ANTIFUNGAL ACTIVITY AGAINST POSTHARVEST FUNGI BY EXTRACTS FROM COLOMBIAN PROPOLIS

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ANTIFUNGAL ACTIVITY AGAINST POSTHARVEST FUNGI BY EXTRACTS FROM COLOMBIAN PROPOLIS. The aims of the present study were to evaluate the antifungal properties of Colombian propolis extracts against *Collectorichum gloeosporioides* and *Botryodiplodia theobromae*, and to isolate and identify the main constituents from the active extracts. Therefore, propolis samples were thoroughly extracted with *n*-hexane/methanol (EPEM), dichloromethane, ethyl acetate, and methanol. Experimental results indicated that mycelial growth of all selected microorganisms was reduced in culture media containing EPEM and dichloromethane fractions. Furthermore, through antifungal bioassay-guided fractionation, three known labdane-type diterpenes: isocupressic acid (1), (+)-agathadiol (2) and epi-13-torulosol (3) were isolated as the main constituents from the active fractions.

Keywords: antifungal activity; diterpenes; Colletotrichum.

INTRODUCTION

Anthracnose (caused by *Colletotrichum gloeosporioides*) and stem-end rot (caused by *Botryodiplodia theobromae*) are two important post-harvest diseases, which affects many tropical and subtropical fruits,^{1,2} including papaya (*Carica papaya* L.), avocado (*Persea americana*) and mango (*Mangifera indica* L.). The use of synthetic chemicals as fungicides is the primary method of control of postharvest fungal decay caused by both diseases. However, several fungicides are not used for postharvest treatment or have been removed from the market due to possible toxicological risks.³ Therefore, there is a need to develop new and acceptable post- and pre-harvest disinfestation methods. In this way, the use of natural products, either directly as crude preparations, or as pure compounds, can be a very attractive method for postharvest disease control of mango, papaya and avocado fruits.

Propolis (or bee glue) is a natural resinous substance produced by Apis mellifera bees made up from parts of the plants, buds and exudates. In the beehive, propolis is thought to be used to seal holes, exclude draught and protect against external invaders. Its main function, however, is to prevent the decomposition of organic matter (i.e. creatures that have been killed by bees after an invasion) within the hive by inhibiting microbial growth.⁴ Therefore, the presence of propolis may provide an environment not suitable for the growth of fungi and other microorganisms, and consequently to keep the hive environment aseptic.⁵ In this way, one of the most frequent applications of bee glue is its antimicrobial activity against many gram-positive and gram-negative bacteria, yeasts and fungi, most of them associated with varying degrees of pathogenicity in humans.^{6,7} However, the action of propolis against phytopathogenic fungi has received little attention.4 Thus, this natural antifungal agent could be potentially exploited in controlling the growth of phytopathogenic fungi during post- and pre-harvest.

Reports by several authors support the fact that the chemical composition and biological activities of propolis depends on many different factors such as the geographical region, collecting time, and plant source.^{8,9} Thus, propolis originating from temperate zones (West Asia, Europe, and North America) possesses a quite

different chemistry from that of tropical propolis.¹⁰ In temperatezone propolis, exudates of poplar trees (Populus spp.) and horsechestnut trees (Aesculus hippocastanum) are mentioned as the main sources of European and North American propolis. However, in tropical regions, there are no Populus spp., and bees use other plant sources for the production of propolis.^{11,12} Thus, different compounds have been reported in tropical propolis, such as terpenoids and prenylated derivatives of p-coumaric acids in Brazilian propolis,^{13,14} lignans in Chilean propolis,¹⁵ and polyisoprenylated benzophenones in Venezuelan, Brazilian, and Cuban propolis.^{10,16-19} Propolis from Colombia, however, has only recently begun to be studied; therefore, information concerning to its chemical composition, phytochemical origins, and phytotherapeutic properties is still especially limited. The Colombian flora presents a great biodiversity with a high percentage of endemic plant species and consequently the chemical investigation of Colombian propolis appeared to be very interesting. Moreover, although propolis is produced and used in Colombia in folk medicine, in cosmetology and in the food industry, its chemical composition and biological activities have little been evaluated.

Considering that only a few studies have been reported on the composition of Colombian propolis, and even less about its *in vitro* effect against phytopathogenic fungi, the objective of this work was to evaluate the chemical composition and antifungal potential of their constituents on the growth of *Colletotrichum gloeosporioides* (Penz.) Sacc and *Botryodiplodia theobromae*, two well-known preand postharvest pathogens that give rise to important plant diseases and considerable crop economic losses.

EXPERIMENTAL

Propolis samples

Crude propolis of bee *Apis mellifera* was collected from an experimental apiary (Apiary of the National University of Colombia, LIMA) located in the city of Medellín, Antioquia, Colombia. Bee glue samples were gathered along the dry (April-October, 2005, 62.0 g, PROP1) and rainy (December-March, 2005, 145.0 g, PROP2) seasons. Samples were obtained from plastic nets and were subsequently frozen to promote propolis removal.

