spectrophotometer at 470 nm. A  $\beta$ -carotene/linoleic acid emulsion without antioxidant was used for control samples, and a 1 mg mL<sup>-1</sup> solution of 2,6-tert-butyl-1-hydroxy-toluene (BHT) was used as a positive control. The antioxidant activity (AA%) was expressed as the percentage of inhibition compared with that of the control after 120 min.

### Antimicrobial activity

The gram-positive bacteria *Bacillus cereus* INCQS 00003 (ATCC 11778) and *Staphylococcus aureus* INCQS 0057 (ATCC 43300), the gram-negative bacteria *Pseudomonas aeruginosa* INCQS 00025 (ATCC 15442) and *Escherichia coli* INCQS 00051 (ATCC 13863), and the yeasts *Candida albicans* (ATCC 90028) and *Candida tropicalis* (ATCC 28707) were utilized for antimicrobial tests. The strains were supplied by the Coleção de Microrganismos de Referência em Vigilância Sanitária (CMRVS, FIOCRUZ-INCQS, Rio de Janeiro, RJ, Brazil). The bacteria were cultivated in brain-heart infusion medium (BHI) at 36 ± 1 °C for 24 h and the yeasts were cultivated in Sabouraud agar at 36 ± 1 °C for 36 h.

The cultures were adjusted to 0.5 McFarland (1 × 10<sup>8</sup>) standard turbidity scale equivalents (10<sup>5</sup> CFU mL<sup>-1</sup>), then a dilution (1:10) was performed to achieve a dilution of 10<sup>7</sup> CFU mL<sup>-1</sup>. After the suspension was inoculated into the broth, the final bacterial concentration of the test was 10<sup>5</sup> CFU mL<sup>-1</sup>, as recommended by CLSI (2012). The minimum inhibitory concentration values (MICs) of the ethanolic extracts were determined with 96-well plates as per a method established by the Clinical and Laboratory Standards Institute (CLSI).<sup>18</sup> The negative control was 80% ethanol and the positive controls were ampicillin and fluconazole. The extract concentrations tested were 1000, 500, 250, 125, 62.5, 31.3, 15.6, and 7.8 µg mL<sup>-1</sup> and 2,3,5-triphenyl tetrazolium chloride (TCC) was used to assess the viability of the microorganisms.

## RESULTS AND DISCUSSION

### Volatiles

The relative abundances of essential oils in the *F. longipes* propolis varied between the colonies sampled. FL-1 had an essential oil percentage of 0.07%, while FL-2 and FL-3 had much higher values of 0.47 and 0.78%, respectively. In contrast, *A. mellifera* contains only 0.12% essential oils.

The essential oil constituents of propolis from *F. longipes* and *A. mellifera* are listed in Table 1. In the propolis of *F. longipes*, around 40 compounds were identified as mono- and sesquiterpenes. There is much less variety in the compounds found for in *A. mellifera* propolis, which comprises exclusively sesquiterpenes.

Six monoterpenes were identified in *F. longipes*. Sample FL-1 presented lower concentration of monoterpenes (1.40%) compared to those of FL-2 and FL-3 (14.58 and 10.74%, respectively). The most abundant monoterpene was (*Z*)-ocimene (7.43%, in FL-2).

Among the sesquiterpenes,  $\beta$ -caryophyllene presented the highest percentages in all the processed samples (19.6% in FL-1, 20.99% in FL-2, 34.52% in FL-3 and 31.42% in AM). The sesquiterpenes that presented contents of > 5% in *A. mellifera* were  $\alpha$ -copaene (9.43%), cyperene (6.24%),  $\alpha$ -humulene (7.06%),  $\gamma$ -gurjunene (5.55%),  $\beta$ -bisabolene (5.53%), and  $\delta$ -cadinene (8.61%). Of these, cyperene and  $\gamma$ -gurjunene were not detected in *F. longipes* propolis. *F. longipes* propolis presented significant percentages of  $\alpha$ -copaene (3.87-5.99%),  $\alpha$ -humulene (4.38-6.16%), germacrene D (2.98-6.95%),  $\delta$ -cadinene

(6.95-7.45%), and  $\gamma$ -cadinene (3.38–5.46%). Of these, germacrene D was not detected in *A. mellifera* propolis.

