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► To cite this version:

Auriane Dudoit, Christian Mertz, Marc Chillet, Nicolas Cardinault, Pierre Brat. Antifungal activity of Brazilian red propolis extract and isolation of bioactive fractions by thin-layer chromatography-bioautography. *Food Chemistry*, 2020, 327, pp.127060 -. 10.1016/j.foodchem.2020.127060 . hal-03490752

HAL Id: hal-03490752

<https://hal.science/hal-03490752v1>

Submitted on 22 Aug 2022

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1 **Antifungal activity of Brazilian red propolis extract and isolation of**
2 **bioactive fractions by thin-layer chromatography-bioautography**

3
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12

13 **Abstract**

14 **Objectives.** This study set out to highlight the *in vitro* and *in vivo* antifungal activity of an
15 Ethanollic Extract of Red Brazilian Propolis (EERBP) and identify bioactive fractions
16 effective against *Colletotrichum musae*. **Methods.** Active fractions were detected by the thin-
17 layer chromatography-bioautography method and characterised by HPLC-MSⁿ. **Results.** The
18 *in vitro* results showed that EERBP had strong antifungal properties against *C. musae* ($81 \pm$
19 1% inhibition at 1.6 g GAE L⁻¹). Medicarpin, (3*S*)-vestitol and (3*S*)-neovestitol were the main
20 compounds identified in the EERBP extract (45% of all detected peaks). Two isolated
21 fractions displayed inhibition percentages of 35 ± 4 and $42 \pm 1\%$, respectively, on *C. musae*
22 mycelial growth compared to the EERBP extract. The biological activity of the two fractions

23 displayed an additive effect. **Conclusion.** A further *in vivo* investigation revealed that EERBP
24 is a potential natural alternative for controlling banana crown rot.

25 **Keywords**

26 Propolis; Antifungal; Polyphenols; Thin-layer chromatography-bioautography;
27 *Colletotrichum musae*; Banana

28

29 1. Introduction

30 Storage disease of fruits and vegetables cause highly significant economic losses each year.
31 *Colletotrichum* sp. causes substantial damage throughout the world in tropical, subtropical
32 and temperate regions. It colonizes a wide range of hosts, such as cereals, vegetables, coffee,
33 or tropical fruits (banana, mango, papaya, etc.) (Latunde-Dada, 2001). *Colletotrichum musae*
34 (Berk. and Curt.) is the most important pathogen on green fruits and ripe bananas
35 (Priyadarshanie & Vengadaramana, 2015). Crown rot is caused by the development of a
36 parasitic complex, mainly of mycotic origin, but other microorganisms, such as bacteria, may
37 also control the spread of crown rot. Many fungi are involved in this pathology. They include
38 *C. musae*, *Fusarium*, such as *Fusarium moniliforme*, *Fusarium oxysporum*, *Fusarium*
39 *verticillioides*, or *Botryodiplodia theobromae*. Around twenty plant pathogenic fungi have
40 been isolated from banana crown rot (Wallbridge, 1981). Symptoms usually appear when the
41 fruit ripens, and losses can be huge.

42 The plant protection products currently used to limit and prevent losses caused by plant
43 pathogenic fungi are synthetic fungicides. Risks to the environment and to their users, plus the
44 fact that no resistance phenomena have been noted in the targeted pathogens, are major issues
45 for the development of these natural products.

46 Propolis, a complex, natural resinous substance, is harvested by honeybees (*Apis mellifera* L.)
47 from exudates of leaves, buds, bark, or leaves of trees and shrubs. Its chemical composition is
48 complex and mainly depends on the botanical species and geographical origin from which the
49 resinous substances are collected. It is rich in polyphenolic compounds (mainly flavonoid
50 compounds and phenolic acids) and has many scientifically demonstrated pharmacobiological
51 properties associated with it, such as its antibacterial, anti-inflammatory, antitumour or
52 antimicrobial properties (Burdock, 1998). Despite the diversity of compound classes with
53 antifungal potential, all the propolis extracts described in the literature prove to be effective

54 against various phytopathogenic moulds, such as *Aspergillus* sp., *Fusarium* sp., *Penicillium*
55 sp., *Botrytis cinerea*, or *Colletotrichum gloeosporioides*. Consequently, propolis would appear
56 to be a potentially interesting candidate for postharvest treatment.

57 Despite its great biodiversity, five types of propolis predominate in the research and industrial
58 sector. Of them, Brazilian red propolis from *Dalbergia ecastophyllum* has attracted
59 considerable attention for its biological properties, which include antioxidant (Alencar et al.,
60 2007), antibacterial (Rufatto et al., 2018), antimicrobial (Alencar et al., 2007) and antitumour
61 (Rosales et al., 2019) activities. The antifungal properties of Brazilian red propolis (mainly on
62 *Candida* species) have also been studied (das Neves, da Silva, de Oliveira Lima, da Cunha, &
63 Oliveira, 2016; Pippi et al., 2015).

64 Two other research studies highlighted the marked antifungal activity of an ethanolic extract
65 of propolis (China & commercial ethanolic extract from São Paulo, Brazil) against 2 strains of
66 *C. gloeosporioides* (Ali, Cheong, & Zahid, 2014; Mattiuz et al., 2015).

67 Postharvest spoilage of citrus fruits is most commonly caused by *Penicillium* species. Iraqi
68 EEP (2 and 3%) inhibited the growth of *Penicillium digitatum* on oranges (Matny, 2015).
69 (Matny, Al-warshan, & Ali, 2015) also noted the efficacy of Iraqi EEP (3%) against
70 *Penicillium* apple decay. A green Brazilian EEP treatment reduced the moulded area
71 (*Penicillium expansum*) to 66.8% compared to untreated fruits. Similar results have been
72 found for the efficacy of postharvest treatments using extracts of green propolis (Brazil)
73 against powdery mildew on naturally inoculated tomato leaves (Moraes, Jesus Junior, Belan,
74 Peixoto, & Pereira, 2011). The main and original characteristic of this type of red propolis is
75 that it is rich in specific isoflavones, isoflavonoids (isoflavans, isoflavones and pterocarpan),
76 triterpenic alcohols, phenylpropene derivatives (Sforcin & Bankova, 2011; Trusheva et al.,
77 2006) chalcone (Piccinelli et al., 2011) and polyprenylated benzophenones (Piccinelli et al.,
78 2011; Trusheva et al., 2006).