General experimental procedure

Thin-layer (TLC) and column (CC) chromatography

TLC was achieved on precoated Merck Kieselgel 60 F_{254} (0.25 or 0.50 mm thickness); the compounds were visualized by spraying plates with aqueous solution 5% FeCl₃ and/or 1% vanillin in 96% sulfuric acid followed by brief heating (~120 °C, 1 min). Column chromatography (CC) was performed over Sephadex LH-20 (Amersham Bioscience, Sweden) or Silica gel 60 (0.040-0.063 mm; Merck).

Preparative high performance liquid chromatography (HPLC)

Preparative HPLC (Agilent 1100 UV/Vis, model LC-20) was used with a Zorbax SB-CN column for the separation of two of the isolated main compounds. HPLC separation was done with an isocratic method using *n*-hexane-EtOAc, 85:15 at 4.0 mL/min as eluent. Detection was achieved at 254, 280 and 310 nm.

Gas chromatography-mass spectrometry (GC-MS) analysis

GC-MS analysis was performed on a Hewlett-Packard 6890 gas chromatograph (Agilent Technologies) coupled to an Agilent MSD 5973 mass detector under electron impact ionization. The separation was carried out using a 30 m x 0.25 mm x 0.25 µm DB-35MS column (Agilent Technologies). Propolis samples were analyzed after derivatization using a method described by Silici and Kutluca.20 Briefly, about 1mg of the propolis samples was mixed with 50 µL of dry pyridine and 100 µL bis(trimethylsilyl)trifluoracetamide (BSTFA) and 1% trimethylchlrosilane (TMCS) in a sealed glass tube for 30 min at 100 °C. Sample volume of 1 µL was injected and analyzed by GC-MS with the column held initially at 100 °C for 5 min and then increased to 150 °C and then kept at 150 °C for 2 min. Finally, temperature was increased to 280 °C with a 2 °C/min heating and the temperature was kept 280 °C for 60 min for samples. The injection was carried out in split mode at 250 °C. The GC was operated in constant flow mode (1 mL/min) with helium (99.996% purity) as carrier gas. The GC-MS peaks were identified by comparison with data from literature and the profiles from the Nist 02 mass spectral database.

Structure elucidation

Infrared (IR) spectra of the isolated compounds were recorded on a Bruker spectrophotometer (model IFS 28/55), in the frequency range 4000-400 cm⁻¹. Nuclear magnetic resonance, NMR, spectra (¹H, ¹³C, COSY, DEPT, HMQC and HMBC) were determined with a Bruker AMX 300 spectrometer with 300.12 MHz for ¹H- and 75.42 MHz for ¹³C-nuclei. Tetramethylsilane (TMS) was used as internal standard for NMR spectra measured in deuterochloroform (CDCl₃); chemical shifts are expressed in ppm units downfield from TMS and coupling constants (*J*) are in Hz. Electron impact mass spectra (EI-MS) measurements of isolated and derivatized compounds were obtained using GC-MS. Chromatographic conditions were the same as described above. All chemicals were analytical grade and used without further purification.

Extraction and isolation of the main compounds from active extracts

Propolis samples were ground and thoroughly extracted (in the absence of bright light, at room temperature) successively with *n*-hexane, dichloromethane, ethyl acetate and methanol for 24 h by percolation. The extract in *n*-hexane was further fractionated with methanol and filtered through Whatman no. I filter paper, to obtain the methanolsoluble fraction from *n*-hexane extract (EPEM). This treatment allowed discarding a wax, insoluble in methanol. All extracts were evaporated to dryness under vacuum using a rotary evaporator at 40 °C and weighed. The CH₂Cl₂-soluble fraction (10.26 g), which showed the greatest antifungal activity according to established statistics parameters, was subjected to Si gel column chromatography with *n*-hexane-EtOAc gradient and further then ethanol and methanol to give six fractions [fraction A, *n*-hexane and 90% *n*-hexane-EtOAc eluate, 2.6710 g; fraction B, 80% *n*-hexane-EtOAc eluate, 1.5214 g; fraction C, 70% *n*-hexane-EtOAc eluate, 0.4796 g; fraction D, 50% *n*-hexane-EtOAc eluate, 1.2483 g; fraction E, ethanol eluate, 2.1456 g; fraction F, methanol eluate, 1.5688 g].

In the same way, the methanol-soluble fraction from *n*-hexane extract (EPEM, 10.29 g) was chromatographed on silica gel; elution was performed with a *n*-hexane-EtOAc gradient system and further ethanol and methanol. Several fractions were collected and separated into ten groups (A-J), by TLC: fraction A and B: *n*-hexane eluate, 0.1119 g; fraction C, D and E: 5% EtOAc-*n*-hexane eluate, 4.9506 g; fraction F: 10% EtOAc-*n*-hexane eluate, 0.5871 g; fraction G: 20% EtOAc-*n*-hexane eluate, 0.342 g; fraction H e I, ethanol eluate, 2.3443 g; fraction J: MeOH eluate, 1.129 g. Fractions were monitored by TLC and antifungal assay.

