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ANTIFUNGAL BIOASSAY-GUIDED FRACTIONATION OF AN OIL EXTRACT OF PROPOLIS

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ABSTRACT

The study aimed to evaluate the antifungal potential of an extract of propolis obtained with edible vegetable oil (ODEP) and to identify antifungal compounds in this extract. Propolis sample was extracted with canola oil. After filtration, the oily liquid extract was submitted to solvent partition and dried to obtain ODEP, which was tested *in vitro* against *Candida albicans* strains. ODEP was fractioned on Sephadex and by high-performance liquid chromatography (HPLC). Active fractions and/or a pure compound were analyzed by nuclear magnetic resonance, liquid chromatographic mass spectrometry (LC–MS) and liquid chromatography-tandem mass spectrometry (LC–MS/MS) to characterize their chemical composition. Bioassay-guided fractionation allowed the isolation of dihydrokaempferide, which was also quantified in ODEP by HPLC together with the total flavonoid content. Kaempferide and isosakuranetin were identified by LC–MS and LC–MS/MS in fractions with a potential activity against *C. albicans*. *In vitro* assays showed that some fractions from ODEP produced better inhibition of *C. albicans* than the propolis extract itself.

PRACTICAL APPLICATIONS

Aqueous or ethanolic extracts of propolis (EEP) are widely used in alternative homemade medicine products mainly because of their antimicrobial and healing properties. Edible vegetal oils are known to extract bioactive compounds from propolis. The resulting extract (ODEP) has several advantages over the common EEP, such as the possibility of new pharmaceutical presentations for topical or internal applications. Because of the absence of ethanol in its formulation, edible ODEP are expected to be more tolerated. In this study, ODEP was active *in vitro* against *Candida albicans* strains. This supports the use of ODEP as an alternative to EEP and also its potential for topical application in combination with antifungal drugs. In addition, the ingestion of ODEP (e.g., contained in gelatin capsules), which have a high percentage of flavonoids, could be of nutritional value, helping to reach the recommended daily amounts of flavonoids and phenolic compounds.

INTRODUCTION

Aqueous or ethanolic extracts of propolis are widely used as an alternative medicinal product mainly because of their antimicrobial and anti-inflammatory properties as well as the characteristic of enhancing body resistance to illness. Because propolis, regardless of its chemical composition, possesses antimicrobial activity (Kujumgiev *et al.* 1999), in the beehives, propolis is primarily used to keep an aseptic environment, which is essential to the colony's survival.

The *in vitro* antimicrobial activity of ethanolic extracts of propolis (EEP) is among its most investigated biological activities, but due to the complex mixture in propolis extracts, few studies relate the antimicrobial activity of pure isolated compounds (Yang *et al.* 2011). The qualitative composition of propolis extracts varies with geographical origin, season and bee species. In addition, the potency of the biological activities also depends on the quantitative composition of the extracts, which is strongly influenced by the solvent and the experimental variables during extraction (Sawaya *et al.* 2002, 2011).

In a review, Sforcin and Bankova (2011) discussed the potential of propolis for the development of new drugs. They concluded that in spite of the abundant data collected about the pharmacological properties of propolis, there is a substantial lack of clinical research. Furthermore, it is essential to establish the chemical nature of the propolis extracts under pharmacological study.

Recently, we reported the *in vivo* anti-tumoral activity and composition of an extract of Brazilian propolis obtained with an edible vegetable oil (Carvalho *et al.* 2011). The oil extract (ODEP) was as effective as the EEP at inhibiting tumoral growth in mice. Few studies have reported the antimicrobial activity of ODEP, although it has shown promising *in vitro* antibacterial activity (Tosi *et al.* 1996; Buriol *et al.* 2009). As oil extracts present advantages over the more commonly used EEP, it is important to assess their pharmacological potential.

The aim of the present study was to evaluate the *in vitro* antifungal activity of ODEP and its fractions against *Candida albicans.* Altogether, we pursued to identify antifungal constituents in this functional extract. Chromatographic fractionation and isolation were bioassay-guided to seek antifungal compounds against *C. albicans*.

MATERIALS AND METHODS

Propolis and Extracts

Samples. Propolis samples were collected at the end of the summer season in March 2006 and supplied by Campolin & Schmidt Company from Prudentópolis city (Paraná State, Brazil). Propolis was stored at −18C until extraction.

Extracts. Propolis samples were extracted with Canola oil as described by Carvalho *et al.* (2011) and Buriol *et al.* (2009). Fifty grams of propolis was extracted in a shaker with 500 mL of Canola oil for 24 h at room temperature. After that period, the extractive solution was filtered and partitioned into 80:20 (v/v) methanol : water. The aqueous methanolic phase was dried in rotatory evaporator, yielding an oil-extracted dry extract of propolis (ODEP).

Chromatographic Fractionation

ODEP was separated by chromatography on Sephadex LH-20 with methanol obtaining six fractions labeled as OLSx1 to OLSx6 (Fig. 1), as described by Carvalho *et al.* (2011).

