Chemical composition and antimycoplasma activity of a brown propolis from southern Brazil

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Abstract Extracts of a sample of brown propolis produced in the district of Itapará (Southern Brazil) were obtained with solvents with increasing polarity. The extracts were analyzed by RPHPLC–DAD–ESI–MS/MS and evaluated toward activity against *Mycoplasma bovis*, *M. gallisepticum*, *M. genitalium*, *M. hominis*, *M. hyorinis*, *M. penetrans* and *M. pneumoniae*. Typical components of “alecrim-do-campo” propolis (e.g. prenylated phenylpropanoids and caffeoyl-quinic acids) were characterized in the analyzed extracts, in addition to several flavonols. Less polar extracts showed higher anti-mycoplasma activity (MIC value commonly 3.9 µg/mL) than alcoholic and aqueous extracts (MIC value often 7.8–250 µg/mL). The results indicate that Itapará propolis is a promising source for the development of therapeutic drugs.

Keywords Antibacterial activity · Artepillin C · *Baccharis dracunculifolia* · Caffeoyl-quinic acids · Propolis

Introduction

Propolis is a beehive product containing chiefly beeswax and resins obtained from plants. Most propolis types have resinous flavor and some of them exhibit characteristic smell. Propolis is used medicinally and as a complementary food in several parts of the world, particularly Eastern Europe, China and Japan (Hata et al. 2012). It has been used popularly to treat and prevent diseases, as well as to enhance the resistance against infections, presumably with low or no risks of side effects (Berretta et al. 2017; Machado et al. 2016). Propolis is available in a diversity of food and pharmaceutical products and used industrially for production of cosmetics, such as creams and toothpastes. Many classes of secondary metabolites, including hydrocarbons, fatty acids, fatty esters, flavonoids, benzoic acids, phenylpropanoids, phenolic esters and lignans have been detected in propolis from several parts of the world (Berretta et al. 2017; Zhang et al. 2017). A wide diversity of biological activities has been reported for propolis, such as antioxidant, antimicrobial, anti-inflammatory, antiulcer, antitumor and immunomodulatory (Berretta et al. 2017; Hata et al. 2012).

Propolis composition depends on the resin plant source. Propolis types are characterized by the corresponding chemical composition and resin plant source (Popova et al. 2017). Several types of Brazilian propolis have been characterized. The Brazilian propolis most commercialized is the green type, derived from shoot apices of *Baccharis dracunculifolia* (“alecrim-do-campo” plant) and produced mostly in central and southeast Brazil (Teixeira et al. 2005). Brazilian red propolis is derived from *Dalbergia ecastaphyllum* and produced in the littoral of northeast Brazil (Daugusch et al. 2007; Alencar et al. 2007). A new type of green propolis from northeast Brazil, derived from

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shoot apices of *Mimosa tenuiflora*, was reported recently (Ferreira et al. 2017). Other Brazilian propolis types are known, such as the yellow and brown propolis, so far with undetermined plant origin (Machado et al. 2016).

Propolis from south of Brazil may have distinct botanical origins. Thus, “alecrim-do-campo” propolis has been reported for the states of Paraná and Santa Catarina, often with characteristics similar with the typical product from the state of Minas Gerais, although with lower contents of phenolic compounds (Coelho et al. 2017). Itapará is a district of the municipality of Irati (state of Paraná; south of Brazil), one of the most important apicultural centers in Paraná. Local propolis production already represents a relevant means of income for both large and small neighboring entrepreneurs.

Propolis has long been known to exert antimicrobial activity (Castaldo and Capasso 2002). Assays involving antibacterial activity have been included among the parameters to be considered toward propolis type standardization (Bankova et al. 2016). Many studies have shown that “alecrim-do-campo” propolis is active against a diversity of pathogenic microorganisms, including Gram-positive bacteria, e.g. *Kocuria rhizophila*, *Staphylococcus aureus* (Lupatini et al. 2016), *Streptococcus epidermidis*, *S. pneumoniae* and *S. mutans* (De Luca et al. 2014) and Gram-negative bacteria, e.g. *Haemophilus influenzae*, *Porphyromonas gingivalis*, *P. endodontalis* and *Prevotella denticola* (Jorge et al. 2008). Antibacterial activity has also been reported regarding Brazilian red propolis (Righi et al. 2011) and propolis from Brazilian Amazon (Ishida et al. 2011). Antimycoplasma activity of propolis of the stingless bee *Melipona quadrifasciata* has been reported (dos Santos et al. 2013). To our knowledge, nothing has been published about antimycoplasma activity of honeybee propolis. Mycoplasmas are the smallest self-replicating organisms and constitute a genus of bacteria with no cell wall outside the cellular membrane. Because of this, they are resistant to many commonly used antibiotics (Dybvig and Voelker 1996). Most mycoplasmas are parasitic and many are pathogenic to plants and animals, including humans (Kahane 1992).