Sub-fractions selected on the basis of its activity in the antifungal bioassays were: fraction C from CH_2Cl_2 extract and fraction I from EPEM. The fraction C (379.6 mg) from dichlorometane extract was fractionated by size-exclusion column chromatography over Sephadex LH-20 (100 x 2 cm) using *n*-hexane- CH_2Cl_2 -MeOH (50:25:25, v/v) as eluent (10 fractions were obtained). Samples 5, 6 and 7 were assembled (88.2 mg) according with its similar composition and repeated column chromatography of this resultant fraction over silica gel, followed by preparative TLC (*n*-hexane, EtOAc, ethyl ether: 85:15:40 as eluent), yielded the compounds: (1) (11.7 mg) and (2) (6.5 mg).

Fraction I (0.5818 g) obtained from EPEM extract through CC using ethanol as eluent was applied in succession to column chromatography on Sephadex LH-20 with *n*-hexane-CH₂Cl₂-MeOH 2:1:1 (20 fractions) and further on Si gel with a *n*-hexane-EtOAc gradient (0, 1, 2, 5, 10, 20, 50, 100% EtOAc) of sub-fractions 11-14 (43.5 mg) yielding seven fractions of increasing polarity. Subsequent purification from sub-fractions 3-5 by preparative HPLC using *n*-hexane, EtOAc, 85:15 as solvent system, afforded the compounds (1) (7.0 mg) and (3) (14.3 mg).

Isocupressic acid (1) was obtained as a colorless semisolid. FT-IR (KBr, cm⁻¹): 3376, 3084, 2933, 2849, 1701, 1643, 1445, 1377, 1226, 1179, 887. ¹H NMR (CDCl₃, 300 MHz): δ 5.39 (1H, t, *J* = 6.9, H-14), 4.86 (1H, s, H-17b), 4.53 (1H, s, H-17a), 4.16 (2H, d, *J* = 6.9, H-15), 2.39 (2H, m, H-7), 2.13 (2H, m, H-12), 1.90-1.83 (6H, m, H-1, H-2, H-3, H-4, H-5, H-9), 1.68 (3H, s, Me-16), 1.63-1.25 (3H, m, H-2, H-11), 1.24 (3H, s, Me-18), 1.05-0.65 (2H, m, H-1, H-3), 0.60 (3H, s, Me-20); ¹³C NMR (CDCl₃, 75 MHz): δ 184.1 (C-19), 148.4 (C-8), 140.9 (C-13), 123.3 (C-14), 106.9 (C-17), 59.8 (C-15), 56.7 (C-5), 56.0 (C-9), 44.6 (C-4), 40.9 (C-10), 39.6 (C-1), 39.2 (C-7), 38.8 (C-3), 38.4 (C-12), 29.4 (C-18), 26.5 (C-6), 22.4 (C-11), 20.3 (C-2), 16.8 (C-16), 13.2 (C-20); EIMS: *m/z* (%) 302 (M⁺-18: 5), 287 (M⁺-18-15: 30), 121 (100), 107 (65), 81 (74).

Agathadiol (2) was obtained as a white semisolid. FT-IR (KBr, cm⁻¹): 3287, 2931, 1646, 1457, 1027. ¹H NMR (CDCl₃, 300 MHz): 5.39 (1H, t, J = 6.9, H-14), 4.83 (1H, s, H-17b), 4.52 (1H, s, H-17a), 4.16 (2H, d, J = 6.9, H-15), 3.76 (1H, d, J = 10.8, H-19a), 3.39 (1H, d, J = 10.8, H-19b), 2.37 (1H, m, H-7), 1.93 (1H, m, H-12), 1.85-1.80 (6H, m, H-1, H-2, H-3, H-6, H-7, H-9), 1.67 (3H, s, Me-16), 1.60-1.28 (6H, m, H-1, H-2, H-5, H-6, H-11), 0.98 (3H, s, Me-18), 0.88-0.85 (1H, m, H-3), 0.65 (3H, s, Me-20); ¹³C NMR (CDCl₃, 75 MHz): 148.3 (C-8), 140.2 (C-13), 123.5 (C-14), 107.0 (C-17), 65.5 (C-19), 59.9 (C-15), 56.8 (C-5), 56.7 (C-9), 41.1 (C-10), 39.4 (C-12), 39.0 (C-1), 38.8 (C-4), 35.8 (C-7), 27.5 (C-18), 24.9 (C-6), 22.3 (C-11), 19.4 (C-2), 16.8 (C-16), 15.8 (C-20). EIMS: m/z (%) 291 (M⁺-15; 10), 257 (50), 189 (37), 121 (40), 95 (100), 81 (78).