Patricio *et al.* identified components in the tibia of three *Frieseomelitta* species collected in different places.<sup>19</sup> When comparing the data obtained in their study to that obtained in the current study for the propolis of *F. longipes*, it is confirmed that no component is present in all the samples analyzed for this genus of bees, demonstrating that there is no uniformity in their compositions.  $\alpha$ -phellandrene in *F. longipes*, *F. silvestrii*, and *F. s. languida*, and  $\alpha$ -cubebene and  $\alpha$ -copaene in *F. longipes*, *F. silvestrii*, and *F. varia* were detected. Comparing *F. longipes* and *F. silvestrii*, eight common components were identified:  $\alpha$ -phellandrene,  $\alpha$ -cubebene,  $\alpha$ -copaene,  $\beta$ -caryophyllene,  $\gamma$ -muurolene,  $\alpha$ -muurolene,  $\gamma$ -cadinene, and  $\delta$ -cadinene. When *F. longipes* was compared with *F. s. languida*, only one component was identified in both. In a comparison between *F. longipes* and *F. varia*, six common components were identified:  $\alpha$ -cubebene,  $\gamma$ -yalangene,  $\alpha$ -copaene,  $\alpha$ -gurjunene, E-  $\beta$ -farnesene, and germacrene D.

There are far fewer studies on the volatile compounds from stingless bees compared to those on the volatile compounds from honey bees. Studies performed with the propolis of *A. mellifera* and *Melipona beecheii* obtained in Yucatan, Mexico and extracted with a Likens-Nickerson micro-apparatus identified similar compositions with some qualitative differences. Some compounds were only present in the propolis of *A. mellifera* and others in that of *M. beecheii*. The authors concluded that the behavior of these two bee species must be different.<sup>9</sup>

Leonhardt *et al.* showed that the resin from certain tree species did not attract bees, although these species produced large amounts of resin and were often located close to trees from which bees collected resin.<sup>20,21</sup> Leonhardt *et al.* showed that Borneo stingless bees use olfactory cues to find trees from which to collect resins. Specifically, they use mono- and sesquiterpenes to localize and recognize the source of resins.<sup>22</sup> In the current study, different components were found between the propolis of *F. longipes* and *A. mellifera*, even though they are exposed to the same flora in the same period. Thus, they must have different preferences regarding the collection of resins.

### Chemical profiles of the extracts by APCI-MS

Figure 1 shows profiles of the propolis extracts obtained by direct injection of samples from honeybees and stingless bees in negative ion mode.

The chemical profile of propolis from *F. longipes* showed greater variety of compounds compared to that of *A. mellifera*. However there were common ions in all samples, as is the case for *m/z* 109, 533, 601, and 669. FL-1 and FL-2 exhibit very similar profiles, although FL-3 presents the same ions. Ishida *et al.*,<sup>23</sup> in studies with propolis samples from *A. mellifera* of Amazonia, identified the polyprenylated benzophenones 7-*epi*-nemorosone (*m/z* 501) and xanthochymol (*m/z* 601) as the main constituents. These compounds have already been isolated from species of the family Clusiaceae.<sup>24,25</sup> The ion at *m/z* 669 has been previously identified in the red propolis of Alagoas as a mixture of the isomers guttiferone C and D by Mendonça *et al.*,<sup>26</sup> confirming the predominance of benzophenones in propolis. In terms of the propolis of stingless bees, mainly of the genus *Frieseomelitta*, there are little data relating to the composition. However, the existing data confirm the relationship of propolis with floral resins of *Clusia*. Using HPLC analysis Tomás-Barberán *et al.* identified prenylated benzophenones in the propolis of *Frieseomelitta varia*, *Melipona favosa*, *Melipona compressipes*, and *Scaptotrigona depilis* from Venezuela, and verified that *Clusia* was the resin source.<sup>27</sup>