OLSx4 was chosen to further purification due to its high antifungal activity and because it had a less complex chromatogram than the other bioactive fractions. High-performance liquid chromatography (HPLC) fractionation of OLSx4 was performed in a Waters 600 system using guard column (μBondapak C18 Waters 2PK, 3.9 × 20 mm, 10 μm, Waters Technologies do Brasil Ltda, Barueri, SP, Brazil) and a C18 reversed-phase semi-preparative column (Phenomenex, 10 × 250 mm, 10 μm, Phenomenex, Torrance, CA). Elution was performed with a gradient between 30 and 100% acetonitrile in aqueous acetic acid (1% v/v) during 30 min and flow rate of 2 mL/min to produce six subfractions (OLSx4-F1 to OLSx4-F6). Detection was accomplished in a photodiode array detector (UV/PDA, Waters 2696) at 244 and 290 nm. A pure compound was crystallized from fraction OLSx4-F2 and was identified by nuclear magnetic resonance (NMR), high-resolution mass spectrometry (HRMS), tandem mass spectrometry (MS/MS) and by comparison with data from literature. The major compound from fraction OLSx4-F4 was identified by HRMS and MS/MS.

Mass Spectrometry Analysis and NMR Spectra Acquisition

Chemical composition of fractions OLSx1 to OLSx6 was described previously by Carvalho *et al.* (2011). Identification was carried out by liquid chromatographic mass spectrometry analysis (LC–MS) with electrospray ionization in the negative ion mode ESI(−) and by tandem mass spectrometry ESI(−)–MS/MS.

Herein, we report the structural identification of the compound isolated from subfraction OLSx4-F2 and the major compound in subfraction OLSx4-F4. Structural elucidation of compounds in subfractions was accomplished by ESI–MS/MS with collision-induced dissociation (CID). Spectra were acquired using a hybrid high-resolution and high-accuracy (5 ppm) Micromass Q-TOF mass spectrometer.

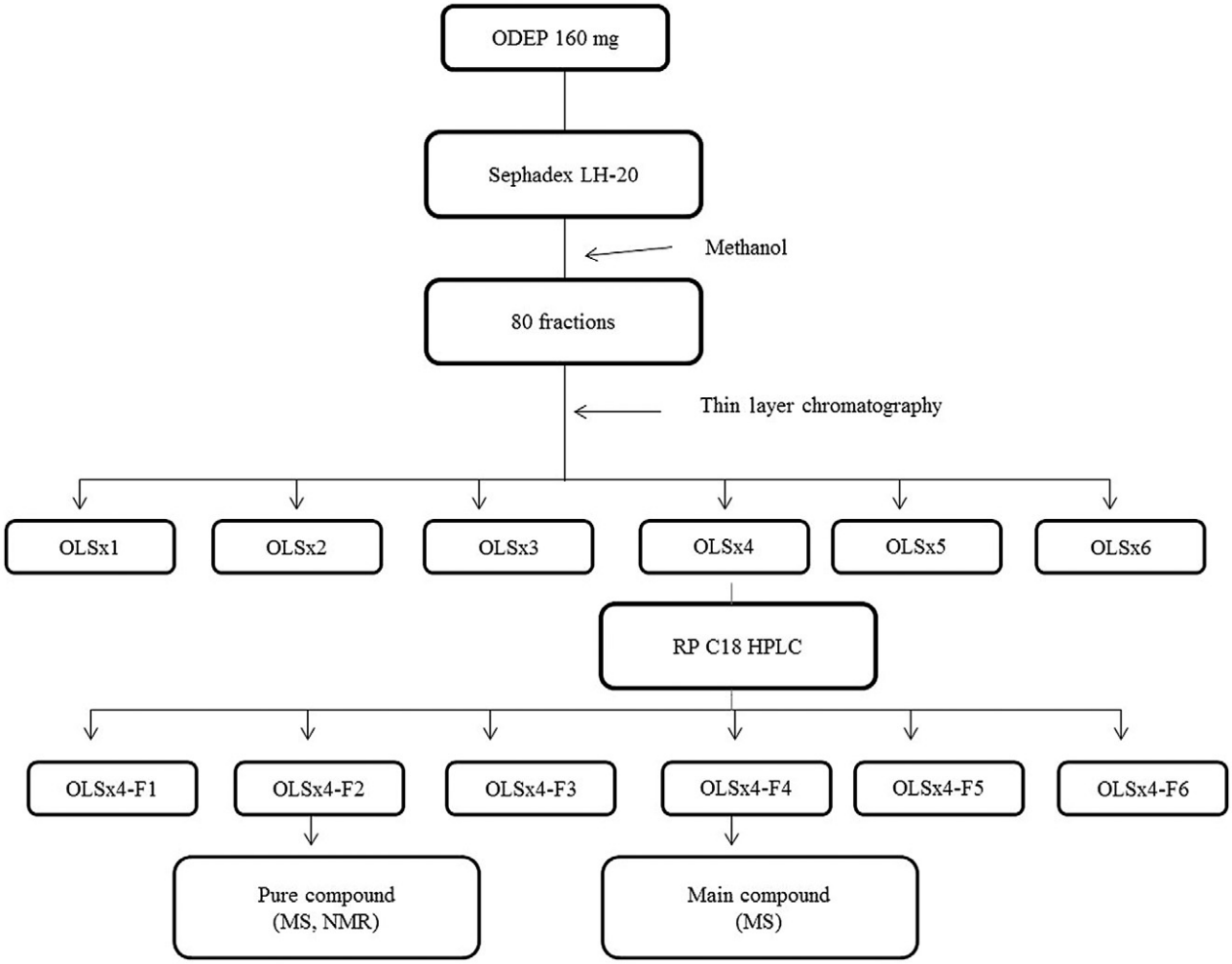

FIG. 1. ANTIFUNGAL BIOASSAY-GUIDED FRACTIONATION OF THE OIL EXTRACT OF PROPOLIS

Capillary and cone voltages were set to ±3000 and ±40 V, respectively, for the negative ESI(−) or positive ESI(+) mode. Desolvation temperature was 100C and nitrogen and argon were used as desolvation or collision gas, respectively.