79 Thin-Layer Chromatography (TLC)-bioautography can be used to carry out phytochemical
80 screening of complex plant extracts with a view to identifying biologically active compounds.
81 It is a planar chromatographic analysis coupled with a method of demonstrating biological
82 activity (antifungal, antibacterial, antiprotozoal, antitumour) (Marston, 2011). Direct
83 bioautographic assaying using TLC plates was chosen because it plays an important role in
84 the search for active compounds from plants, providing quick access to information about
85 both the activity and the localisation of the activity in complex plant matrices (Marston,
86 2011). Yang et al. (2011) used a bioassay-guided fractionation technique to show the
87 antifungal activity of four compounds present in an ethyl acetate fraction (E-Fr) of Chinese
88 propolis. Bioautographic assays of an E-Fr extract (200 mg L⁻¹, weight of crude
89 propolis/volume of solvent) led them to identify four active zones. Among them, in the most
90 active band, pinobanksin, pinocembrin, chrysin and galangin were then identified by HPLC-
91 MS/MS and were shown to be effective against *P. digitatum*, citrus blue mould.

92 To our knowledge, there are no reports in the literature on the antifungal activity of Brazilian
93 red propolis against *C. musae*. The aim of this study was to highlight the *in vitro* and *in vivo*
94 antifungal activity of an Ethanolic Extract of Red Brazilian Propolis (EERBP). In an attempt
95 to go further in the mechanistic understanding, isoflavonoid compounds were chemically
96 characterised by HPLC-MS, and bioactive fractions were isolated/identified by TLC-
97 bioautography on *C. musae*.

98

99 **2. Materials and methods**

100 **2.1. Reagents and chemicals**

101 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), formic acid, petroleum
102 ether, sodium carbonate, Folin-Ciocalteu reagent, saccharose, magnesium sulfate (Mg₂SO₄),

103 potassium dihydrogen phosphate (KH₂PO₄), glycerol, ethanol and acetonitrile were purchased
104 from SIGMA-ALDRICH (Saint-Quentin Fallavier, France) and the purity of both solvents
105 was of HPLC quality. Bacto-Peptone, bacteriological agar type E and Yeast Extract were
106 obtained from (Difco, Saint-Ferréol, France). Liquiritigenin, isoliquiritigenin, formononetin
107 and biochanin A were purchased from Extrasynthese (Genay, France), vestitol and medicarpin
108 from *Centre Technique de la Conservation des Produits Agricoles* (CTCPA, Avignon,
109 France), and Ortiva[®] and Fungaflor[®] 75C from *Les producteurs de Guadeloupe* (LPG,
110 Guadeloupe, France).

111

112 **2.2. Propolis samples**

113 Crude red propolis was provided by the Pollenergie company (Agen, France). The botanical
114 origin of this propolis sample, collected in Brazil, was *Dalbergia ecastophyllum*. A frozen,
115 raw propolis sample was homogenised into a fine powder in a mixer (Thermomix Vorwerk,
116 France) with liquid nitrogen (Air Liquide, Paris, France). Aliquots (100 g) were then stored at
117 -80°C pending processing.

118

119 **2.3. Fungal strain and culture preparation**

120 Fungal strains of *C. musae* (Co-GLP 40), provided by UMR BGPI (CIRAD, Montpellier,
121 France), were isolated from crown rot disease of Guadeloupean bananas. *C. musae* was
122 maintained on Potato-Dextrose Agar plates (PDA) (SIGMA-ALDRICH, Saint-Quentin
123 Fallavier, France) at 25°C. To obtain conidia, the fungus was grown to sporulate on sterilised
124 modified Mathur's medium containing 10 g saccharose, 1 g bacto peptone, 15 g
125 bacteriological agar type E, 1 g yeast extract, 2.5 g Mg₂SO₄ x 7 H₂O and 2.7 g KH₂PO₄ per
126 litre of distilled water, for 14 days at 25°C (Lassois et al., 2010). A conidial suspension was

127 cryogenically stored in water-diluted glycerol (15%) in sterilised cryotubes at -80°C pending
128 biological assays.

129 **2.4. Preparation of EERBP**

130 An aliquot of crude propolis (10 g) was dissolved with 50 mL of 70% ethanol. The mixture
131 was protected from light and subjected to moderate shaking for 1 h, at room temperature. The
132 resulting aqueous ethanolic extract was filtered by Whatman filter paper (No. 4) (Dutscher,
133 Issy-les-Moulineaux, France). The residue underwent secondary extraction with the same
134 proportions as the first. Lastly, the two extracts were mixed and topped up to 100 mL with
135 70% ethanol. This final solution, called Ethanolic Extract of Red Brazilian Propolis (code
136 name: EERBP) was stored at -20°C pending use for analytical and antifungal assays. For a
137 polyphenol analysis using HPLC, an aliquot of EERBP was filtered through a membrane
138 (0.45 µm pore size) before injection.

139

140 **2.5. Qualitative analysis: HPLC-ESI/MS assay for polyphenolic compounds**

141 The analysis conditions were based on the Biesaga (2011) study, with modifications. The
142 system used to analyse polyphenolic compounds was composed of a high-performance liquid
143 chromatograph (model: Finnigan Surveyor, Thermo Electron, San Jose, CA, USA), with a
144 Diode Array Detector (DAD) (model: UV6000LP, Thermo Electron, San Jose, CA, USA), LC
145 pumps (model: P4000, Thermo Electron, San Jose, CA, USA) and an autosampler (model:
146 AS3000, Thermo Electron, San Jose, CA, USA). A C18 ACE column (250 x 4 mm, 5 µm
147 particle size) (AIT, Houilles, France) was used as a stationary phase, and a mixture of water
148 with 0.1% formic acid (v/v) (solvent A) and acetonitrile (solvent B) as a mobile phase. The
149 initial injection conditions were 3% B. A gradient programme was then run: 0-10 min, 15%
150 B; 10-25 min, 30% B; 25-40 min, 40% B; 40-60 min, 60% B; 60-80 min, 90% B; 80-85 min,

151 90% B; 85-87 min, 100% B; 87-92 min, 100% B; 92-95 min, 25% B; 95-97 min, 3% B; 97-
152 110 min, 3% B. The flow rate was fixed at 1 mL min⁻¹ and the column temperature was set at
153 30°C. The injection volume was 10 µL and detection was monitored at 280, 330 and 360 nm.
154 After passing through the flow cell of the DAD, the column eluate was split and 0.5 mL min⁻¹
155 was directed to an LCQ ion trap mass spectrometer equipped with an electrospray ionisation
156 (ESI) interface (Thermo Electron, San Jose, CA, USA). Experiments were carried out in
157 negative ion mode. The scan range was 90 to 1500 Da. The desolvation temperature was set
158 to 300°C. Both apparatus were controlled and monitored by XCalibur acquisition software
159 (Thermo Fischer Scientific, San Jose, CA, USA). Polyphenols were identified on the basis of
160 diode array spectral characteristics, retention times and relative elution order compared to
161 standards and literature data. The identification of some polyphenols was confirmed using the
162 standard addition method.

163

164 **2.6. Quantitative analysis: HPLC-DAD assay for polyphenolic compounds**

165 Polyphenols were analysed by HPLC using an Agilent Technologies 1200 Series (Santa
166 Clara, CA, USA). The column, solvents, detection and gradient conditions were the same as
167 those used in the mass spectrometry analysis. The injection volume was 20 µL. Absorbance
168 was performed with a G1315 photodiode array detector. Agilent Chemstation (Rev.B.02.01)
169 software was used for data analysis. Polyphenols were quantified using external
170 standardisation. Each analysis was carried out in triplicate.