Epi-13-torulosol (**3**) was isolated as a white amorphous powder. FT-IR (KBr, cm⁻¹): 3425, 3293, 3068, 2936, 1600, 1585, 1239. ¹H NMR (CDCl₃, 300 MHz): δ 5.92 (1H, dd, *J* = 17.4, *J* = 10.8, H-14), 5.21 (1H, dd, *J* = 17.4, *J* = 1.2, H-15a), 5.06 (1H, dd, *J* = 10.8, *J* = 1.2, H-15b), 4.82 (1H, s, H-17b), 4.53 (1H, s, H-17a), 3.76 (1H, d, *J* = 10.9, H-19a), 3.39 (1H, d, *J* = 10.9, H-19b), 1.29 (3H, s, Me-18), 0.98 (3H, s, Me-16), 0.65 (3H, s, Me-20); ¹³C NMR (CDCl₃, 75 MHz): δ 148.6 (C-8), 145.7 (C-14), 112.1 (C-15), 107.2 (C-17), 74.1 (C-13), 65.5 (C-19), 57.8 (C-9), 56.8 (C-5), 41.8 (C-12), 40.2 (C-1), 39.4 (C-4), 39.3 (C-10), 39.1 (C-7), 35.9 (C-3), 28.1 (C-16), 27.5 (C-18), 24.9 (C-6), 19.4 (C-2), 18.3 (C-11), 15.7 (C-20). EIMS: *m/z* (%) 288 (M⁺-18: 5), 257 (53), 107 (79), 95 (97), 81 (100), 43 (89).

Antifungal assays of extracts and fractions

Colletotrichum gloeosporioides was originally obtained from anthracnose diseased fruits of papaya (Carica papaya) and mango (Mangifera indica). Botryodiplodia theobromae was isolated from avocado (Persea americana) fruits infected with stem-end rot. Fungi were morphological and molecularly characterized and pleasantly provide by Phytopathology Laboratory of National University of Colombia (Medellín). The microorganisms used for the bioassays were selected among some of the most economically important fungus to control in Colombia. The effects of plant crude extracts and fractions on colony growth of C. gloeosporioides and B. theobromae were performed by the agar dilution method. Briefly, antifungal activity was evaluated on these fungi at propolis extract concentrations of: 100, 250, 500 and 1000 mg of propolis extract/L of culture medium (all subfractions, however, were only evaluated at the concentration of 250 mg/L against phytopathogenic fungus C. gloeosporioides isolated from mango). Fungal plugs (0.4mm in diameter) were obtained and placed at the center of Petri dishes in a potato-dextrose-agar (PDA) culture media with extracts (fractions or sub-fractions) dissolved in ethanol:tween 60, 1:1 (v/v) at the various concentrations. PDA plates containing only ethanol:tween 60, 1:1 (v/v) were used as control plates. The cultures were incubated at 24 ± 2 °C and the radial growth of mycelia measured daily during 7 days for C. gloeosporioides and 2 days for B. theobromae. The second one presented a fast radial growth of mycelium and reached the edge of the plates after 2 d of inoculation. The radial mycelial growth was measured daily, and the percentage of inhibition was calculated on the basis of growth in control plates as:

Percentage of mycelial growth inhibition

$$= \frac{\text{mycelial growth in control} - \text{mycelial growth in propolis}}{\text{mycelial growth in control}} \times 100$$

The antifungal effect was measured under a random design with three replications.

Antifungal assays of isolated compounds

Antifungal activity of isolated compounds was evaluated by the paper disk-agar diffusion method. Test plates (diameter: 15 cm) were prepared with PDA agar medium and inoculated in surface with a spore suspension of fungi (the concentration was adjusted to 10^6 CFU/mL). Sterile paper disks (diameter: 1 cm, Whatman no 1) were impregnated with the substances until to reach concentrations of 1.0 x 10^4 and 5.0 x 10^4 mg/kg of paper, or with the commercial fungicides, Chlorothalonil (50% active ingredient) and Mancozeb (80% active ingredient) as standard antifungal agents. Impregnated disks were placed on the assay plates. Discs soaked in sterilised distilled water without extracts were used as negative control. The fungi were cultivated in Potato Dextrose (PD) agar at 25 °C. After 24-72 h incubation, zones of inhibition (mm in diameter) were recorded. For each compound, two replicate trials were conducted against each fungus.

Statistical analysis

Analysis of variance was performed. To determine differences in radial growth between samples and controls, Duncan's and Tukey's multiple pairwise comparisons tests were applied to the results (*p*-levels at 0.01 and below were considered significant).