The compound isolated in a pure form was also analyzed by NMR. The NMR spectra were recorded in a Bruker DRX 9.4 T instrument (Billerica, MA), operating at 400.23 MHz for ^{1}H and 100.23 MHz for ^{13}C, respectively. All NMR spectra were obtained at 298 K using tetramethylsilane as an internal reference. For the ^{1}H and ^{13}C NMR, ^{1}H-^{1}H correlated spectroscopy (^{1}H-^{1}H COSY), heteronuclear multiple bond correlation (HMBC), heteronuclear single quantum correlation (HSQC) and UV/PDA data for dihydrokaempferide (see the Supporting Information).

Antimicrobial Assays

This study involved six strains of *Candida*: one ATCC 40175 *Candida albicans* strain obtained from INCQS/FIOCRUZ (National Institute for Health Quality Control, Brazil) and five strains of *Candida albicans* (PT*1–5*) isolated from vaginal exudates of patients attending the Clinical Analysis Laboratory (CAL) of the Faculty of Pharmacy, Federal University of Pará/UFPA (Brazil). The strains were identified in the Evandro Chagas Institute (IEC/Brazil). These fungi were maintained in Sabouraud dextrose agar (SDA) (Difco Laboratories, Detroit, MI) at room temperature and subcultured monthly in the Laboratory of Microbiology in the Pharmacy Faculty at UFPA.

Yeast inoculum was prepared according to the Clinical and Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards, NCCLS) microdilution protocol (M27-A3) (CLSI 2008). Briefly, three to four colonies of yeast organisms, each 1 mm or more in diameter, kept for 24 h at 25C on SDA (Himedia M063) subcultures were suspended in 2 mL of Müller Hinton broth (Merck, Darmstadt, Germany). The resulting suspension was mechanically mixed and the cell

turbidity adjusted to correspond to a 0.5 McFarland standard. This procedure yielded a stock suspension containing 1×10^6 cfu/mL and then diluted until 1×10^3 cfu/mL. Ninety microliters was removed from this suspension and mixed with 10 μL of ODEP in dimethylsulfoxide (DMSO) or the fractions resulting from chromatographic fractioning. The solutions were incubated aerobically for 24 h at 37C.

Negative control tube consisted of 90 μL of the yeast inoculum in 10 μL of DMSO. Fluconazole (FLC), itraconazole (ITRA) and ketoconazole were used as positive controls. After incubation, the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) for each isolate were determined by subculturing 10 μL aliquots from each well of the broth dilution onto Müller Hinton agar plates (Himedia M 173). The plates were incubated for 24 h at 37C. The number of colonies growing in the presence of each concentration was determined by counting of unit of colony forming (UCF). MIC was reported as MIC_{90}, the concentration that prevented in 90% the growth of different *C. albicans* strains. MFC was defined as the lowest antimicrobial concentration that resulted in either no growth or fewer than three colonies (99.9% death) according to Quadros *et al.* (2011). Both MIC and MFC tests were carried out concurrently in triplicate.

Quantitative HPLC analysis of Flavonoids in ODEP

Flavonoids in ODEP were quantified by the external standard method. An analytical curve was plotted (peak area versus concentration) using dihydrokaempferide, isolated from ODEP, as reference standard flavonoid in six concentration levels (2–70 μg/mL). Dihydrokaempferide purity was assessed by NMR inspection. Flavonoid peaks in ODEP were identified by checking their UV/PDA spectra under the chromatographic conditions employed for the quantitative analysis.

Quantitative HPLC analysis employed a Waters 600 system with a thermostatized (30C ± 1C) analytical μBondapak C-18 column (Waters 3.9 × 300 mm, 10 μm). Elution gradient was performed as described in the chromatographic fractionation section but with a flow rate of 1 mL/min. Samples were injected in triplicates using a 20 μL loop and detected at 292 nm and by recording UV spectra between 210 and 400 nm. Data were processed by Empower 2 software (Waters Corporation, Milford, MA).

Statistical Analysis

MIC and MFC data were analyzed by a one-way analysis of variance (ANOVA). Tukey's post hoc tests was carried out to identify the average values, considering the results against all *C. albicans* strains, which statistically differ from each other at the 95% confidence level ($P \leq 0.05$). The statistical package BioEstat 5.0 (statistical development: Ayres and Ayres Jr; software development: Ayres and dos Santos; Belém, Pará, Brazil, 2007) was used.

RESULTS AND DISCUSSION

Antifungal Assays

Microdilution in broth, followed by fungal growth in plaque, was used to evaluate antifungal activity. In this study, antifungal activity of fractions and subfractions from ODEP were evaluated against six *C. albicans* strains; one sample of ATCC and five clinical isolates. MIC_{90} and MFC were determined for ODEP and its chromatographic fractions (Table 1) and subfractions (Table 2). Table 1 shows that ODEP and its fractions were able to inhibit the growth of *C. albicans* at low concentrations and at different statistically significant fungicidal potencies ($P < 0.05$). Only OLSx5 and OLSx6 had similar fungicidal activity. OLSx3, OLSx4, OLSx5 and OLSx6 fractions were more powerful in inhibiting the yeast growth with MIC_{90} values ranging from 50 to 1,200 μg/mL and MFC from 600 to 1,200 μg/mL. Furthermore, the fungicidal activity of OLSx3 to OLSx6 was significantly higher than that activity for ODEP.