So far, there are no available data about chemical composition and antimicrobial activity propolis from Itapará. The aim of the present work was to characterize the chemical constituents of a brown propolis produced in the district of Itapará and evaluate their in vitro activity against species of mycoplasma recognized as pathogenic to humans and domestic animals.

Materials and methods

Materials

The propolis sample was kindly provided by Breyer e Cia Ltda. It was produced by apiaries in the district of Itapará, municipality of Irati, Paraná State, Brazil (25° 27' 56" S, 50° 37' 51" W). The sample had brown color and firm, although not hard texture.

Chemicals and mycoplasma strains

Solvents, reagents and media used in the experiments were *n*-hexane, ethyl acetate, methanol and ethanol (p.a., Synth, Brazil), methanol—HPLC (Merck, Germany), formic acid (J.T.Baker, Mexico), SP4 with arginine (Tully 1993), FREY (Frey et al. 1968) and FRIIS (Kobisch and Friis 1996) media, enrofloxacin (Sigma-Aldrich-Merck, Germany), phenol red pH indicator (P3532, Sigma-AldrichMerck, Germany).

Seven reference mycoplasma strains were used for the antimicrobial assays. Strains of *Mycoplasma bovis* AN5 (ATCC 19,210), *M. genitalium* G37 (ATCC 33,530), *M. hominis* PG-21 (ATCC 23,114), *M. penetrans* GTU 54 (ATCC 55,252) and *M. pneumoniae* M129 (ATCC 15,531) were cultured in liquid medium SP4 with arginine (Tully 1993). *M. gallisepticum* PG31T (ATCC 19,610) was grown in FREY medium, and *M. hyorhinis* (ATCC 17,981), in FRIIS medium (Kobisch and Friis 1996).

Sample preparation and extraction

The propolis sample was pulverized with mortar and pestle, with the aid of liquid nitrogen. The powdered material was treated in Soxhlet for sequential extraction during 12 h with solvents of increasing polarity: *n*-hexane, *n*-hexane:ethyl acetate (1:1, v/v), ethyl acetate and methanol. The extracts were concentrated in rotary evaporator under reduced pressure and the remaining solvent was evaporated to dryness under nitrogen flow. The powder remaining in the Soxhlet cartridge was extracted three times with distilled water on water bath at 80 °C during 3 h (E). The residue obtained after solvent elimination was resuspended in ethanol, filtered and the concentration of the extract solution adjusted to 10 mg/mL.

RPHPLC–DAD–ESI–MS/MS analyses

The ethanol solutions of the extracts were filtered with a 0.45 µm PTFE filter, prior to injection of 30.0 µL for liquid chromatography analysis.

Five analyses were carried out, corresponding to the extracts from *n*-hexane (**A**), *n*-hexane:ethyl acetate (**B**), ethyl acetate (**C**), ethanol (**D**) and water (**E**). The flow rate was 1.0 mL/min, and the temperature of the column 28 °C. The mobile phases comprised eluents A (0.1% aq. formic acid) and B (methanol), according to the following program of B concentrations: 0 min: 20%; 10 min: 30%; 20 min: 50%; 30 min: 70%; 40 min: 90%; 45 min: 40%; return to 20% over indefinite time to re-equilibrate the column. The ionization conditions involved electrospray voltage of the ion source −38 V, capillary voltage 4400 V and capillary temperature 325 °C. Helium and nitrogen were used as collision gas and nebulizing gas, respectively. Nebulization was aided with a coaxial nitrogen sheath at 26 psi. Dessolvation was facilitated by a counter current nitrogen flow of 7.0 L/min. The collision energy was set with voltage ramping cycles in the range 0.5–1.3 V. MS data were obtained in the mass range 100–1000 Da with resolution set at 30,000, using a normal scan rate tube lens −110.0 V. The characterization of the constituents was based on MS and UV-DAD spectral data, by analysis of the spectra and comparison with reference compounds and mass spectral data available in the literature (Righi et al. 2011) and on-line chemical databases Scifinder® (<https://www.scifinder.org>), Riken MSn Spectral database for Phytochemicals (Respect) (<https://spectra.psc.riken.jp/>), Phenol-Explorer (www.phenol-explorer.eu), Chem. Spider (<https://www.chemspider.com>) and HMDB (www.hmdb.ca).