**Table 1.** Volatile constituents of *F. longipes* and *A. mellifera* propolis

CONSTITUENTS	IR <sub>L</sub>	IR <sub>C</sub>	FL-1	FL-2	FL-3	AM
			Area (%)			
<b>MONOTERPENES</b>						
$\alpha$ -Phellandrene	1003	1008	ND	0.20	t	ND
Pseudolimonene	1004	1001	t	3.87	2.13	ND
$\beta$ -Phellandrene	1030	1020	0.55	0.08	3.34	ND
( <i>Z</i> )- $\beta$ -Ocimene	1037	1039	0.85	7.43	3.23	ND
Unknown 1	-	1049	ND	2.57	1.86	ND
Camphor	1146	1144	t	0.43	0.15	ND
<b>SESQUITERPENES</b>						
$\alpha$ -Cubebene	1351	1350	3.21	1.89	1.42	0.39
$\alpha$ -Ylangene	1375	1372	0.30	0.26	0.12	0.28
$\alpha$ -Copaene	1377	1376	5.99	4.32	3.87	9.43
$\beta$ -Bourbonene	1388	1385	1.86	0.84	0.21	ND
$\beta$ -Cubebene	1388	1391	1.20	0.68	0.16	ND
$\beta$ -Elemene	1391	1393	0.48	0.22	t	ND
<i>Iso</i> -Italicene	1402	1396	0.37	0.22	0.14	ND
Cyperene	1399	1398	ND	ND	ND	6.24
$\beta$ -Longipinene	1401	1400	1.43	1.55	1.49	ND
$\alpha$ -Gurjunene	1410	1410	1.10	1.22	0.33	1.09
$\alpha$ - <i>cis</i> -Bergamotene	1413	1416	2.58	1.43	0.90	ND
$\beta$ -Caryophyllene	1419	1420	19.60	20.99	34.52	31.42
$\beta$ -Copaene	1432	1430	1.35	1.02	1.00	ND
$\beta$ - <i>trans</i> -Bergamotene	1435	1436	4.32	4.13	3.43	2.95
$\alpha$ -Guaiene	1440	1439	0.58	0.45	0.43	ND
Aromadendrene	1441	1444	0.46	0.35	0.11	1.48
$\alpha$ -Himachalene	1451	1449	0.57	0.45	0.21	0.35
$\alpha$ -Humulene	1455	1454	4.50	4.38	6.16	7.06
<i>E</i> - $\beta$ -Farnesene	1457	1458	1.81	1.44	0.65	4.22
9- <i>epi</i> -( <i>E</i> )-Caryophyllene	1466	1461	0.48	0.42	0.26	ND
<i>allo</i> -Aromadendrene	1460	1461	ND	ND	ND	0.51
<i>cis</i> Muurolo-4(14),5-diene	1467	1463	0.45	0.37	0.51	ND
<i>trans</i> Cadina-1(6),4-diene	1477	1474	ND	ND	ND	0.92
$\gamma$ -Gurjunene	1477	1476	ND	ND	ND	5.55
$\gamma$ -muurolene	1480	1477	3.06	2.41	3.81	0.58
Germacrene D	1485	1481	6.95	4.37	2.98	ND
$\beta$ -Chamigrene	1478	1484	4.55	1.30	1.52	ND
$\alpha$ -Curcumene	1481	1483	ND	ND	ND	0.38
Unknown 2	-	1485	ND	1.96	2.21	ND
$\beta$ -Selinene	1490	1486	ND	ND	ND	3.22
Unknown 3	-	1488	1.80	ND	ND	ND
<i>cis</i> $\beta$ -Guaiene <i>cis</i>	1493	1491	ND	ND	ND	0.29
$\gamma$ -Amorphene	1493	1492	0.47	0.52	0.36	4.40
Valencene	1496	1495	2.06	1.52	3.25	ND
Bicyclogermacrene	1500	1497	1.75	1.13	ND	ND
$\alpha$ -Muurolene	1501	1501	0.80	0.47	0.63	0.82
$\beta$ -Bisabolene	1506	1509	4.03	1.57	2.37	5.53
$\gamma$ -Cadinene	1514	1515	7.10	6.95	7.45	3.33
$\delta$ -Cadinene	1523	1524	4.05	3.38	5.46	8.61
( <i>E</i> )- $\gamma$ -Bisabolene	1531	1533	1.38	1.52	0.94	ND
<i>trans</i> Cadina-1(2),4-diene	1535	1532	ND	ND	ND	0.60
$\alpha$ -Cadinene	1539	1538	0.45	t	0.23	ND
Germacrene B	1561	1557	4.33	1.61	1.01	ND
Caryophyllene oxide	1583	1583	0.54	0.51	0.43	0.35
1,10-di- <i>epi</i> -cubenol	1619	1616	0.40	0.40	0.20	ND

Legend: IR<sub>L</sub>: literature retention index; IR<sub>C</sub>: calculated retention index; t: trace; ND: not detected. Unknown 1 = MS data: 43 (100 %), 55(40 %), 71(60 %), 81(95 %), 93(80 %), 108(65 %), 139(55 %), 154(40 %). Unknown 2 = MS data: 41(51 %), 55(25 %), 69(100 %), 105(30 %), 120(35 %), 133(45 %), 161(35 %). Unknown 3 = MS data: 41(50 %), 55(53 %), 67(40 %), 79(80 %), 91(95 %), 93(100 %), 119(80 %), 121(45 %), 147(45 %), 161(95 %), 189(20 %), 204(15 %).