TABLE 1. MINIMUM FUNGICIDAL CONCENTRATION (MFC) AND MINIMUM INHIBITORY CONCENTRATION (MIC_{90}) OF THE OIL EXTRACT OF PROPOLIS (ODEP) AND SIX FRACTIONS (OLSX1 TO OLSX6) AGAINST *CANDIDA ALBICANS* STRAINS

	ODEP and fractions (μg/mL)*													
	ODEP		OLSx1		OLSx2		OLSx3		OLSx4		OLSx5		OLSx6	
Yeast	MFC[a]	MIC_{90}[a]	MFC[b]	MIC_{90}[b]	MFC[c]	MIC_{90}[a]	MFC[d]	MIC_{90}[a]	MFC[e]	MIC_{90}[a]	MFC[f]	MIC_{90}[a]	MFC[f]	MIC_{90}[a]
C. albicans ATCC 40175	1,700	150	3,000	>300	2,700	>200	1,100	100	600	50	800	70	800	>70
C. albicans PT1	1,900	200	3,300	>300	2,900	>300	1,200	<1,200	700	>60	900	>80	900	>80
C. albicans PT2	1,900	>200	3,300	<3,200	2,900	300	1,200	120	700	60	900	<900	900	<900
C. albicans PT3	1,900	200	>3,300	3,200	2,900	<2,900	1,200	120	700	60	900	<900	900	<800
C. albicans PT4	1,900	<1,900	3,300	<3,200	2,900	300	1,200	>120	700	<700	900	<900	900	80
C. albicans PT5	>1,900	1,900	3,300	<3,200	2,900	300	>1,200	1,200	700	<700	900	<860	900	80

* Different letters represent statistical differences between average MFC or MIC_{90} values ($P < 0.05$) by Tukey's test.
PT, patients.

TABLE 2. MINIMUM FUNGICIDAL CONCENTRATION (MFC) AND MINIMUM INHIBITORY CONCENTRATION (MIC_{90}) OF SUBFRACTIONS OLSX4-F1 TO OLSX4-F6 AGAINST *CANDIDA ALBICANS* STRAINS

	OLSx4 subfractions (μg/mL)*											
	F1		F2		F3		F4		F5		F6	
Yeast	MFC	MIC_{90}	MFC	MIC_{90}	MFC	MIC_{90}	MFC	MIC_{90}	MFC	MIC_{90}	MFC	MIC_{90}
C. albicans ATCC40175	920	460	810	>400	>80	80	120	60	>80	90	>130	130
C. albicans PT1	30	<30	260	130	>80	80	100	50	>450	>450	200	100
C. albicans PT3	>2,300	>2,300	1,050	520	>80	80	200	100	>450	>450	200	>100
C. albicans PT4	70	30	520	260	>80	80	>300	>300	>450	>450	>650	>650

* No statistical differences between mean MFC and MIC_{90} values ($P > 0.05$) by Tukey's test.
PT, patients.

Because OLSx4 fraction had the best fungicidal activity (average MFC 683 ± 40 μg/mL), the lowest MIC_{90} against most of the *Candida* strains tested and also because OLSx4 had a simpler chromatographic profile by HPLC than the other bioactive fractions, it was subfractioned into OLSx4-F1 to OLSx4-F6. The antifungal activity of these subfractions was tested against some strains of *C. albicans*. Table 2 shows that all subfractions were able to inhibit the yeast growth. In addition, all subfractions had significantly higher fungicidal activity (lower average MFC) than ODEP.

Several studies have been conducted on the antifungal activity of EEP against *C. albicans* (Kujumgiev *et al*. 1999; Sawaya *et al*. 2002). Stepanović *et al*. (2003) evaluated the antimicrobial activity of 13 EEP from different regions of Serbia against 39 microorganisms. *C. albicans* was the most resistant yeast but an important synergistic effect between propolis and nystatin was reported. Taking this into account, the combination of ODEP with antifungal drugs may be of great medical interest for topical application.

On the other hand, Ota *et al*. (2001) related that several strains of *Candida* (*C. albicans*, *Candida guilliermondii*, *Candida tropicalis* and *Candida krusei*) were sensitive to the alcoholic extract of propolis. The most sensitive strain was *C. albicans*, which showed fungicidal activity at 8 mg/mL. Furthermore, Pepeljnjak and Kosalec (2004) found that propolis extracts at concentrations of 15–30 mg/mL inhibited the growth of *Candida albicans*, *Aspergillus flavus*, *Aspergillus ochraceus*, *Penicillium viridicatum* and *Penicillium notatum*. The ethanolic extract of propolis was also found to inhibit 60 strains of yeasts (Cizmárik and Trupl 1975). The fungicidal activity of ethanolic extracts of Brazilian propolis against *Paracoccidioides brasiliensis*, *C. albicans* and *Sporothrix schenckii* has already been reported (Sforcin *et al*. 2001; Salomão *et al*. 2004). In the current study, we demonstrate that extracts of propolis made with Canola oil, instead of ethanol, have potent fungicidal activity at MFC ≤ 1.9 mg/mL.