RESULTS AND DISCUSSION

Extraction yield

Table 1 shows an overview of the propolis used, as well as the extraction yields, for the extracts. Bee glue samples, PROP1 and PROP2, were collected during the dry (April-October, 2006) and rainy (December-March, 2006) seasons, respectively. The extraction yields ranged from 0.4% for EtOAc-extract to 36.1% for CH₂Cl₂-extract, both proceeding from PROP 1. EtOAc-extracts from both propolis samples gave the lowest yields; 0.4 and 0.5% for PROP1 and PROP2, respectively. According to the results of the TLC and GC screening, the qualitative chemical composition for both propolis was very similar. EPEM fractions revealed a complex chemical composition with various peaks at different retention times whereas CH2Cl2 fraction presented few compounds (Figure 1S, supplementary material). However, the GC chromatogram pattern of EPEM and CH₂Cl₂ extracts from dry season was very similar from those of samples collected on rainy season. Thus, seasonal variations in bee glue composition are not significant and only minor quantitative differences were detected. Some main peaks observed on GC-MS were identified by comparison with Nist database corresponding to aromatic acids, fatty acids, sesqui- and diterpenes, flavonoids, among others. The most significant constituents detected in propolis extracts using GC-MS were: fatty acids and their esters (palmitic acid, stearic acid, oleic acid), aromatic acids (cinnamic acid, dihydrocinnamic acid, and p-methoxycinnamic acid), sesquiterpenes (δ -cadinene, β -caryophyllene, β -gurjunene, aromadendrene), diterpenes ((+)-totarol, ferruginol, manool), triterpenes (lupeol), flavonoids (naringenin, sakuranetin) among others. Consequently, propolis extracts proceeding from propolis collected during the dry and rainy seasons were combined according to polarity in order to obtain only four extracts in a suitable amount for subsequent analysis.

 Table 1. Extraction yield for Colombian propolis collected during the dry (PROP1) and rainy seasons (PROP2)

Extracts	PROP1 Comp	(145 g) osition	PROP2 (62 g) Composition		
	Grams	Percent	Grams	Percent	
Waxes	40.60	28.0	18.15	29.3	
Fraction EPEM	31.80	21.8	15.80	25.5	
Fraction CH ₂ Cl ₂	52.60	36.1	17.00	27.5	
Fraction EtOAc	0.52	0.4	0.30	0.5	
Fraction MeOH	5.00	3.4	3.60	5.8	
Inert material	15.00	10.8	7.10	11.4	

In vitro antifungal activity

The effect of different concentrations of the four extracts of Colombian propolis against phytopathogenic fungi is shown in Table 2. The reduction in radial mycelial growth of fungi in culture PDA media with CH₂Cl₂ and EPEM extracts was higher than those with EtOAc and MeOH extracts. The phytopathogenic fungi, *C. gloeosporioides*, isolated from papaya and mango, as well as *B. theobromae* were sensitive to both active extracts. In addition, antifungal activities of CH_2Cl_2 and EPEM samples were found to enhance with the increase in concentration of the extracts. The maximum inhibition level was 47.6% achieved by CH₂Cl₂-soluble fraction against *C. gloeosporioides* from mango.

The dichloromethane extract was found to possess higher degree of antifungal activity than EPEM extract against *C. gloeosporioides* (both strains) at each concentration between 250 and 1000 mg/L. Particularly, at 500 mg/L, CH₂Cl₂ fraction was observed to possess significantly (Tukey, $p \le 0.01$) higher (30.8%) antifungal activity against the strain proceeding from papaya than that of EPEM extract (24.0%). The phytopathogenic fungus *B. theobromae*, however, was more sensitive to EPEM extract.

Culture PDA media with CH₂Cl₂-soluble fraction resulted in significant (Tukey and Duncan, $p \le 0.01$) reduction on colony development of C. gloeosporioides isolated from mango (Figure 1) between 100 and 1000 mg/L and for the fungus C. gloesporioides from papaya, the same parameter was also significant between 250 and 1000 mg/L. However, comparing the myceliar growth inhibition for each concentration evaluated revealed that the strain derived from mango was the most sensitive fungus to the treatments with the above mentioned propolis sample. Otherwise, CH₂Cl₂ extract showed lower degree of antifungal activity against B. theobromae than that observed with both strains of C. gloeosporioide; at 1000 mg/L concentration, phytopathogenic fungi from mango and papaya were inhibited 47.6 and 38.1%, respectively, while that proceeding for avocado had the lower inhibition at this concentration, being 23.5%. Several common compounds, corresponding mainly to aromatic acids, were identified in CH₂Cl₂ soluble fraction by GC-MS analysis: the three majority peaks were identified as cinnamic acid (Rt = 20.48 min), *p*-methoxycinnamic acid (Rt = 57.46 min) and dihydrocinnamic acid (Rt = 34.28 min).