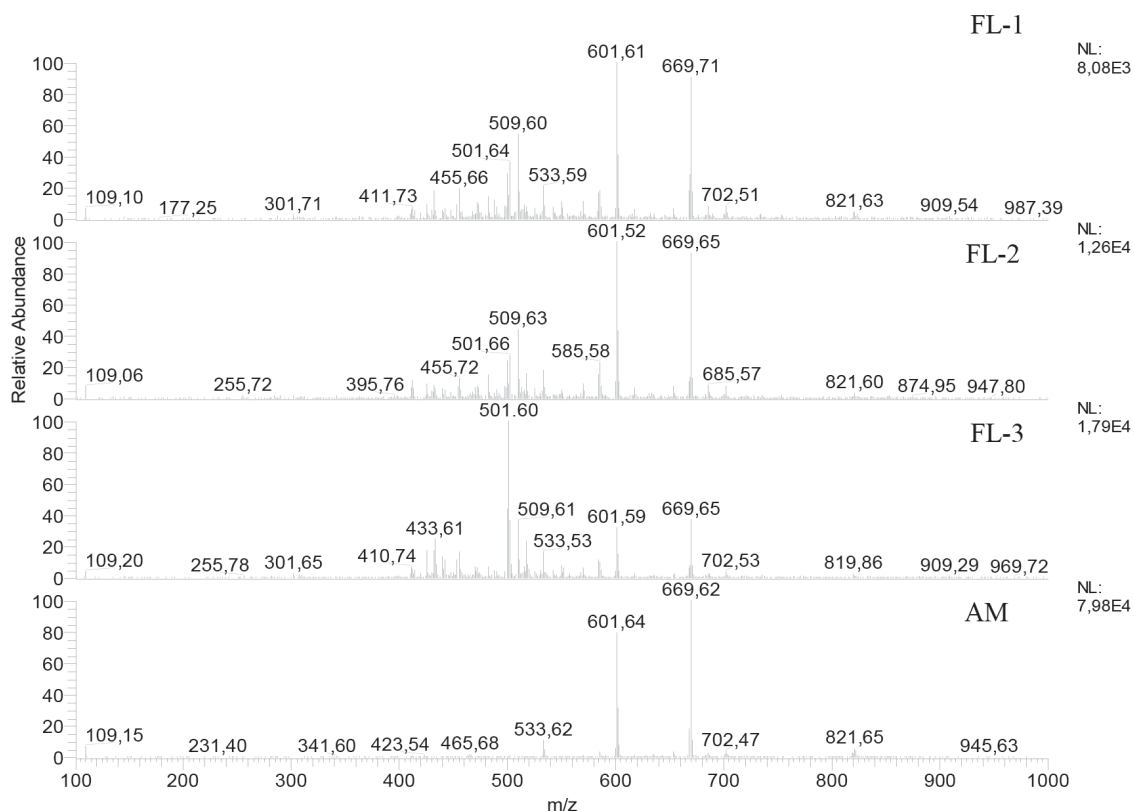


Figure 1. APCI-MS fingerprints of *F. longipes* and *A. mellifera* propolis extracts in negative ion mode

### LC-ESI-MS/MS analysis

The LC-ESI-MS/MS analyses were performed in a negative ionization mode to obtain more information about the chemical compositions of the propolis displayed on APCI-MS fingerprint. Ions

with  $m/z$  451, 501, 533, 601, and 669 were fragmented, and their fragmentation profiles were compared with data described in the literature.<sup>23-26</sup> This evidence suggests the presence of benzophenones derivatives in all propolis samples (Table 2).

In the sample FL-1, a peak with retention time (RT) 11.65 min

Table 2. Compounds detected in the propolis samples using LC-ESI-MS/MS in negative ion mode.