Sforcin *et al*. (2001) also reported that *C. albicans* were susceptible to low concentrations of an ethanolic extract of Brazilian propolis from the state of São Paulo. It seems therefore that the degree of susceptibility is related to both qualitative and quantitative variability of the propolis extracts (Cafarchia *et al*. 1999; D'Auria *et al*. 2003; Salomão *et al*. 2004; Silici and Kutluca 2005), consequently reinforcing the need to carry out *in vitro* and *in vivo* assays in chemically characterized extracts (Sforcin and Bankova 2011). Additionally, Silici and Kutluca (2005) showed that minor differences in antibacterial activity of propolis extracts were due to different races of honeybees.

In face of the present results, the edible ODEP may be considered a promising alternative to the EEP for the treatment of several conditions associated with *Candida* infections such as denture stomatitis and vaginitis (Casaroto and Lara 2010). Shokri *et al*. (2011) showed that an EEP effectively inhibited the growth of fluconazole-resistant *Candida glabrata* isolates obtained from women with recurrent vulvovaginal candidiasis.

Fractionation and Identification of Antifungal Compounds from ODEP

To characterize antifungal constituents from the extract, the most active fractions OLSx3 to OLSx6 were analyzed by LC–MS and LC–MS/MS. It was possible to establish the presence of 3,4-dihydroxi-5-prenyl-cinnamic acid (*m/z* 247, OLSx3); dihydrokaempferide (*m/z* 301, OLSx3 and OLSx4); 3-prenyl-4-hydroxicinnamic acid (*m/z* 231, OLSx3); (E)-3-{4-hydroxi-3-[(E)-4-(2,3-diidrocinamoyloxi)-3-metil-2-butenil]-5-prenyl-fenil}-2-propenoic acid (*m/z* 447, OLSx3); isosakuranetin (*m/z* 285, OLSx4 and OLSx6) and kaempferide (*m/z* 299, OLSx5) by their fragmentation patterns (Carvalho *et al*. 2011). In addition, all sub-fractions OLSx4-F1 to OLSx4-F6 present a strong effect against *C. albicans* strains. In subfraction OLSx4-F2, a pure compound was crystallized and analyzed for secure structural elucidation.

The compound from fraction OLSx4-F2 displayed in its high resolution ESI(−)–MS a deprotonated molecules $[M - H]^-$ of *m/z* 301.0658 corresponding to the molecular formula $C_{16}H_{14}O_6$ (calculated *m/z* 301.0718). Upon CID, a fragment ion $[M - H - 18]^-$ of *m/z* 283 due to the loss of

TABLE 3. ^{1}H NMR, ^{13}C NMR AND HMBC DATA OF DIHYDROKAEMPFERIDE

Position	δ ^{13}C[a]	δ ^{1}H (mult, *J* in Hz)[a]	^{1}H-^{1}H COSY	HMBC	δ ^{1}H (mult, *J* in Hz)[b]
2	84.7	5.00 (d, 11.84)	H3	H2′, H6′, H3	5.0 (d, 11)
3	73.7	4.50 (d, 11.84)	H2	H2	4.5 (d, 11)
4	197.9			H2, H3	
5	165.4			H6	
6	97.8	5.90 (d, 1.78)	H8	H8	5.8
7	170.3			H8, H6	
8	96.8	5.84 (d, 1.78)	H6	H6	5.8
9	164.5			H8, H2	
10	101.5			H6, H8	
1′	130.7			H3, H2, H3′, H5′, H2′, H6′	
2′ e 6′	130.3	7.45 (d, 8.91)	H3′, H5′	H3′, H5′, H2	7.4
3′ e 5 ′	114.8	6.96 (d, 8.91)	H2′, H6′	H2′, H6′	6.8
4′	161.7			H2′, H6′, H3′, H5′, H11	
11	55.8	3.80 (s)			

a Assignments by inverse detection at 400 MHz (heteronuclear single quantum correlation).
b Data from Malterud *et al*. (1985) for dihydrokaempferide taken in acetone-d_6 at 60 MHz.
HMBC, heteronuclear multiple bond correlation.

water was observed. Another ion of *m/z* 255 was formed probably due to subsequent losses of water and C_2H_4.

Flavonoid fragment ions can be designated according to the nomenclature proposed by Ma *et al*. (1997), where ^{i,j}A and ^{i,j}B labels refer to the fragments containing intact A- and B-rings, respectively. Superscripts *i* and *j* indicate the C-ring bonds that have been broken (Cuyckens and Claeys 2004). Considering this nomenclature, fragment ions of *m/z* 151 and 152 could be represented as $^{1,3}[A-H]^-$ and $^{1,3}[A]^-$, respectively (Fig. 2).

Comparison with ^{1}H NMR data from literature (Malterud *et al*. 1985) and ^{13}C NMR, ^{1}H-^{1}H COSY, HSQC and HMBC data confirmed that the isolated compound was 2*R*,3*R*-dihydrokaempferide (Table 3).

NMR analysis indicated the presence of two aromatic rings. A *para*-substitution pattern was evident for B-ring with ortho coupling hydrogens at δ 6.96 and δ 7.45 ($J = 8.91$ Hz). Constant coupling of 1.78 Hz for aromatic Hs at δ 5.84 and δ 5.90 pointed to a meta-coupling in A-ring. Two doublets at δ 4.50 and δ 5.00 with constant coupling of 11.84 Hz made clear a trans-diaxial coupling and a configuration 2*R*,3*R* at the stereogenic centers in C-ring (van Reensburg *et al*. 1997). Methoxyl group was identified by the singlet with typical shift at δ3.80.