171

172 **2.7. Determination of total polyphenol content**

173 The total polyphenols in the ethanolic extract of propolis were estimated by an optimised
174 Folin-Ciocalteu colorimetric method, as described by Georgé, Brat, Alter, & Amiot (2005). A
175 2.5 mL sample of water-diluted Folin-Ciocalteu reagent (1/10: v/v) was added to the water-

176 diluted EERBP (1/50: v/v). The mixture was incubated for 2 min at room temperature, and 2
177 mL of sodium carbonate (75 g L^{-1}) was added. The mixture was incubated for 15 min at 50°C
178 and finally cooled in a water-ice bath. Specific absorbance was immediately measured at 760
179 nm using a Specord S600 UV-Vis spectrophotometer (Analytik jena – Saint Aubin, France).
180 All tests were carried out in triplicate and the results were expressed as Gallic Acid
181 Equivalents (GAE).

182

183 **2.8. TLC bioautography study: detection and identification of antifungal compounds** 184 **in propolis extract**

185 Preliminary tests showed that a toluene/acetone mixture of medium polarity (3/1: v/v) was the
186 appropriate elution solvent system to achieve good separation of the compounds present in
187 our extract on TLC plates. Development was carried out after spraying a universal chemical
188 developer (vanillin) on the surface of the developed TLC plate, after heating for 10 min at
189 110°C (Figure 2 - a).

190

191 **2.8.1. TLC analysis**

192 The isolation of antifungal compounds from the ethanol extract was guided using the
193 TLC-bioautography agar overlay method, as described by Dissanayake, Ito, & Akakabe
194 (2015), with modifications. Five μL of EERBP solution ($12.8 \pm 0.4 \text{ g GAE L}^{-1}$) was directly
195 deposited on normal phase Silica Gel 60 F254 TLC plates (10 x 20 cm) (CAMAG, Muttenz,
196 Switzerland), using a CAMAG LINOMAT 5 automatic sample applicator (Muttentz,
197 Switzerland). The application (in bands) was carried out on 6 mm long strips, 7 mm from the
198 lower edge of the plate. All these application parameters were controlled using WinCATS
199 Planar Chromatography Manager software (Muttentz, Switzerland).

200 The TLC plates were developed in a CAMAG ADC2 automatic development chamber
201 (Muttentz, Switzerland). Twenty-five mL of elution solvent (toluene/acetone: 3/1: v/v) was
202 first introduced into the automatic development chamber for the first saturation step for 20
203 min. Then, 10 mL of the same solvent was introduced into a second chamber for the isocratic
204 development (or elution) step, until the solvent front reached 2 cm from the top of the plates.
205 The developed TLC plates were then removed from the chamber and allowed to air-dry for 48
206 hours.

207 A hundred mL of a 1% (w/v) vanillin solution was dissolved in 95% ethanol, then 5 mL of
208 concentrated sulphuric acid was added dropwise to the solution (universal sulphuric vanillin
209 reagent). TLC plates can be chemically revealed by spraying this reagent and heating to
210 110°C for 10 minutes before reading.

211

212 **2.8.2. Detection of active fractions by the bioautographic agar overlay method**

213 The TLC plates were each covered with the agar inoculum to obtain the bioautograms. This
214 was done by adding 90 mL of fungal inoculum (3.3×10^6 conidia mL⁻¹) to 210 mL of sterile
215 PDA agar medium while still liquid (~40°C), and homogenising by magnetic stirring. The
216 developed TLC plates were each placed in the centre of a 25 x 25 cm Petri dish. The fungal
217 suspension, in still liquid agar, was poured into the Petri dish. A thin layer of 1 to 2 mm then
218 covered the TLC plates and quickly gelled. The TLC plates thus in contact with the inoculum
219 (bioautogram) were incubated for 4 days at 25°C ± 1°C in a climatic chamber with 85%
220 relative humidity. Lastly, the bioautograms were chemically developed by spraying a MTT
221 solution (2.5 g L⁻¹) and incubating for 4 h at 25°C. MTT, an aqueous solution of tetrazolium
222 salt (yellow solution), is reduced by the succinate dehydrogenase (a mitochondrial enzyme)
223 from living fungal cells of *C. musae* to purple formazan crystals (Lim, Loh, Ting, Bradshaw,
224 & Allaudin, 2015). The intensity of the purple coloration is proportional to the concentration

225 of living cells. In the absence of mitochondrial activity due to cell lysis, the tetrazolium salt is
226 not reduced. The colour of this area remains yellowish-white, indicating the presence of
227 biologically active compounds.

228 Active bands were thus observed as white bands on a purple background. The relative front
229 value (Rf) was calculated as $Rf = \text{distance travelled by solute} / \text{distance travelled by solvent}$.

230

231 **2.8.3. Desorption of bioactive compounds identified by bioautography**

232 One part of the TLC plate was set aside (non-bioautographed/uncoated with an agar medium
233 containing inoculum) and a second was used to highlight the areas of the biologically active
234 plate by bioautography (Supplementary material). The Rf values of the white areas
235 highlighted by MTT on the treated plates were used to identify the active areas on the
236 untreated plates. Bioactive compounds identified in this area were then isolated by desorption
237 of the non-bioautographed TLC plates.

238 Eighty 5 μL samples of EERBP solution were deposited on TLC plates and developed. Once
239 the active areas were identified by bioautography, silica areas corresponding to active
240 fractions were carefully scraped with a sterilised cutter. In each zone, compounds were then
241 desorbed from the silica by maceration for 45 min in 4 mL of petroleum ether/ethanol solvent
242 (1/1: v/v). The bioactive solution was then filtered on No. 4 Whatman paper (SIGMA-
243 ALDRICH, Saint-Quentin Fallavier, France). All the desorption solvent was removed by
244 nitrogen bubbling. The pellet was finally taken up in 4 mL of 70% ethanol.

245 The bioactive ethanol fractions were then divided into two parts. The first was analysed by
246 HPLC-DAD and the second was used to study the inhibitory effect on *C. musae* mycelial
247 growth.

248

249 **2.9. Antifungal activity assay**

250 **2.9.1. Preparation of conidial suspension**

251 Before use, the homogeneous *C. musae* conidial suspension was revived by subcultures in
252 PDA plates at 25°C for 10 days. After incubation, fungal strains were re-inoculated under the
253 same conditions. Plates were stored at 4°C pending use.

254 A suspension of spores was prepared by washing a 10-day-old culture of *C. musae* strains
255 with sterilised, distilled water. The number of conidia in suspension was estimated using a
256 Malassez haemocytometer (Dutscher, Issy-les-Moulineaux, France). Inoculum concentration
257 was adjusted to 10⁶ conidia mL⁻¹ with sterilised, distilled water for *in vitro* assays and
258 10⁴ conidia mL⁻¹ for *in vivo* assays.