Peak	Retention Time (min)	Sample	Putative Identification	Molecular Weight	Precursor Ion (m/z)	Error (ppm)	Fragment Ions
1	11.65	FL-1, FL-2, FL3, AM	7- <i>epi</i> -Nemorosone	C <sub>33</sub> H <sub>42</sub> O <sub>4</sub>	501.3002	0.59	432.2296 [M-H-C <sub>5</sub> H <sub>9</sub> ] <sup>-</sup> , 417.2064 [M-H-C <sub>5</sub> H <sub>9</sub> -CH <sub>3</sub> ] <sup>-</sup> , 363.1586 [M-H-2(C <sub>5</sub> H <sub>9</sub> )] <sup>-</sup> , 327.1952 [M-H-C <sub>7</sub> H <sub>5</sub> O-C <sub>5</sub> H <sub>9</sub> ] <sup>-</sup> , 309.1128 [M-H-C <sub>7</sub> H <sub>5</sub> O-C <sub>5</sub> H <sub>9</sub> -H <sub>2</sub> O] <sup>-</sup>
2	14.74	FL-1, FL-2, FL3, AM	Xanthochymol	C <sub>38</sub> H <sub>50</sub> O <sub>6</sub>	601.3520	0.49	465.3363 [M-H-C <sub>7</sub> O <sub>3</sub> H <sub>4</sub> ] <sup>-</sup> , 449.1949 [M-H-C <sub>10</sub> H <sub>16</sub> O] <sup>-</sup> , 177.1040 [M-H-C <sub>10</sub> H <sub>16</sub> OC <sub>15</sub> H <sub>12</sub> O <sub>5</sub> ] <sup>-</sup> , 109.0288 [C <sub>6</sub> O <sub>2</sub> H <sub>3</sub> ] <sup>-</sup>
3	16.57	FL-1, FL-2, FL3, AM	Guttiferone C or D	C <sub>43</sub> H <sub>58</sub> O <sub>6</sub>	669.4147	0.29	533.3986 [M-H-2(C <sub>5</sub> H <sub>9</sub> )] <sup>-</sup> , 177.0185 [M-H-C <sub>10</sub> H <sub>16</sub> O-C <sub>15</sub> H <sub>12</sub> O <sub>5</sub> ] <sup>-</sup> , 109.0295 [C <sub>6</sub> H <sub>3</sub> O] <sup>-</sup>
4	10.61	FL-1, FL-2, FL-3	Gambogenone	C <sub>27</sub> H <sub>32</sub> O <sub>6</sub>	451.2099	4.87	315.1945 [M-H-C <sub>7</sub> O <sub>3</sub> H <sub>4</sub> ] <sup>-</sup> , 109.0279 [C <sub>6</sub> O <sub>2</sub> H <sub>3</sub> ] <sup>-</sup>
5	11.37	FL-1, FL-2, FL-3, AM	Aristophenone A	C <sub>33</sub> H <sub>42</sub> O <sub>6</sub>	533.2898	3.37	464.2198 [M-H-C <sub>5</sub> H <sub>9</sub> ] <sup>-</sup>
6	12.95	FL-1, FL-2, FL3, AM	Polyprenylated benzophenone derivative	C <sub>33</sub> H <sub>42</sub> O <sub>4</sub>	501.3003	*	363.1597 [M-H-2(C <sub>5</sub> H <sub>9</sub> )] <sup>-</sup> , 309.1127 [M-H-C <sub>7</sub> H <sub>5</sub> O-C <sub>5</sub> H <sub>9</sub> -H <sub>2</sub> O] <sup>-</sup>
7	13.16	FL-1, FL-2, FL3, AM	(1R,5R,7R)-3-Benzoyl-7-[(2E)-3,7-dimethyl-2,6-octadien-1-yl]-4-hydroxy-8,8-dimethyl-5-(3-methyl-2-buten-1-yl)bicyclo[3.3.1]non-3-ene-2,9-dione	C <sub>33</sub> H <sub>42</sub> O <sub>4</sub>	501.2998	0.59	433.2365 [M-H-C <sub>5</sub> H <sub>9</sub> ] <sup>-</sup> , 336.1356 [M-H-C <sub>10</sub> H <sub>17</sub> -C <sub>2</sub> H <sub>5</sub> ] <sup>-</sup> , 309.1126 [M-H-C <sub>7</sub> H <sub>5</sub> O-C <sub>5</sub> H <sub>9</sub> -H <sub>2</sub> O] <sup>-</sup>

\*Dashes: unidentified compound.