Figure 1. Inhibitory effect of CH_2Cl_2 extract on mycelial growth of C. gloeosporioides from mango at 5 days after incubation

Additionally, EPEM fraction demonstrated significant mycelial growth inhibition of the fungus B. theobromae (Tukey and Duncan, $p \le 0.01$) at concentrations between 250 and 1000 mg/L. The concentration of 100 mg/L was not statistically different from the control. On the other hand, concentrations from 100 to 1000 mg/L of EPEM, showed significant antifungal activity against both strains of *C. gloeosporioides*, (Tukey and Duncan, $p \le 0.01$). Radial growth was inhibited above 39 and 23% at 500 mg/L against the phytopathogenic fungus C. gloesporioides, from mango and papaya, respectively. The maximum inhibition level achieved by EPEM fraction was 39.8% against C. gloeosporioides from mango. The chromatographic profiles of the EPEM extract showed a wide range of compounds of varying polarity. The GC-MS analysis allowed the identification of fatty acids and their esthers (peaks with retention times, Rt, from 35.0 to 53.0 min), sesqui- and diterpenes, principally. EPEM sample obtained during rainy season presented as main components fatty acid methyl esters (palmitic acid methyl ester, Rt =

Table 2. Effect of propolis extracts on mycelial growth of C. gloeosporioides from mango and papaya, and B. theobromae from avocado

Propolis extracts	Concentration (mg/L)	Radial mycelial growth (mm)			Inhibition of mycelia growth (%)		
		C. gloeosporioides (mango)	C. gloeosporioides (papaya)	B. theobromae	C. gloeosporioides C (mango)	C. gloeosporioides (papaya)	B. theobromae
	100	40.1*	40.1*	43.2	17.0	13.4	10.0
EPEM	250	32.3*	38.1*	36.0*	33.1	17.7	25.0
	500	29.2*	35.2*	35.1*	39.5	24.0	26.9
	1000	29.1*	34.2*	34.1*	39.8	26.1	29.0
	control	48.3	46.3	48.0	0	0	0
CH ₂ Cl ₂	100	34.2*	49.0	42.0	31.6	5.8	8.7
	250	33.4*	40.1*	40.1*	33.2	22.9	12.8
	500	29.2*	36.0*	38.2*	41.6	30.8	17.0
	1000	26.2*	32.2*	35.2*	47.6	38.1	23.5
	control	50.0	52.0	46.0	0	0	0
EtOAc	100	48.1	52.0	36.0	2.6	4.4	3.0
	250	48.1	50.0	36.1	2.6	8.1	2.7
	500	47.1	48.1*	34.1	4.7	11.6	8.1
	1000	46.2	48.1*	35.0	6.5	11.6	5.7
	control	49.4	54.4	37.1	0	0	0
МеОН	100	41.1	43.1	43.1	3.3	2.9	0
	250	40.2*	43.0	43.1	4.4	3.2	0
	500	40.1*	42.0	41.0	5.7	5.4	4.9
	1000	38.3*	42.1	41.0	9.9	5.2	4.9
	Control	42.5	44.4	43.1	0	0	0

Values represent means of measurements made on three independent plates per treatment. * $p \le 0.01$ vs. respective control.

36.05 min and stearic acid methyl ester, Rt = 44.69 min). Otherwise, EPEM extract obtained from propolis collected in the dry period presented as majority peaks those corresponding to (+)-totarol (Rt = 57.88 min) and manool (Rt = 52.98 min).

However, EtOAc and MeOH extracts demonstrated a slight fungistatic activity even at the highest concentrations evaluated, 500 and 1000 mg/L. Results showed that, for these fractions, there were no significant differences between samples with 100 and 250 mg/L of extracts and the control. Therefore, high polarity fractions obtained from the partition between immiscible solvents showed the lowest values of percentage of inhibition.

The dichloromethane fraction that showed the highest antifungal activity was further fractioned (6.0 g) into six fractions (fractions A-F) on silica gel CC eluting with petroleum ether-ethyl acetate (9:1, 8:2, 7:3, 1:1, v/v), ethanol and methanol. All sub-fractions were controlled by TLC (Figure 2, right) and samples C-G were only tested for antifungal activities against C. gloeosporioides isolated from mango (fractions A and B collected with petroleum ether-ethyl acetate, 9:1 and 8:2, were discarded because they were mainly constituted of waxes). The antifungal effect of the fractions was only measured against this fungus because its radial growth was shown to be more homogeneous (colonies were completely circular and compact) than that on C. gloesporioides proceeding from papaya, and B. theobromae. Activity was determined under a random design with two replications. TLC plates showed few spots for each subfraction evaluated with retention factor values of components varying in a narrow range. As can be seen from Figure 2 (left), all subfractions (C-F) exhibited considerable antifungal activity; culture PDA media with samples C-F, resulted in significant (Tukey and Duncan, $p \le 0.01$) reduction on colony development of fungus at 250 mg/L. However, the sub-fraction C collected with the elution system petroleum ether-ethyl acetate, 7:3 v/v, demonstrated the highest antifungal activity (40% of mycelial growth inhibition).



