

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

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Abstract

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

1. Introduction

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

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

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

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

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

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

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

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

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

2. Materials and methods

2.1. Reagents and chemicals

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

potassium dihydrogen phosphate (KH_2PO_4), glycerol, ethanol and acetonitrile were purchased from SIGMA-ALDRICH (Saint-Quentin Fallavier, France) and the purity of both solvents was of HPLC quality. Bacto-Peptone, bacteriological agar type E and Yeast Extract were obtained from (Difco, Saint-Ferréol, France). Liquiritigenin, isoliquiritigenin, formononetin and biochanin A were purchased from Extrasynthese (Genay, France), vestitol and medicarpin from *Centre Technique de la Conservation des Produits Agricoles* (CTCPA, Avignon, France), and Ortiva[®] and Fungaflor[®] 75C from *Les producteurs de Guadeloupe* (LPG, Guadeloupe, France).

2.2. Propolis samples

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

2.3. Fungal strain and culture preparation

Fungal strains of *C. musae* (Co-GLP 40), provided by UMR BGPI (CIRAD, Montpellier, France), were isolated from crown rot disease of Guadeloupean bananas. *C. musae* was maintained on Potato-Dextrose Agar plates (PDA) (SIGMA-ALDRICH, Saint-Quentin Fallavier, France) at 25°C. To obtain conidia, the fungus was grown to sporulate on sterilised modified Mathur's medium containing 10 g saccharose, 1 g bacto peptone, 15 g bacteriological agar type E, 1 g yeast extract, 2.5 g $\text{Mg}_2\text{SO}_4 \times 7 \text{H}_2\text{O}$ and 2.7 g KH_2PO_4 per 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.

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,

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

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

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

2.7. Determination of total polyphenol content

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

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

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

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

2.8.1. TLC analysis

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

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

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

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

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

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

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

2.8.3. Desorption of bioactive compounds identified by bioautography

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

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

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

2.9. Antifungal activity assay

2.9.1. Preparation of conidial suspension

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

A suspension of spores was prepared by washing a 10-day-old culture of *C. musae* strains with sterilised, distilled water. The number of conidia in suspension was estimated using a Malassez haemocytometer (Dutscher, Issy-les-Moulineaux, France). Inoculum concentration was adjusted to 10^6 conidia mL⁻¹ with sterilised, distilled water for *in vitro* assays and 10^4 conidia mL⁻¹ for *in vivo* assays.

2.9.2. In vitro assay: mycelial growth inhibitory effects

The *in vitro* inhibitory effects of EERBP against *C. musae* were assessed by mycelial growth inhibition testing using the agar dilution method, as described by Stepanović, Antić, Dakić, & Švabić-Vlahović (2003), with some modifications. For the assay, 19 mL of sterilised PDA medium (temperature < 50°C) was dissolved in 1 mL of EERBP (0.1, 0.2, 0.4, 0.8 and 1.6 g GAE L⁻¹). Twenty mL of the homogeneous mixture was dispensed into Petri dishes. Plates were dried and stored for a day at room temperature. Each Petri dish plate was then inoculated with a vortexed suspension of *C. musae* (10 µL - 10^6 conidia mL⁻¹). Incubation was carried out at 25°C for a week. The first negative control sets were prepared using 70% ethanol instead of EERBP, and the second with agar culture medium only. The two perpendicular diameters (mm) of fungal colonies were measured. Percentage mycelial inhibition was calculated by the following formula. Percentage inhibition = $(D_0 - D_{\text{EERBP}})/D_0 \times 100$, where

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

2.9.3. *In vivo* assay: plant material

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

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

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

Fifty μL of *C. musae* spore suspension (10^4 conidia mL^{-1}) was deposited on each crown. The spore suspension was stirred manually between inoculations (Lassois et al., 2010). The 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

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

2.10. Statistical data analysis

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

3. Results and discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

**3.3.2. Antifungal properties of isolated fractions identified by the bioautography
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,

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

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

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

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

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

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

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

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

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

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

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

4. Conclusion

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

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

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648

Figure captions

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.