showed a deprotonated molecular ion at  $m/z$  501.3002 [M-H]<sup>-</sup>, and a fragments at  $m/z$  432.2296,  $m/z$  417.2064, and  $m/z$  363.1586 (Table 2), which is consistent with the presence of 7-*epi*-Nemorosone considering a 0.59 ppm mass error.<sup>23</sup> This ion was common to all samples from *F. longipes* and *A. mellifera* and was detected at similar retention times. In the samples FL-1, FL-2, FL-3, and AM, another benzophenones were detected by ESI-MS/MS. The signal at  $m/z$  601.3520 (C<sub>38</sub>O<sub>6</sub>H<sub>49</sub>) corresponds to a deprotonated molecular ion [M-H]<sup>-</sup>, with a similar retention time (14.74 min) and the same fragmentation pattern in all four samples, producing daughter ions at  $m/z$  465.3363,  $m/z$  449.1949,  $m/z$  177.1040, and  $m/z$  109.0288, which is consistent with the xanthochymol molecule considering a mass error of 0.49 ppm (Table 2).<sup>23</sup> The molecular ion [M-H]<sup>-</sup> at  $m/z$  669.4147 (16.57 min) was tentatively identified as guttiferone C or D derivatives,<sup>26</sup> corresponding to the mass of the xanthochymol plus an isopentenyl unit (C<sub>5</sub>H<sub>8</sub>, 68 Da) showing the same fragmentation pattern, within a 0.29 ppm mass error (Table 2).

Other probable benzophenone was detected in FL-1, FL-2 and FL-3 samples (RT 10.61 min) consistent with gambogone, with deprotonated molecular ion at  $m/z$  451.2093 [M-H]<sup>-</sup> (C<sub>27</sub>H<sub>32</sub>O<sub>6</sub>), producing daughter ions at  $m/z$  315.1945 and  $m/z$  109.0279, with mass error of 4.87 ppm.<sup>23</sup> The peak at retention time 11.37 min present in all samples was tentatively characterized as aristophenone A, which produced a deprotonated ion  $m/z$  533.2898 [M-H]<sup>-</sup> (C<sub>33</sub>O<sub>6</sub>H<sub>41</sub>) and a fragment at  $m/z$  464.2198 (loss of the prenyl group), with mass error of 3.37 ppm.<sup>23</sup> In addition, other probable polyprenylated benzophenones were detected by LC-ESI-MS/MS. These compounds showed a deprotonated molecular ion [M-H]<sup>-</sup> at  $m/z$  501 (C<sub>33</sub>H<sub>42</sub>O<sub>4</sub>), and in all cases, the fragmentation pattern showed ions at  $m/z$  309, probably indicating the presence of a prenyl portion. The ion at  $m/z$  501.3003 with RT 12.95 min (Table 2) yielded fragments at  $m/z$  363.1587 and 309.1127. The compound at  $m/z$  501.2996 with RT 13.16 min produced fragments at  $m/z$  433,2365 [M-H-C<sub>5</sub>H<sub>9</sub>]<sup>-</sup>, 336,1356 [M-H-C<sub>10</sub>H<sub>17</sub>-C<sub>2</sub>H<sub>5</sub>]<sup>-</sup> e 309.1126 [M-H-C<sub>7</sub>H<sub>5</sub>O-C<sub>5</sub>H<sub>9</sub>-H<sub>2</sub>O]<sup>-</sup> (Table 2) was tentatively characterized as (1*R*,5*R*,7*R*)-3-benzoyl-7-[(2*E*)-3,7-dimethyl-2,6-octadien-1-yl]-4-hydroxy-8,8-dimethyl-5-(3-methyl-2-buten-1-yl)bicyclo[3.3.1]non-3-ene-2,9-dione. The fragmentation pattern of both compound showed the loss of C<sub>5</sub>H<sub>9</sub> group, indicating the presence of a prenyl moiety. Polyprenylated benzophenones predominated in these samples making up a chemical profile very few reported for Brazilian propolis. The chromatograms of LC-ESI-MS/MS analysis and the mass spectra of the detected benzophenones are shown in Supplementary material (Figures 1S–8S).

#### Determination of the antioxidant activity of the extracts

The antioxidant activities of the propolis extracts were evaluated by different methods. The results of the analyses are listed in Table 3.