^{13}C NMR showed 14 carbon signals. A carbonyl at δ 197.9 due to unsaturated ketone, six signals for quaternary aromatic carbons at δ 170.3; 165.4; 164.5; 161.7; 130.7 and 101.5, two more intense resonances at δ 130.3 and δ 114.8 corresponding to CH carbons in the *para*-substituted B-ring, and two resonances at δ 97.8 and δ 96.8 for CH carbons in the *meta*-substituted A-ring. Additionally, signals at δ 84.7 and δ 73.7 were assigned to metine sp^3 carbons in central C-ring. Carbon methoxyl signal was found at δ 55.8.

HMBC correlations were crucial for the correct assignment of signals found in ^{1}H NMR and ^{13}C NMR. For instance, long range coupling between carbon at δ 164.5 and hydrogens at δ 5.84 and δ 5.00, and between carbon at δ 84.8 and aromatic hydrogens at δ 7.45 enabled to establish the position of attachment of C-ring and B-ring. Quaternary carbon at δ 161.7 was assigned to C-4' position due to its correlation with metoxyl hydrogens and with aromatic hydrogens at δ 7.45 and δ 6.96. Similarly, correlation between quaternary carbon at δ 101.5 and aromatic hydrogens at δ 5.90 and δ 5.84 placed this carbon in the A-ring. Key correlations are found in Fig. 3. Each spectrum obtained is available in the Supporting Information.

As a major compound in fraction OLSx4-F4, isosakuranetin was identified by MS/MS and by comparison with an authentic isosakuranetin standard. ESI(−) and ESI(+) was applied, and because isosakuranetin has a MW of 286 Da, the deprotonated molecule $[M-H]^-$ of *m/z* 285 was observed via ESI(−)–MS. Upon CID, a fragment ion of *m/z* 270 was formed due to the loss of a methyl radical. Several other ions were originated by fragmentation at the central ring C such as that of *m/z* 243 $[M-H-42]^-$, *m/z* 107 $^{0,4}[A-H]^-$, and *m/z* 164 $^{0,4}[B-CH_3]^-$ (Fig. 2). Similarly, the ion of *m/z* 151 may be originated by simultaneous fragmentation at bonds 1 and 3, that is $^{1,3}[A-H]^-$, whereas the ion of *m/z* 136 may be formed by fragmentation at bonds 0 and 2.

CID of isosakuranetin via ESI(+)–MS/MS formed fewer ions (see Supporting Information). The protonated molecule $[M+H]^+$ of *m/z* 287 confirmed the formula assignment, and the base peak due to the fragment ion of *m/z* 153 as well as the ion of *m/z* 133 were possibly formed by fragmentation at the central ring C $^{1,3}[A+H]^+$.

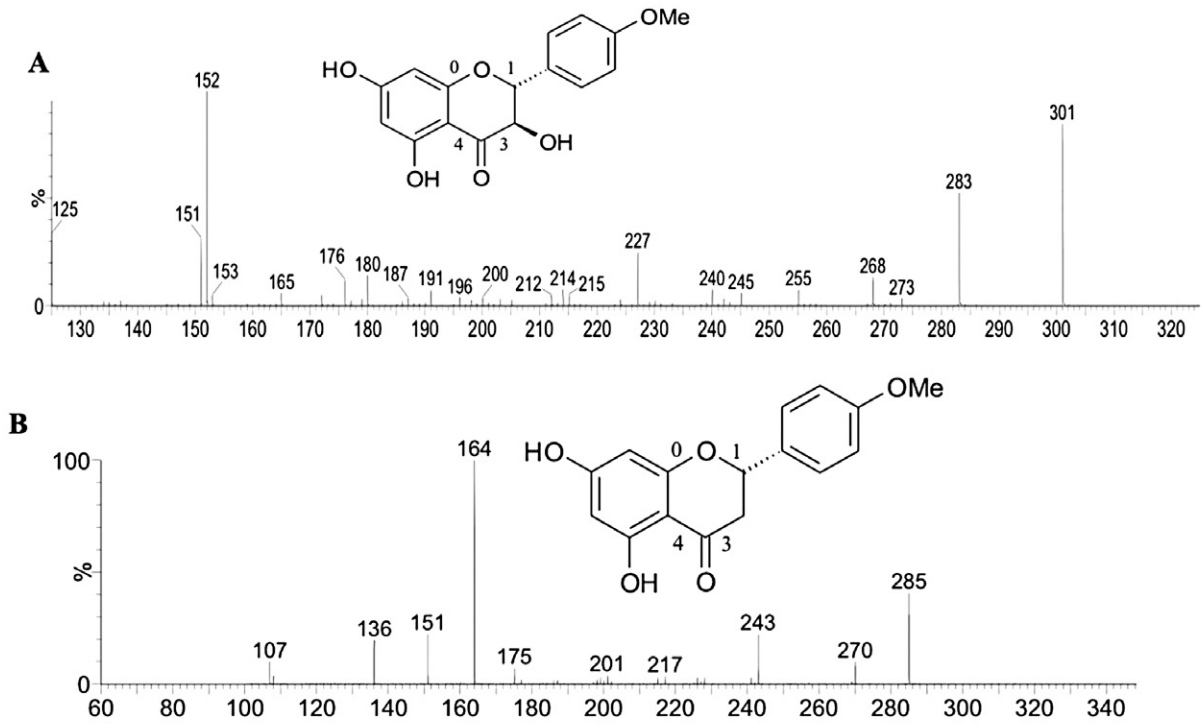

FIG. 2. ESI(-)-MS/MS OF (A) DIHYDROKAEMPFERIDE AND (B) ISOSAKURANETIN FROM THE OIL EXTRACT OF PROPOLIS

The compounds identified in the present study have been already found in propolis from Brazil and other countries and are related to many biological activities (Malterud *et al*. 1985; Banskota *et al*. 1998; Sawaya *et al*. 2002, 2004a,b; Lustosa *et al*. 2008).