259

260 **2.9.2. In vitro assay: mycelial growth inhibitory effects**

261 The *in vitro* inhibitory effects of EERBP against *C. musae* were assessed by mycelial growth
262 inhibition testing using the agar dilution method, as described by Stepanović, Antić, Dakić, &
263 Švabić-Vlahović (2003), with some modifications. For the assay, 19 mL of sterilised PDA
264 medium (temperature < 50°C) was dissolved in 1 mL of EERBP (0.1, 0.2, 0.4, 0.8 and 1.6 g
265 GAE L⁻¹). Twenty mL of the homogeneous mixture was dispensed into Petri dishes. Plates
266 were dried and stored for a day at room temperature. Each Petri dish plate was then inoculated
267 with a vortexed suspension of *C. musae* (10 µL - 10⁶ conidia mL⁻¹). Incubation was carried
268 out at 25°C for a week. The first negative control sets were prepared using 70% ethanol
269 instead of EERBP, and the second with agar culture medium only. The two perpendicular
270 diameters (mm) of fungal colonies were measured. Percentage mycelial inhibition was
271 calculated by the following formula. Percentage inhibition = (D₀ - D_{EERBP})/D₀ × 100, where

272 D_0 is the colony diameter of the control sets and D_{EERBP} the colony diameter of treated
273 samples. All tests were carried out in triplicate and the results were averaged.

274

275 **2.9.3. *In vivo* assay: plant material**

276 Fruits (Cavendish var.) were supplied by a local producer in Manapany (Reunion Island).
277 Sampling was carried out on the same day at CIRAD in Saint-Pierre (Reunion), at the Ligne
278 Paradis station, and stored in a coldroom at 13°C for 16 hours. The bananas came from hands
279 II and III (comprising between 18 and 22 fruits) of 30 different bunches. The two external
280 fruits of each hand were discarded. Each hand was cut into five bunches of three fruits. They
281 were used to study the effectiveness of the antifungal treatment against crown rot. Each hand
282 II and III of a plant was considered identical and each bunch was considered as a replicate
283 (Jullien, Malézieux, Michaux-Ferrière, Chillet, & Ney, 2001). For each assay, nine bunches of
284 three fingers were formed, so a bunch constituted a Fisher block (with one replication of each
285 treatment).

286

287 **2.9.4. Controlled inoculation of a banana crown by *C. musae***

288 Bunches of three fingers were cut off the day before the antifungal treatment. The day of the
289 experiment, crowns were refreshed by cutting all their sides cleanly with a sterile scalpel. The
290 cuts were made in such a way that all the crowns were square and had the same penetration
291 surface. The bunches were then placed in distilled water for a few minutes to allow the latex
292 to drain off.

293 Fifty μL of *C. musae* spore suspension (10^4 conidia mL^{-1}) was deposited on each crown. The
294 spore suspension was stirred manually between inoculations (Lassois et al., 2010). The
295 bunches were then stored for 4 hours at room temperature and the crowns were treated later.

296

297 **2.9.5. Application of antifungal treatments on bananas**

298 Seven treatments were applied against crown rot. A control inoculated by *C. musae*, but not
299 treated, was carried out with crowns soaked in water only. The solvent, chemical fungicide
300 and EERBP control treatments were carried out by dipping and spraying, to compare the
301 effectiveness of the two application methods.

302 The polyphenol content of the ethanolic extract of propolis was 10 g GAE L⁻¹. Lastly, a
303 commercial chemical antifungal agent (Ortiva[®], 2 mL L⁻¹ + Fungaflor[®] 75C, 0.5 g L⁻¹)
304 (Guadeloupe Island, France) was tested against banana crown rot.

305 The antifungal treatments applied by dipping were carried out for 2 minutes. Spray treatments
306 were carried out with a hand-held sprayer. Three successive sprays were applied to each
307 crown.

308

309 **2.9.6. Banana storage and transport simulation**

310 Bunches were stored for 10 days in a climatic chamber at 13°C. These special storage
311 conditions thus simulated the shipping of bananas for export from the West Indies to
312 mainland France. Afterwards, to initiate fruit ripening, the bananas were exposed to ethylene
313 treatment at 1000 ppm for 24 hours in a thermoregulated chamber at 19°C, and finally the
314 fruits were stored at 20°C pending evaluation.

315

316 **2.9.7. Assessment of crown rot development**

317 After 10 days of fruit storage, a white cottony mycelial down sometimes appeared on the
318 surface of the crowns. The first assessment of the spread of *C. musae* could begin. This stage

319 corresponded to fruit ripening. Thus, external mycelium development was determined with
320 the External Lesion Surface index (SEL). Either no necrosis had developed (no visible
321 mycelium development) (Level 0), or necrosis was less than 25% of the crown surface area
322 (Level 1), or it was between 25% and 50% (Level 2), or between 50% and 75% (Level 3), or
323 lastly, necrosis had developed over more than 75% of the total crown surface area (Level 4)
324 (de Lapeyre de Bellaire, Chillet, & Chilin-Charles, 2005).

325

326 **2.10. Statistical data analysis**

327 The inhibitory effects of EERBP on mycelial growth were tested by an analysis of variance
328 (ANOVA) on all the data using XLSTAT software (Addinsoft version 19.01, Paris, France).
329 A Fisher LSD test was carried out and considered $P < 0.05$ as significant.

330

331 **3. Results and discussion**

332 **3.1. Characterisation of the main compounds by HPLC-ESI/MS**

333 A polyphenolic analysis of EERBP (botanical origin: *Dalbergia ecastophyllum*) revealed 38
334 compounds, as shown in Table 1. Twenty-six of the 38 compounds detected could not be
335 identified.

336 Peaks 21 and 23 were respectively identified as (3*S*)-vestitol and (3*S*)-neovestitol, two
337 isoflavans, a subclass of the isoflavonoids. Peak 26 was medicarpin, a natural pterocarpan.
338 These phenolic compounds were the main compounds (on the basis of the surface area)
339 identified in the extract, in agreement with Inui et al. (2014).

340 Peak 6 had a UV-visible spectrum ($\lambda_{\text{max}} = 278\text{-}314$) and a molecular ion at $m/z = 255$,
341 characteristic of liquiritigenin, an isoflavanone. The secondary ions detected (m/z 153 and

342 m/z 135 ((C₇O₃H₃)⁺) corresponded to fragment-ions obtained following a retro-Diels-Alder
343 rearrangement (Fabre, Rustan, de Hoffmann, & Quetin-Leclercq, 2001). The identification of
344 liquiritigenin was confirmed by co-injection with an authentic standard. Peak 19 had the same
345 characteristic fragmentations as liquiritigenin. Only the maximum absorbance at wavelengths
346 248 nm and 373 nm differed (presence of a hydroxyl group at position 4 of the B ring). The
347 compound identified and confirmed by co-injection was isoliquiritigenin, a position isomer of
348 liquiritigenin.