Antimycoplasm activity

The minimal inhibitory concentration (MIC) of the extracts **A–E** was evaluated. To determine Color Change Units (CCU), samples were diluted at 1:10 ratio and incubated at 37 °C under microaerophilic conditions (5% CO₂) in 96 well sealed plate to prevent moisture loss (Hannan 2000). The cultures were diluted, if necessary, to obtain inoculums containing 10³ to 10⁵ CCU/mL. Thus, tests were performed following a standardized broth micro-dilution method in 96-well plates (Hannan 2000). Fractions were diluted in ethanol 5% to a final concentration in the range 1000 µg/mL–3.9 µg/mL. Mycoplasma culture inoculums (190 µL) in log phase (10⁴–10⁵ CCU/mL) were added to all wells, followed by 10 µL of extracts **A–E** at each concentration. The negative control was carried out with the culture medium alone. As growth control, one inoculum without ethanol or propolis extract was used. As positive control, the antibiotic enrofloxacin (Hannan 2000) was used at 2 µg/mL. The plates were sealed, incubated at 37 °C and observed daily. The growth was determined by the color change of the culture medium, which contained the pH indicator red phenol. Minimum inhibitory concentration

value (MIC) was defined as the lowest concentration of the extract at which the metabolism of the organism was inhibited, as evidenced by no medium color change. All procedures were performed in triplicate on different days, in a laminar flow booth.

Results and Discussion

Propolis constituents

Relatively high contents of drupanin (**33**), 2,2-dimethyl chromene-6-propenoic acid (**35**), artemillin **C** (**40**) and baccharin (**42**) were detected in the extracts **A–E** (Table 1). These constituents are prenylated phenylpropanoids, a class of substances characteristic of Brazilian “alecrim-do-campo” propolis (Berretta et al. 2017; Veiga et al. 2017). Other prenylated phenylpropanoids detected were 2,2-dimethyl chromene-6-propenoic acid (**35**) and the 2,2-dimethyl-8-prenyl chromene-6-propenoic acid derivatives **28**, **36** and **39**, as well as the prenylated cinnamic acid derivative **37** (Table 1). All these prenylated cinnamic acids exhibited UV maximum absorption at 310–330 nm and MS/MS base peak resulting from loss of a COO group (44 Da) (Bankova et al. 2016).

Another class of abundant constituents of Brazilian “alecrim-do-campo” propolis is represented by chlorogenic acids, which are phenols containing quinic acid esterified by one or more units of cinnamic, *p*-coumaric, caffeic or ferulic acids. Caffeoylquinic and feruloylquinic acids are phenylpropanoids with maximum UV absorption at 330 nm. Compounds **5–8**, **12–16**, **22**, **24** and **25** were characterized as caffeoylquinic acids, based on caffeoyl neutral losses (162 Da) (Alakolanga et al. 2014). Compounds **9**, **10**, **17** and **26** were characterized as feruloyl quinic acids, due to feruloyl neutral losses (176 Da) (Alakolanga et al. 2014; Carazzone et al. 2013). By reverse phase HPLC, the order of elution of isomers of monocaffeoylquinic acids is 5-, 4- and 3- isomers (compounds **5–7**, respectively, Table 1). Regarding dicaffeoylquinic acids, the elution order is 3,4-, 3,5- and 4,5-isomers (compounds **12–14**, respectively, Table 1) (Alakolanga et al. 2014; Karar and Kuhnert 2015). Compounds **19–21** were characterized as caffeoylquinic acid glucosides, based on UV spectra and comparison of fragmentation patterns with data of Karar and Kuhnert (2015). Flavonoids were detected as minor constituents. This is a known characteristic of Brazilian green, as opposed to Brazilian red and European propolis (Silva-Carvalho et al. 2015). Flavonoids were characterized by UV/vis bands at 345–360 nm (band I) and 255–280 nm (band II). Compound **18** has UV spectrum consistent with a quercetin glycoside and MS/MS fragmentation indicating loss of a glucuronic acid moiety.