In the free radical DPPH sequestration method, the extract of

**Table 3.** Antioxidant activities of *F. longipes* and *A. mellifera* propolis extracts (mean ± SD; n = 3).

Propolis	DPPH IC <sub>50</sub> (µg mL <sup>-1</sup> )	β-carotene/linoleic acid (%)
FL-1	8.47±0.00040	75.6±0.7
FL-2	8.64±0.00001	75.1±1.1
FL-3	8.81±0.00002	73.1±1.1
AM	3.74±0.00020	75.5±1.1
BHT	6.00±0.00020	86.4±0.7

the propolis from *A. mellifera* presented high antiradical action, with values above those for the propolis of *F. longipes*. Using the β-carotene/linoleic acid oxidation method, both the propolis of *A. mellifera* and *F. longipes* exhibited strong protection capacity of a lipid substrate, presenting comparable percentages to that of the reference standard, BHT. Mendonça *et al.*<sup>26</sup> in a study on red propolis of *A. mellifera* determined the IC<sub>50</sub> using the radical DPPH sequestration method for several extracts among which the ethanolic extract presented a value of 8.01 µg mL<sup>-1</sup>, similar to those found for *F. longipes*. Campos *et al.*<sup>28</sup> evaluated the biological activities of *Melipona orbignyi* propolis and obtained an IC<sub>50</sub> of 40.0 µg mL<sup>-1</sup>, a much greater value than those for the samples from *F. longipes* and *A. mellifera*. Propolis has been reported to be a source of compounds with phenolic functionalities. Consequently, its antioxidant activities have been intensely investigated because phenolic substances are recognized as potent antioxidants. The antioxidant activity of phenolic compounds is related to their structural arrangements, which promote the inactivation of free radicals through the donation of hydrogen atoms or in the complexation of metals, which occurs due to the presence of hydroxyl groups, conjugated double bonds, and carbonyl groups.<sup>29,30</sup>

The results obtained in this study can be attributed to the presence of prenylated benzophenones, and the antioxidant activities of 7-*epi*-nemorosone, xanthochymol, gambogone and aristophenone A identified in this study have already been determined.<sup>31,32</sup> Trusheva *et al.*<sup>32</sup> have isolated several compounds with high antioxidant activities, among them prenylated benzophenones, from red propolis. The data obtained in the present study show the potential antioxidant properties of propolis by two separate methods used to measure antioxidant activity.

#### Antimicrobial activities of the extracts

Table 4 shows the results regarding the antimicrobial activities of ethanolic propolis extracts. All extracts are active against all microorganisms used.

There is currently no antimicrobial activity data for *F. longipes* propolis in the literature. The sample FL-3 exhibited activity against *B. cereus* (ATCC 11778) that is comparable to that of

**Table 4.** Antimicrobial activities of *F. longipes* and *A. mellifera* propolis extracts.

	<i>B. cereus</i>	<i>E. coli</i>	<i>S. aureus</i>	<i>P. aeruginosa</i>	<i>C. albicans</i>	<i>C. tropicalis</i>
	CIM (µg mL <sup>-1</sup> )					
FL-1	15.6	125	125	31.3	62.5	250
FL-2	15.6	125	125	62.5	62.5	250
FL-3	7.8	125	125	62.5	250	250
AM	62.5	62.5	125	31.3	250	500
Ampicillin	7.8	7.8	15.6	62.5	-	-
Fluconazole	-	-	-	-	31.3	12.5

ampicillin. *A. mellifera* (AM) showed the highest activity against *E. coli* (ATCC 13863) of all the samples. *S. aureus* (ATCC 43300) is resistant to methicillin and oxacillin but was inhibited by all the extracts tested. All extracts exhibited strong antimicrobial activity for all microorganisms and new studies must be carried out to determine active compounds.<sup>33</sup>

## CONCLUSIONS

The study revealed that the volatile compositions of propolis were very different between the two different bee species examined, as *F. longipes* propolis contained both mono- and sesquiterpenes, whereas the propolis of *A. mellifera* contained only sesquiterpenes. The chemical profiles of the extracts showed a greater diversity of ionizable compounds in the samples of the stingless bee, and although the colonies sampled were found in the same environment, the individual samples had several constituents that were unique to a particular sample. The antioxidant and antimicrobial activities of the extracts were significant, and could be better explained upon the determination of the biological activities of isolated constituent compounds in future studies.

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## SUPPLEMENTARY MATERIAL

Figures 1S to 8S are available for free download at <http://quimicanova.sbq.org.br> in pdf format.

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