349 Two methoxylated isoflavones were identified and confirmed by co-injection with
350 commercial standards: formononetin (peak 20) and biochanin A (peak 32). The specificity of
351 these two compounds is the loss of the CH₃ group (-15 Da) during MS² type fragmentation.

352 An isoflavone, calycosin (peak 8), and an isoflavane, retusapurpurin A (peak 25), were also
353 identified. In addition, luteolin (peak 11), calycosin isomer (peak 16) and retusapurpurin A
354 isomer (peak 28) were identified in EERBP.

355 The main compounds identified (on the basis of the surface area) in our extract of *Dalbergia*
356 *ecastophyllum* botanical origin were (3S)-vestitol, (3S)-neovestitol and medicarpin, as shown
357 in Table 1, accounting for 45% of all detected peaks. The other two compounds mainly
358 present were liquiritigenin and formononetin (nearly 9% compared to all peaks), as described
359 by Piccinelli et al. (2011). Lastly, the other seven compounds detected only amounted to a
360 small percentage (less than 5%) compared to the rest of the polyphenolic fraction.

361

362 **3.2. Evaluation of antifungal activity on *C. musae*: *in vitro* study**

363 All the controls involving solvent extraction (i.e. 70% ethanol) showed no effect on mycelial
364 growth. The result of this study is presented in Figure 1, where each total polyphenol content

365 studied is represented by the histogram detailing the percentage of mycelial growth inhibition
366 on *C. musae*.

367 The lowest tested content (0.1 g GAE L⁻¹) had no activity. At concentrations of 0.2 and 0.4 g
368 GAE L⁻¹, the inhibition levels observed on *C. musae* mycelial growth were 22 ± 3% and 24 ±
369 4%. However, these two tested concentrations were not significantly different. The antifungal
370 potential of EERBP changed from 37 ± 4% inhibition (0.8 g GAE L⁻¹) to 81 ± 1% at the
371 highest bioactive compound content (1.6 g GAE L⁻¹). The inhibitory power of our EERBP
372 against this plant pathogenic fungus followed an exponential growth trend, as per Mattiuz et
373 al. (2015).

374 A study by Trusheva et al. (2006) reported that medicarpin and isosativan, identified in an
375 extract of red propolis from Brazil, showed strong antifungal action against *Candida albicans*.
376 This investigation demonstrated an inhibitory zone of 26 ± 0 and 15 ± 1 mm, respectively.
377 Medicarpin, but also formononetin, biochanin A and methoxylated isoflavones, have also
378 been identified as being biologically active against fungus strains (Boulogne, Petit, Ozier-
379 Lafontaine, Desfontaines, & Loranger-Merciris, 2012). In addition, biochanin A showed
380 antifungal activity against *Trichoderma harzianum* as described by Weidenböner & Jha
381 (1994). Isolated formononetin demonstrated fungicidal activity against five of a total of six
382 strains tested (2 *C. albicans*, 2 *C. tropicalis* & 2 *C. neoformans*) with a minimum fungicidal
383 concentration of 200 µg mL⁻¹ (crude ethanolic extract/medium volume) (das Neves et al.,
384 2016). This compound could therefore be responsible, at least partially, for the antimicrobial
385 activity of red propolis.

386 Synergistic and/or antagonistic phenomena are potentially the key to the action mechanism of
387 natural complex matrices. To gain a clearer understanding of the action of Brazilian red
388 propolis on *C. musae*, it was fractionated by TLC and its antifungal activity was identified by
389 bioautography agar overlay.

390

391 **3.3. Screening of active fractions by TLC-bioautography and chemical identification**
392 **by HPLC-ESI/MS**

393 **3.3.1. Detection and characterisation of isolated bioactive fractions by TLC-**
394 **bioautography**

395 The bioautogram revealed the presence of fractions with antifungal activity (Figure 2 - b). The
396 emergence of two yellowish-white spots corresponded to the areas of *C. musae* mycelial
397 growth inhibition (zone A & B, Figure 2 - b). The values of the front ratios for the two
398 inhibited zones were between 0.48 and 0.56 for zone A and between 0.27 and 0.39 for zone B.

399 The compounds identified by chromatography (mainly isoflavonoids) in the Fa and Fb
400 fractions isolated by TLC are detailed Table 2.

401 Seven phenolic compounds were detected in the Fa fraction. Five out of seven compounds
402 detected in this fraction could not be identified. Medicarpin and biochanin A were identified
403 in the Fa fraction detected and isolated by TLC bioautography (Figure 3). These two
404 compounds displayed a percentage content in this fraction of 69% and 28%, respectively,
405 compared to their content (based on area) in the initial EERBP. Five out of 13 compounds
406 were identified in the Fb fraction, namely liquiritigenin, isoliquiritigenin, (3S)-neovestitol,
407 (3S)-vestitol, or formononetin, which displayed a percentage content compared to the initial
408 EERBP of 69%, 43%, 70%, 3% and 4%, respectively. The main compounds ((3S)-vestitol,
409 (3S)-neovestitol and medicarpin) present in the initial extract were identified in one and other
410 of the fractions isolated by TLC.

411

412 **3.3.2. Antifungal properties of isolated fractions identified by the bioautography**
413 **method**

414 The antifungal potential of the Fa and Fb fractions, recovered by TLC, was assessed for its
415 inhibitory power on *C. musae* mycelial growth, compared to that of the original total extract.
416 A mixture of these two fractions (ratio of 1:1: v/v) was also tested to evaluate a potential
417 synergetic, additive or antagonistic effect. The percentages of mycelial growth inhibition for
418 the four extracts tested are presented in Table 3.

419 The Fa and Fb fractions displayed mycelial growth inhibition of $35 \pm 4\%$ and $42 \pm 1\%$,
420 respectively. The inhibitory power of the mixture of these two fractions (Fa + Fb) was $75 \pm$
421 2% . The sum of the activity values obtained by the two separate fractions resulted in a value
422 of around 77% inhibition. This value tallied completely with the inhibitory power of the
423 mixture of the two fractions ($75 \pm 2\%$). These theoretical fungal inhibitory values were
424 similar to that of the original total extract ($81 \pm 1\%$). These results therefore led us to suppose
425 that the two fractions would have an additive effect on antifungal activity rather than a
426 synergistic effect.

427 The study by Oldoni et al. (2011) on the antimicrobial action of red propolis from Brazil
428 suggested that the activity did not seem to be due to a synergistic effect between isoflavonoid
429 ((3S)-vestitol) and chalcone (isoliquiritigenin), but more to individual compound activity.