Several studies have demonstrated that flavonoids with a free hydroxyl group at C-7 in ring A, as found in dihydrokaempferide (dihydroflavonol) and isosakuranetin (flavanone), have antimicrobial activity. In a screening of antimicrobial activity of crude extracts and pure natural products from Mexican medicinal plants, Rojas *et al*. (1992) reported that among the 44 natural products (and two derivatives) tested, only 23 showed significant antimicrobial activity in diffusion experiments for strains of *Staphylococus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *C. albicans*. The most active natural products were flavonoids with a free hydroxyl group at C-7 of the basic skeleton. In that study, isosakuranetin did not show significant inhibition zones (<5 mm) against *C. albicans* in the diffusion experiments and was not further tested in dilution studies against this yeast. Diffusion experiments in agar plates should be seen as a qualitative measure of antimicrobial activity because the results are strongly influenced by solubility of the compounds in the agar (Bosio *et al*. 2000; Sawaya *et al*. 2002, 2011).

Yang *et al*. (2011) recently reported a bioassay-guided isolation and identification of pinobanksin (dihydroflavonol), pinocembrin (flavanone), chrysin (flavone) and galangin (flavonol) from Chinese propolis as antifungal components against *Penicillium italicum*. All these flavonoids possess a free hydroxyl group at C-7 of the basic skeleton.

Uzel *et al*. (2005) evaluated some Anatolian propolis samples against bacteria and yeast. The MIC values against strains of *Candida* ranged from 4.0 to 64 μg/mL. The main compounds of the Anatolian propolis samples determined by GC-MS were flavonoids such as pinocembrin, pinostropin, isalpinin, pinobanksin, quercetin, naringenin, galangin and chrysin.

Kujumgiev *et al*. (1999) tested the antibacterial (*S. aureus* and *E. coli*), antifungal (*C. albicans*) and antiviral (Avian influenza virus) activity of propolis from different geographical origins. Four samples from Brazil were included in this study even a sample from Prudentopolis, Paraná State (the same area as the sample in this study). All samples were equally active against *C. albicans* in spite of the differences in their chemical composition.

The structures of several ions in the MS data of ODEP still remain unknown, indicating the need to carry on the study of the chemical composition of this promising extract