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

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

Graphical illustration

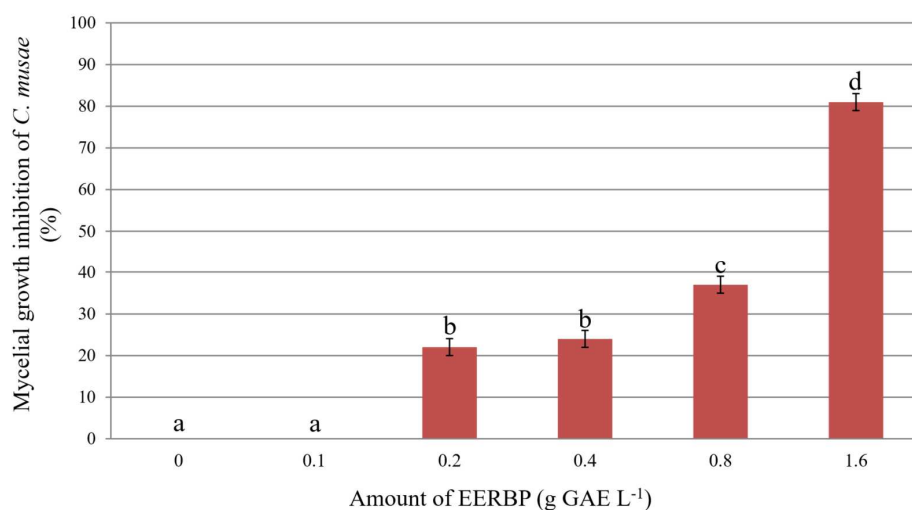


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Graphical illustration

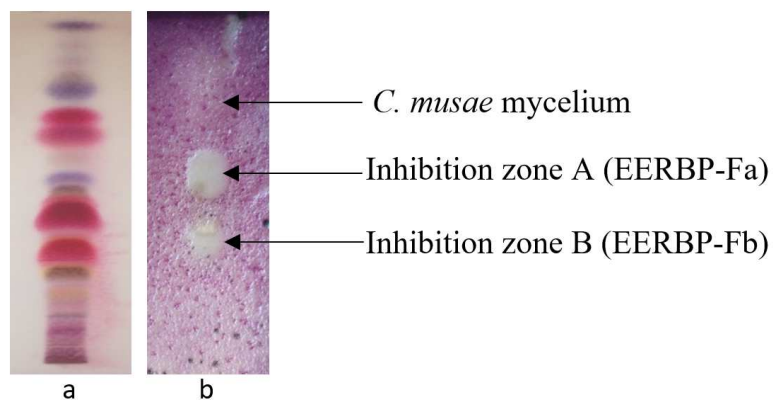


Figure 2. Graphical representation of a chemically revealed TLC plate (a) and bioautogram (b) of Ethanolic Extract of Red Brazilian Propolis (EERBP)

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

Graphical illustration

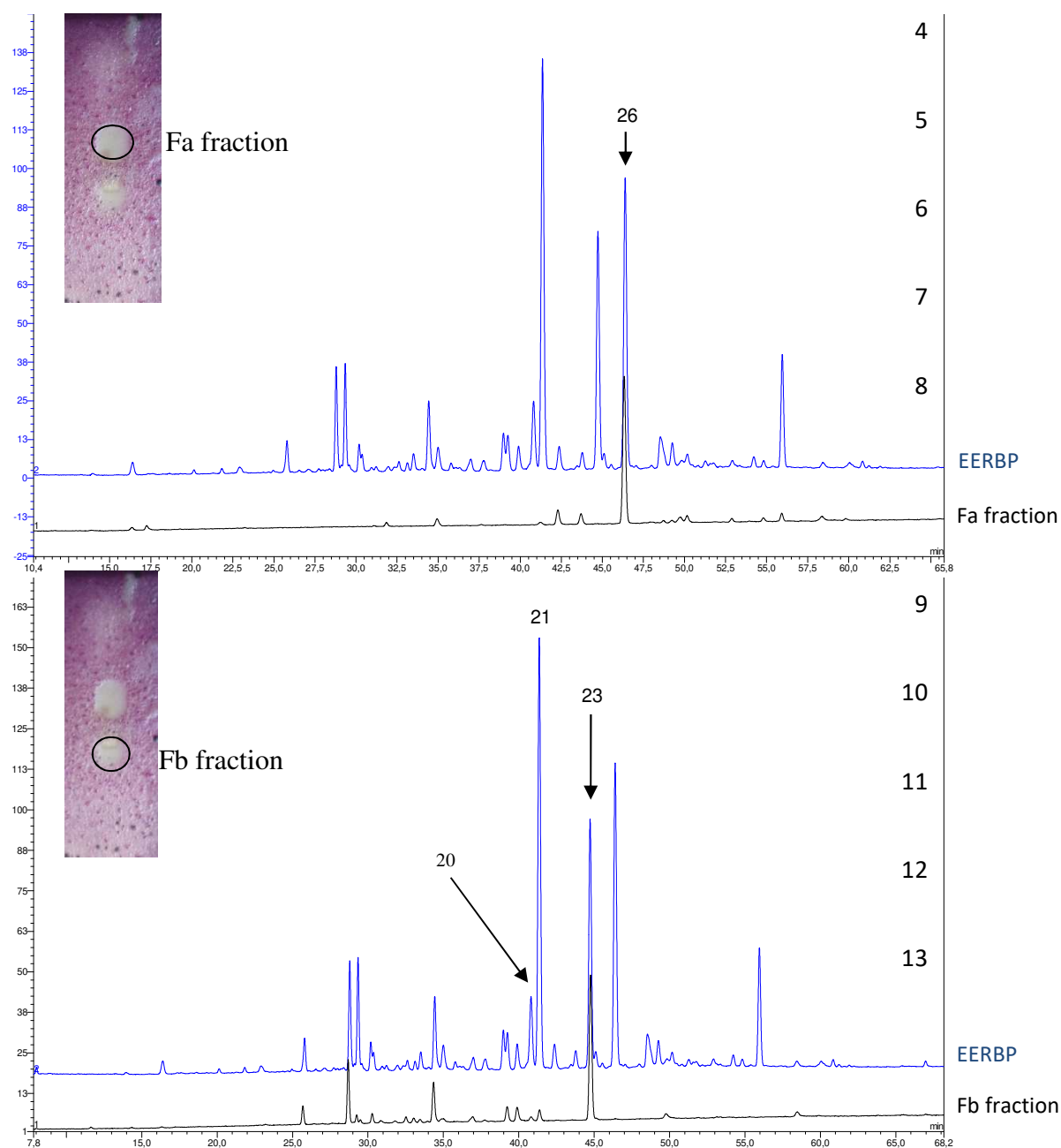


Figure 3. Superposition of HPLC chromatograms of Ethanolic Extract of Red Brazilian Propolis (EERBP), and the Fa and Fb isolated fractions. Peak assignment refers to Table 1.

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

<i>Peak</i>	<i>R_t</i> (<i>min</i>)	<i>λ_{max}</i> (<i>nm</i>)	[<i>M-H</i>] ⁺	<i>MS</i> ²	<i>MS</i> ³	<i>Content</i> (%) [‡]	<i>Compound</i>
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.