430 Indeed, the addition of the inhibitory power over *C. musae* mycelial growth of the two
431 extracts taken separately ($43\% + 52\% = 95\%$), or the inhibitory power of the prior addition of
432 the two extracts (93%), showed that these 2 fractions (Fa and Fb) accounted for almost all the
433 activity compared to the initial extract. The Fa and Fb fractions appeared to account for 95%
434 of the activity of the extract, despite the differences in isoflavonoid content compared to the
435 EERBP extract. Even more interestingly, the few compounds described in the literature as
436 having an antifungal potential (medicarpin, (3S)-vestitol, biochanin A and formononetin) have
437 been identified in these two fractions (Boulogne et al., 2012; das Neves et al., 2016). Based on
438 the initial observations, we suggest that the presence of medicarpin and biochanin A in Fa,

439 and formononetin and (3*S*)-vestitol in Fb, could partially contribute to the antifungal potential
440 of EERBP. Nevertheless, as around half of the compounds isolated from these two inhibition
441 zones have yet to be identified, only a hypothesis can be made. Indeed, it seems obvious that
442 either other compounds are involved in EERBP by potentially inhibiting the active
443 compounds, or unidentified compounds in the Fb fraction play a role in the overall activity.

444 Biochanin A and medicarpin (Fa) potentially resulted in $35 \pm 4\%$ inhibition of mycelial
445 growth, amounting to 43% of the total activity of the initial extract. In addition, the
446 compounds (3*S*)-vestitol and formononetin potentially accounted for 52% of the total activity
447 of the EERBP extract. It is interesting to note that, despite the presence of these two bioactive
448 compounds in very small quantities in the Fb fraction (4 and 3% respectively), compared to
449 their initial content, this fraction demonstrated high activity, exceeding 50%, compared to the
450 activity of EERBP.

451 The differential activity (5%) between the mix of the two isolated fractions and the initial
452 extract is too weak to explain these different properties. Nevertheless, an additive effect was
453 observed through the biological activity of the two fractions and the mixture.

454

455 **3.4. Evaluation of the efficacy of antifungal treatments on banana crown rot**

456 The effectiveness of our EERBP was assessed *in vivo*, in order to confirm our *in vitro* results
457 and determine whether or not its antifungal properties were workable in postharvest treatment
458 against banana crown rot.

459 Although the disease is caused by a parasitic complex, only the *C. musae* strain was
460 inoculated into the crown before the various antifungal treatments. The efficacy of these
461 treatments (commercial chemical fungicide Fungaflor[®] 75C+ Ortiva[®] and EERBP) was
462 assessed using two application methods: dipping and spraying. Thus, the external

463 development of the lesions could be determined for the different treatments and application
464 conditions. The antifungal power of the extraction solvent (70% ethanol) against *C. musae*
465 was also tested. It did not prove to be effective against the strain for mycelium development.

466 The two sets of application conditions (dipping or spraying) for the different fungicides
467 (commercial and EERBP) did not show any greater efficacy for either method. No significant
468 differences were found.

469 All the treatments carried out proved to be effective against *C. musae* compared to the
470 untreated control. Indeed, following the antifungal treatments, two days after the ethylene
471 treatment no bananas showed mycelium development greater than 50% of the total surface
472 area of the crown, whereas more than 65% of the untreated bananas already showed
473 mycelium development greater than 50%. After 10 days of storage, only 20% of the bananas
474 treated by dipping with EERBP showed necrosis greater than 25% of the total surface area of
475 the crown, and 13% by spraying, this difference being however not significant. With
476 application of the chemical antifungal agent, only 20% of the dipped bananas and no sprayed
477 bananas showed necrosis greater than 25% of the crown surface area, respectively. Therefore,
478 as our extract gave equivalent results to the synthetic treatment, we were able to conclude that
479 EERBP is a potentially very interesting candidate as an alternative treatment against banana
480 crown rot diseases.

481 To our knowledge, no studies have assessed the antifungal activity of Brazilian red propolis
482 extract on postharvest diseases of banana. Recent *in vivo* studies have been conducted by
483 applying an ethanolic extract of propolis alone, or film-incorporated, for the bioconservation
484 of fruits in the postharvest stages (Ali et al., 2014; Mattiuz et al., 2015). Mattiuz et al. (2015)
485 reported that the application of a commercial ethanolic extract of propolis from Brazil (1.5%
486 v/v) did not have an impact on the growth of *C. gloeosporioides* on mango fruit after 14 days
487 of incubation. After 7 more days, their treatment led to a significant reduction in the lesion

488 areas on mango compared to the control. Treatment of pepper fruits with a Chinese ethanolic
489 extract of propolis solution (1%, 5% and 10%) affected the development of anthracnose
490 caused by *Colletotrichum capsici* (Ali, Wei, & Mustafa, 2015). While the two controls in our
491 assays (water and 70% ethanol) showed a Disease Incidence (DI = percentage of fruits
492 bearing anthracnose symptoms out of the total number of fruits) of 17.5% and 7.5%,
493 respectively, the propolis extracts showed total efficacy after 28 days of storage (DI = 0%).
494 Moreover, 0.5%, 1%, and 1.5% ethanolic extracts of Chinese propolis also partially inhibited
495 the development of *C. gloeosporioides* on papaya fruit (Ali et al., 2014). However, unlike the
496 work described by Ali et al. (2015), the application of propolis extracts on papayas immersed
497 in a *C. gloeosporioides* spore suspension did not demonstrate total efficacy (20% < DI < 30%)
498 compared to controls (water and 70% ethanol) without propolis (DI > 90%).

499

500 **4. Conclusion**

501 The Brazilian red propolis extract (botanical origin: *Dalbergia ecastophyllum*), showed
502 noteworthy antifungal properties against *C. musae* in *in vitro* assays. This is the first literature
503 report on the efficacy of this type of propolis on plant pathogenic moulds, and more
504 specifically on *C. musae*. An exponential growth trend in antifungal power was observed,
505 ranging from $22 \pm 3\%$ inhibition at a 0.2 g GAE L^{-1} concentration to $81 \pm 1\%$ inhibition at 1.6
506 g GAE L^{-1} . The polyphenolic fraction of EERBP was characterised by HPLC-MSⁿ. This
507 allowed us to identify the isoflavonoid compounds mainly present in the extract: medicarpin,
508 (3S)-vestitol, (3S)-neovestitol, liquiritigenin and formononetin (54% of all detected peaks, on
509 the basis of the surface area). Two fractions of EERBP, detected and isolated by
510 TLC-bioautography, displayed inhibition percentages of 43 and 52%, respectively, on the
511 mycelial growth of *C. musae*, compared to EERBP inhibition. These two isolated fractions
512 accounted for 93% of the activity compared to the total EERBP extract. Medicarpin (69%

513 content compared to EERBP), biochanin A (28%) identified in the Fa fraction by HPLC-MS,
514 along with formononetin (4%) and vestitol (3%) in the Fb fraction, were already known for
515 their antifungal activity. Our results tended to suggest that these compounds have an additive
516 inhibitory effect. Lastly, the *in vivo* results showed that our EERBP could be a very
517 interesting candidate as an alternative treatment to chemical fungicides in controlling banana
518 crown rot, and maybe other types of postharvest decay of fruits and vegetables.