Figure 2. Antifungal activities (left) and TLC chromatogram (right) of dichloromethane subfractions (left). All fractions were evaluated at the concentration of 250 mg/L against phytopathogenic fungus C. gloeosporioides isolated from mango. The results are means of two different experiments. TLC was developed in n-hexane/ethyl acetate 70:30 sprayed with 5% ferric chloride and 1% vanillin in 96% sulfuric acid followed by brief heating

In the same way, the methanol-soluble fraction from *n*-hexane (EPEM) that showed significant antifungal activity against the phytopathogenic fungi evaluated, was fractioned into ten fractions (fractions A–J) on silica gel CC eluting with petroleum ether, petroleum ether–ethyl acetate (9.5:0.5, 9:1, 8:2, v/v), ethanol and methanol. Fraction A and B, collected using the elution system petroleum ether, and fraction C obtained with petroleum ether-ethyl acetate (9.5:0.5, v/v) were not evaluated (mostly waxy nature substances). Samples D-J exhibited significant antifungal activity (Tukey and Duncan, $p \le 0.01$), at the concentration of 250 mg/L, against *C. gloeosporioides*, isolated from mango (Figure 3). The mycelial growth inhibition varies from 39.2 % (fraction J) to 54.9% (fraction I).



Figure 3. Antifungal activities of EPEM subfractions. All fractions were evaluated at the concentration of 250 mg/L against phytopathogenic fungus C. gloeosporioides isolated from mango. The results are averages of two different experiments

Main compounds from antifungal fractions

Sub-fraction C from dichloromethane extract that showed very good antifungal activity at low concentrations was loaded onto silica gel column and the two major compounds were isolated. Their physical and spectral data are as follows.

Compound (1) was isolated as a colorless semisolid. The molecular formula of unknown compound (1) was assigned as $C_{20}H_{22}O_2$ by its NMR spectroscopic analyses and EIMS measurements. Retention time on GC was 26.19 min at the same chromatographic conditions described above. EIMS: m/z (%) 302 (M+-18: 5), 287 (M+-18-15: 30), 121 (100), 107 (65), 81 (74). IR absorptions at 3376 and 1701 cm⁻¹ showed the presence of a carboxylic group. The ¹H NMR spectrum of (1) displayed characteristic signals of a bicyclic labdane-type diterpene, particularly an allylic alcohol moiety R₂C=CHCH₂OH characterized by the olefinic proton signal at δ 5.39 (1H, t, *J* = 6.9), the secondary alcohol resonances appearing at $\delta 4.16$ (2H, d, J = 6.9), three singlet methyl groups at δ 0.60, 1.24 and 1.68, and an exocyclic double bond at δ 4.86 (s) and 4.53 (s) (the HMQC experiment identifies that both protons were directly attached to the same carbon at δ 106.9). In its ¹³C NMR spectrum, 20 resonances were subclassified by DEPT experiments into three methyls, nine methylenes (one sp² methylene), three methines (one sp² methine), and five quaternary carbons (three sp² carbons; two olefinic and one carboxylic carbons). All signals in the above spectra showed that compound (1) had a skeletal structure indicated as 15-hydroxylabda-8(17),13E-dien-19-oic acid. This was further confirmed by HMQC and HMBC experiments. Thus (1) was designated as isocupressic acid. These data are in good agreement with those observed in the literature.21-23

Compound (2) was isolated as a white semisolid. The molecular formula $C_{20}H_{34}O_2$ of compound (2) was assigned by NMR spectroscopic analyses and EIMS measurements. Retention time on GC was 25.08 min at the same chromatographic conditions described above. EIMS: m/z (%) 291 (M+-15; 10), 257 (50), 189 (37), 121 (40), 95 (100), 81 (78). The IR spectrum of (2) showed the absorptions of both hydroxyl groups at 3287 cm⁻¹. The ¹H NMR spectrum of (2) indicated three methyl groups (singlet at δ 1.67, 0.98 y 0.65), an exocyclic methylene group (δ 4.83 and 4.52), an allylic alcohol moiety similar than those on isocupressic acid characterized by the olefinic proton signal at δ 5.39 (1H, t, J = 6.9) and the secondary alcohol resonances appearing at δ 4.16 (2H, d, J = 6.9) (the ¹H-¹H COSY spectrum shows a correlation between these two protons). In addition, the ¹H NMR of compound (2) showed resonances at δ 3.76 (1H, d, J = 10.8) and 3.39 (1H, d, J = 10.8) corresponding to one primary alcohol; the HMQC experiment identifies that both protons were directly attached to the same carbon, C-19. The ¹³C NMR spectrum of (2) displayed 20 resonances, attributable to three methyls, ten methylenes (two oxygenated methylenes and one sp²

methylene), three methines (one sp² methine), and four quaternary carbons (two sp² carbons) through a DEPT experiment. Thus (**2**) was assigned as a labdane-type diterpene. The skeletal structure of (**2**) was confirmed on the basis of its HMQC and HMBC spectra. By spectroscopic methods and comparing the IR, ¹H- and ¹³C-NMR and MS spectral information with known compounds, (**2**) was identified as (+)-agathadiol, [labda-8(17),13-diene-15,19-diol].^{24,25}