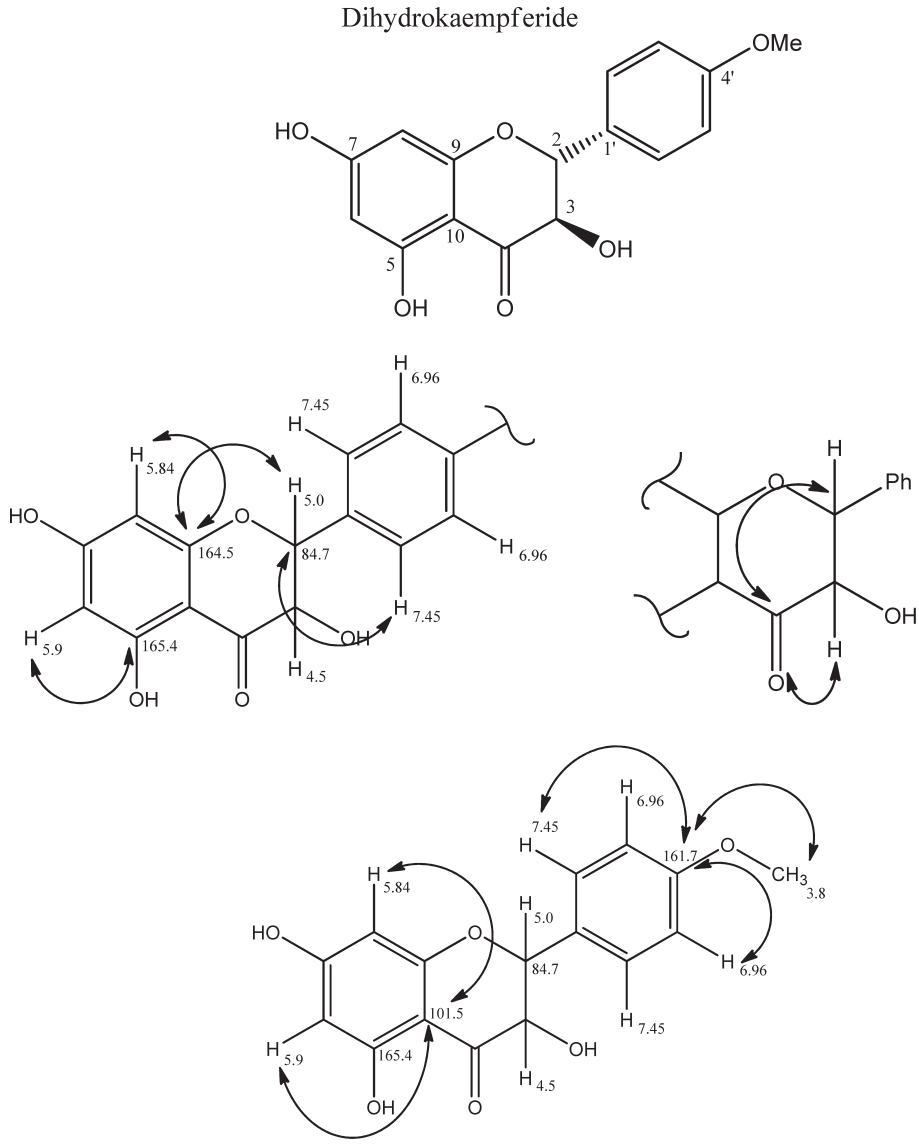

FIG. 3. KEY HETERONUCLEAR MULTIPLE BOND CORRELATION CORRELATIONS FOR DIHYDROKAEMPFERIDE

through chromatography fractionation to allow isolation and identification of ODEP constituents.

Quantitative HPLC Analysis of Flavonoids in ODEP

After HPLC fractionation of OLSx4, dihydrokaempferide was isolated as pure crystals. Its purity and identity were verified by HPLC (PDA peak purity criterion), tandem MS/MS and by NMR spectra. The identified antifungal compounds in ODEP were flavonoids. In green Brazilian propolis, total amount of flavonoids and phenolics compounds are applied for quality control (Sforcin and Bankova 2011). Consequently, the total amount of flavonoids and also of dihydrokaempferide in ODEP was obtained by linear regression ($r^2 = 0.9965$).

The concentration of 23.6 ± 0.3 mg of dihydrokaempferide per gram of ODEP (or 2% w/w) was found after repeated injections of ODEP. Spectrograms were obtained by scanning from 210 to 400 nm, and UV spectra of the main peaks of ODEP were extracted. Adding the areas of peaks that were identified as flavonoids base on their UV spectra and, through the analytical curve of dihydrokaempferide, we obtained a total content of flavonoids in ODEP as 80.2 ± 1.0 mg of flavonoids per gram of ODEP or 8% w/w.

The limit of detection and quantification were 5.21 and 17.4 μg/mL, respectively. Precision of the method was evaluated through the standard deviation and relative standard deviation (RSD) observed for the concentration of dihydrokaempferide in ODEP injected in different amounts and days. RSD values were below 5%. Accuracy was evaluated through recovery experiments of a flavonoid not found in ODEP (quercetin) and in three different concentration levels. Recovery percentages ranged between 95 and 104%, considered suitable for pharmaceutical drugs and natural products (Ribani *et al.* 2004). Thus, the method was sensitive, precise and accurate for the determination of total flavonoids in ODEP.

The amount of total flavonoids in the oil extract of the propolis sample from Prudentopolis was 8% w/w. According to the Brazilian legislation, which accepts minimum flavonoid contents of 0.5% (w/w) and minimum phenolic contents of 5% (w/w) in crude propolis (Ministério da Agricultura e do Abastecimento Secretaria de Defesa Agropecuária 2001), ODEP is therefore of high quality. It is probable that the antifungal activity observed derives from a synergistic action among flavonoids and prenylated derivatives of phenolic acids which were previously identified in this propolis sample (Carvalho *et al.* 2011). Furthermore, Sawaya *et al.* (2002) identified antifungal compounds in a sample of propolis from São Paulo state such as 3,5-diprenyl-4-hydroxycinnamic acid, 3-prenyl-4-hidroxycinnamic acid, *p*-coumaric acid, 2,2-dimethyl-8-prenyl-2*H*-1-benzopyran-6-propenoic acid, pinobanksin and 2,2-dimethyl-6-carboxyethenyl-2*H*-1-benzopyran. All these compounds are also present in the propolis sample from Paraná State evaluated in the present study (Carvalho *et al.* 2011).

Considering a maximum recommended intake of 1 g of flavonoids per day (Arabbi *et al.* 2004), the ingestion of ODEP, e.g., in gelatin capsules could be of nutritional value, helping to reach the recommended daily amounts of flavonoids and phenolic compounds.

CONCLUSIONS

The *in vitro* antifungal properties of a propolis extract obtained with vegetal edible oil against *C. albicans* strains were shown. This supports the use of the oil extract of propolis as an alternative to the ethanolic extract to treat fungal infections bearing in mind that new pharmaceutical presentations are possible. Dihydrokaempferide was isolated as a main active compound, while kaempferide and isosakuranetin were also identified in fractions with a potential activity against *C. albicans*.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. ^{1}H NMR (400 MHz, CD_3OD) spectra of dihydrokaempferide.

Fig. S2. ^{13}C NMR (100 MHz, CD_3OD) spectra of dihydrokaempferide.

Fig. S3. DEPT 135 (100 MHz, CD_3OD) spectra of dihydrokaempferide.
Fig. S4. $^1H - ^1H$ COSY (CD_3OD) spectra of dihydrokaempferide
Fig. S5. HSQC (CD_3OD) spectra of dihydrokaempferide.
Fig. S6. HMBC (CD_3OD) spectra of dihydrokaempferide.
Fig. S7. UV/PDA spectra of dihydrokaempferide isolated from the oil extract of propolis.
Fig. S8. ESI(+)MS/MS spectra of isosakuranetin (A) as a main compound in fraction OLSx4-F4 from the oil extract of propolis and (B) authentic standard.