519

520 **Acknowledgments**

521 This work was funded as part of a CIFRE thesis (No. 2013/1120) in partnership with CIRAD
522 (Centre de coopération Internationale en Recherche Agronomique pour le Développement)
523 and co-financed by the French ANRT (Association Nationale de la Recherche et de la
524 Technologie) and the Pollenergie company.

525

526

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648

649 **Figure captions**

650 **Figure 1.** Effect of amount of Ethanolic Extract of Red Brazilian Propolis (EERBP) on the
651 growth of *C. musae* by the agar dilution method. Values are means of 3 replicates. Significant
652 differences between treatments at $P < 0.05$ are indicated with a letter as measured by Fisher's
653 LSD test.

654

655 **Figure 2.** Graphical representation of a chemically revealed TLC plate (a) and bioautogram
656 (b) of Ethanolic Extract of Red Brazilian Propolis (EERBP).

657

658 **Figure 3.** Superposition of HPLC chromatograms of Ethanolic Extract of Red Brazilian
659 Propolis (EERBP), and the Fa and Fb isolated fractions. Peak assignment refers to Table 1.

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Graphical illustration

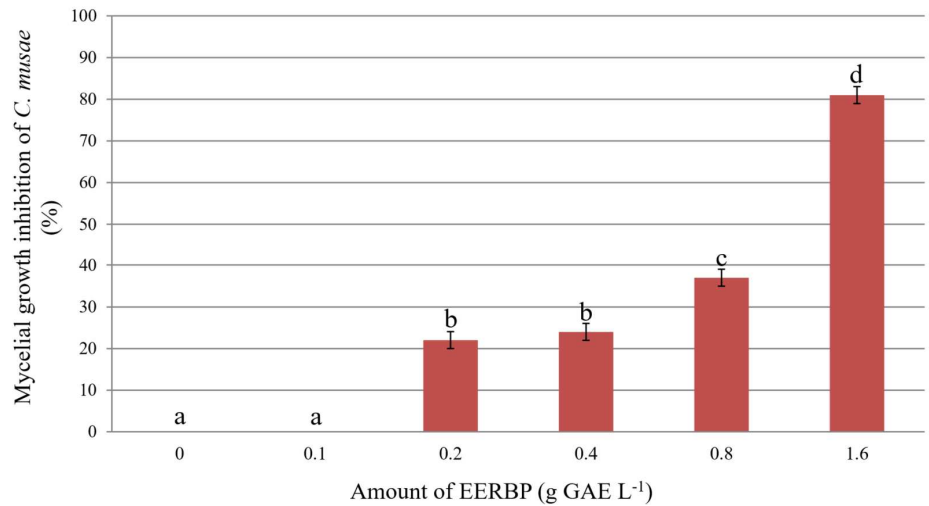


Figure 1. Effect of amount of Ethanolic Extract of Red Brazilian Propolis (EERBP) on the growth of *C. musae* by the agar dilution method. Values are means of 3 replicates. Significant differences between treatments at $P < 0.05$ are indicated with a letter as measured by Fisher's LSD test.

Graphical illustration

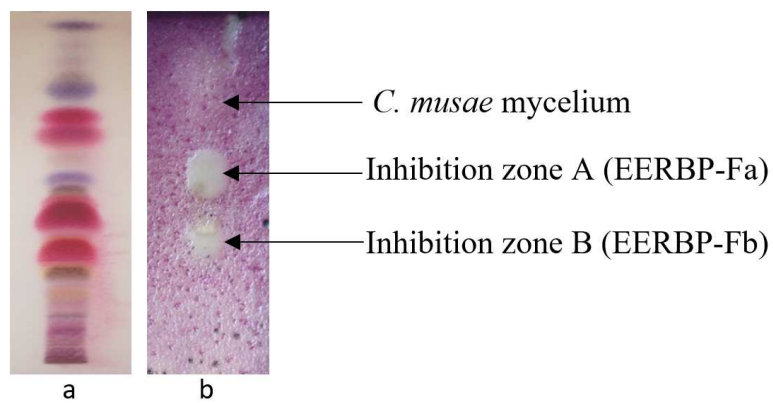
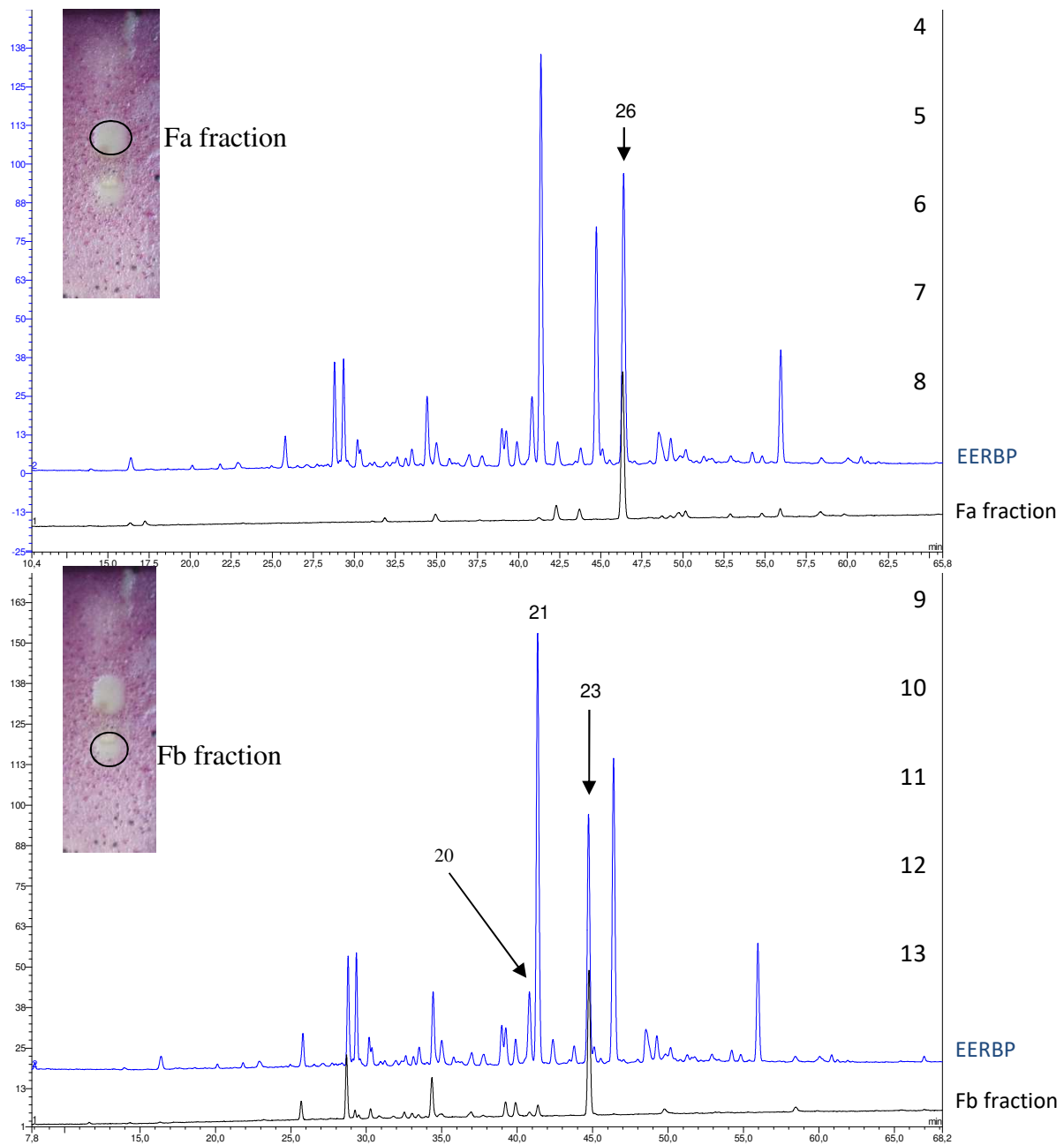