In addition, fraction I from EPEM extract was submitted to fractionation by column chromatography on Sephadex LH-20 and further silica gel, followed by preparative HPLC to yield compounds (1) and (3). The molecular formula $C_{20}H_{34}O_2$ of compound (3) was assigned by NMR spectroscopic analyses. The analyses of ¹H NMR spectrum indicated the existence of mono-substituted olefinic proton resonances at δ 5.92 (1H, dd, J = 17.4, 10.8), 5.21 (1H, dd, J = 17.4, 1.2) and 5.06 (1H, dd, J = 10.8, 1.2), exocyclic olefinic proton signals at $\delta 4.82$ (s) and 4.53 (s), one primary alcohol proton resonance at δ 3.76 (1H, d, J = 10.9) and 3.39 (1H, d, J = 10.9) and three singlet methyls at δ 1.29, 0.98 and 0.65. In its ¹³C NMR spectrum, 20 resonances were subclassified by DEPT experiments into three methyls, ten methylenes (one oxygenated methylene and two sp² methylenes), three methines (one sp² methine), and four quaternary carbons (one oxygenated and one sp² carbons). All signals in the above spectra showed that compound 3 had a bicyclic-labdane diterpenoid skeleton with one allylic hydroxyl group, RC(CH₂)(OH)CH=CH₂, which was further confirmed by HMQC and HMBC experiments. Also the structure was proposed on the basis of mass spectra, using typical fragments and molecular ions, and as well as by comparison of their mass spectra with literature spectra. EIMS m/z (%): 288 (M+-18: 5), 257 (53), 107 (79), 95 (97), 81 (100), 43 (89). On the basis of spectral examination and comparison with literature data, the compound (3) was identified as epi-13-torulosol, [8(17),14-1abdadien-13S, 19-diol].²⁶



Figure 4. Structures of isolated compounds from the antifungal fractions of Colombian propolis

Antifungal activities of isolated compounds

It was not possible to demonstrate antifungal effect of the isolated compounds and at evaluated concentrations by means of the paper disc assay. The mean zone of inhibition of the compounds assayed against the phytopathogenic fungi ranged between 3 and 5 mm. Yet no statistically significant differences were observed with respect to the negative control. Among the commercial fungicides tested as positive control, the mancozeb showed the best inhibition with a significant antifungal activity (Tukey and Duncan, $p \leq 0.01$) against the three phytopathogenic fungi. The produced zones of inhibition using mancozeb ranged from 26 to 32 mm. However, the chloratalonil antifungal positive control produced zones of inhibition that ranged from 6 to 8 mm.

The weak antifungal activity demonstrated by the labdane-type compounds in the paper disc assay can be explained by three potential reasons; firstly, it has been previously shown in the literature that a single propolis component does not have an activity greater than that of the extract and its inhibitory effect depends on the synergism of many compounds.^{13,27} Secondly, due to the low recovery of the labdane-type diterpenes, the compounds were consequently tested at very low concentrations. Thirdly, it can be argued that the lack of effective fungi mycelial growth inhibition is a result of the paper disc retaining the active components and thereby preventing its diffusion into the PDA agar media. This assumption is in agreement with the fact that chloratalonil, a commercial antifungal agent commonly used to control anthracnose, was not active against Colletotrichum sp. in the paper disc assay. Chlorothalonil had previously demonstrated a potent antifungal activity against both strains of Colletotrichum sp. in the agar dilution method with a MIC (minimum inhibitory concentration which completely inhibited mycelial growth) between 400 and 450 mg/L (Data not shown). However, this result was not evident in the paper disc assay presumably for the reasons stated above. Due to the insufficient quantity of the isolated compounds, antifungal assays testing these hypotheses were not performed. Hence, paper disk-agar diffusion method is not suitable for comparison of antifungal activity of labdane-type diterpenes since the concentration of compounds that permitted measurement of the diameter of the inhibition zone is relatively high, and diterpenes samples display deficient diffusion. Due to insufficient quantity of the isolated compounds an antifungal assay considering these factors was not done.

CONCLUSIONS

The increasing social and economic repercussions of fungi food contamination require the development of new antifungal agents to provide safer foods. Thus propolis are promising natural antifungal agents. The present results are the first to show that extracts from Colombian propolis can act as antifungal agents against *Colletotrichum* sp. and *Botryodiplodia* sp. Labdane-type diterpenes could play role in the antifungal effect of EPEM and dichloromethane propolis extracts.

SUPPLEMENTARY MATERIAL

Available in http://quimicanova.sbq.org.br.

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