Figure 2. Graphical representation of a chemically revealed TLC plate (a) and bioautogram (b) of Ethanolic Extract of Red Brazilian Propolis (EERBP)

EERBP-Fa: isolated fraction a; EERBP-Fb: isolated fraction b

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Graphical illustration



14 **Figure 3.** Superposition of HPLC chromatograms of Ethanolic Extract of Red Brazilian
15 Propolis (EERBP), and the Fa and Fb isolated fractions. Peak assignment refers to Table 1.

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17

Table 1. Tentative identification of compounds in the Ethanolic Extract of Red Brazilian Propolis (EERBP)

<i>Peak</i>	<i>Rt (min)</i>	λ_{max} (nm)	[M-H] ⁻	MS ²	MS ³	Content (%) [‡]	Compound
1	20.8	251, 297sh, 327	n.d.	n.d.	n.d.	-	-
2	21.2	251, 279, 308	271	243, 227	MS ³ (243): 225, 199, 109	-	-
3	22.7	249, 279, 344	n.d.	n.d.	n.d.	-	-
4	25.3	267	n.d.	n.d.	n.d.	-	-
5	26.9	252, 301	297	282, 238, 254, 266	n.d.	-	-
6	28.8	278, 314	255	153, 135	MS ³ (153): 135	4.3	liquiritigenin*
7	29.2	281	315	109, 300, 125	n.d.	-	-
8	30.0	251, 293	283	268	224, 240	1.1	calycosin
9	30.3	254, 281, 313	n.d.	n.d.	n.d.	-	-
10	31.1	260	283	268	224, 240	-	calycosin (isomer)
11	31.9	250, 302sh, 347	285	241	n.d.	0.3	luteolin
12	32.5	254, 278, 313	255	237	209, 193	-	-
13	33.3	240, 282, 344	285	270	179	-	-
14	34.5	285	285	109	n.d.	-	-
15	34.9	295, 346sh	301	286	258, 195	-	-
16	35.8	251, 289	283	268	224, 240	0.6	calycosin (isomer)
17	38.9	250, 283, 420, 480	n.d.	n.d.	n.d.	-	-
18	39.3	289	301	125	n.d.	-	-
19	40.0	248, 300sh, 373	255	153	n.d.	1.4	isoliquiritigenin*
20	40.7	252, 303	267	252	224, 208, 196	4.3	formononetin*
21	41.3	284	271	253	235	19.5	(3 <i>S</i>)-vestitol*
22	43.6	250, 328	n.d.	n.d.	n.d.	-	-
23	44.7	284	271	253	n.d.	11.2	(3 <i>S</i>)-neovestitol
24	45.1	265	n.d.	n.d.	n.d.	-	-
25	45.6	251, 282, 471	521	506, 491, 397	MS ³ (506): 491, 397	0.2	retusapurpurin A
26	46.5	289	269	254	226	14.7	medicarpin*
27	47.9	242, 282sh, 340sh	n.d.	n.d.	n.d.	-	-
28	48.2	250, 287, 425sh	521	506, 491, 397	MS ³ (506): 491, 397	-	retusapurpurin A (isomer)
29	48.5	253, 294, 332	n.d.	n.d.	n.d.	-	-
30	49.2	250, 293, 335sh	n.d.	n.d.	n.d.	-	-
31	49.6	250, 293, 336sh	n.d.	n.d.	n.d.	-	-
32	50.2	263, 330sh	283	268	240, 224	0.9	biochanin A*
33	54.8	255, 327sh, 351sh	n.d.	n.d.	n.d.	-	-
34	55.9	284	n.d.	n.d.	n.d.	-	-
35	66.7	264, 244sh, 328	n.d.	n.d.	n.d.	-	-
36	67.6	248	n.d.	n.d.	n.d.	-	-
37	70.9	224, 276	n.d.	n.d.	n.d.	-	-
38	73.0	262	n.d.	n.d.	n.d.	-	-

sh: shoulder; n.d.: not detected

*confirmed by co-injection with authentic standard.

[‡]content of each molecule compared to the total content of all compounds (%).

Table 2. Content of phenolic compounds in Thin-Layer Chromatography isolated fractions

<i>Compound</i>	<i>Content of phenolic compounds^a</i>	
	EERBP-Fa	EERBP-Fb
liquiritigenin	n.d. ^b	69
isoliquiritigenin	n.d.	43
formononetin	n.d.	4
(3 <i>S</i>)-vestitol	n.d.	3
(3 <i>S</i>)-neovestitol	n.d.	70
medicarpin	69	n.d.
biochanin A	28	n.d.

EERBP-Fa: isolated fraction a; EERBP-Fb: isolated fraction b

^aLevels are expressed as a percentage of the initial content in Ethanolic Extract of Red Brazilian Propolis (EERBP).

^bn.d.: not detected.

Table 3. Efficacy of different isolated fractions of Ethanolic Extract of Red Brazilian Propolis (EERBP) on *C. musae* mycelial growth

<i>Extract</i>	<i>Mycelial growth inhibition (%)^a</i>	<i>Mycelial growth inhibition in relation to the total EERBP extract (%)</i>
<i>EERBP-Fa</i>	35 ± 4	43
<i>EERBP-Fb</i>	42 ± 1	52
<i>EERBP-Fa + EERBP-Fb</i>	75 ± 2	93
<i>EERBP (total extract)</i>	81 ± 1	-

EERBP-Fa = isolated fraction a; EERBP-Fb = isolated fraction b

^aAverage of three measurements with standard deviations.