Table 1 Constituents of extracts of a propolis sample from Itapar (state of Paran, Brazil) and corresponding HPLC, UV/vis and MS/MS spectral data. RT: retention time; MS: mass spectra; A-E: extracts; A: hexane; B: hexane:ethyl acetate; C: ethyl acetate; D: methanol; E: aqueous

Comp. code	RT (min)	UV/vis (nm)	MS <i>m/z</i> (%)	Proposed characterization	Extracts				
					A	B	C	D	E
1	2.7	320	[M-H] [−] —341 MS/MS—179 (100), 161 (60)	Caffeoyl glucoside					+
2	3.0	-	[M-H] [−] —191 MS/MS—173	Quinic acid					+
3	3.4	325	[M-H] [−] —195 MS/MS—177	Hydroxy caffeic acid					+
4	5.6	-	[M-H] [−] —183 MS/MS—139	Methyl gallate					+
5	6.1	300(sh), 330	[M-H] [−] —353 MS/MS—191 (100), 179 (50)	5- <i>O</i> -Caffeoyl quinic acid				+	+
6	7.9	300(sh), 330	[M-H] [−] —353 MS/MS—191 (30), 173 (100), 179 (50)	4- <i>O</i> -Caffeoyl quinic acid				+	+
7	8.8	300(sh), 330	[M-H] [−] —353 MS/MS—191 (100), 179 (20)	3- <i>O</i> -Caffeoyl quinic acid				+	+
8	10.5	300(sh), 330	[M-H] [−] —353 MS/MS—191	<i>cis</i> -5- <i>O</i> -Caffeoyl quinic acid				+	+
9	12.0	300(sh), 330	[M-H] [−] —367 MS/MS—191	5- <i>O</i> -Feruloyl quinic acid				+	
10	13.7	300(sh), 330	[M-H] [−] —367 MS/MS—191	4- <i>O</i> -Feruloyl quinic acid				+	
11	13.9	310	[M-H] [−] —163 MS/MS—119	Coumaric acid			+		
12	14.7	300(sh), 330	[M-H] [−] —515 MS/MS—353, 173 (30)	3,4-di- <i>O</i> -Caffeoyl quinic acid			+	+	+
13	15.5	300(sh), 330	[M-H] [−] —515 MS/MS—353	3,5-di- <i>O</i> -Caffeoyl quinic acid			+	+	+
14	16.5	300(sh), 330	[M-H] [−] —515 MS/MS—353, 335, 173 (30)	4,5-di- <i>O</i> -Caffeoyl quinic acid			+	+	+
15	17.2	300(sh), 328	[M-H] [−] —515 MS/MS—353	<i>cis</i> -4,5-di- <i>O</i> -Caffeoyl quinic acid			+	+	+
16	17.5	300(sh), 328	[M-H] [−] —515 MS/MS—353 (100), 173 (40)	<i>cis</i> -3,5-di- <i>O</i> -Caffeoyl quinic acid				+	+
17	17.9	300(sh), 330	[M-H] [−] —529 MS/MS—367 (100), 353 (40)	3- <i>O</i> -Feruloyl-4- <i>O</i> -caffeoylquinic acid				+	
18	18.0	266, 274(sh), 354	[M-H] [−] —477 MS/MS—301	Quercetin glucuronide					+
19	18.3	300(sh), 330	[M-H] [−] —515 MS/MS—353	3- <i>O</i> -(4'- <i>O</i> -caffeoylglucosyl)-quinic acid			+	+	+
20	19.4	300(sh), 330	[M-H] [−] —515 MS/MS—353	4- <i>O</i> -(4'- <i>O</i> -caffeoylglucosyl)-quinic acid			+	+	+
21	22.4	300(sh), 330	[M-H] [−] —515 MS/MS—353	5- <i>O</i> -(4'- <i>O</i> -caffeoylglucosyl) quinic acid			+	+	+
22	24.1	300(sh), 328	[M-H] [−] —677 MS/MS—515 (100), 353 (60)	1,3,4-tri- <i>O</i> -caffeoylquinic acid			+	+	+
23	24.7	-	[M-H] [−] —361 MS/MS—317	15-Acetoxy cupressic acid		+	+	+	
24	24.9	300(s), 328	[M-H] [−] —677 MS/MS—515 (100), 353 (20)	3,4,5-tri- <i>O</i> -Caffeoylquinic acid				+	+
25	25.7	300(sh), 328	[M-H] [−] —677 MS/MS—515 (100), 353 (20)	tri- <i>O</i> -Caffeoylquinic acid				+	+
26	26.0	300(sh), 328	[M-H] [−] —529 MS/MS—367 (100)	4- <i>O</i> -Feruloyl-3- <i>O</i> -caffeoylquinic acid				+	+
27	26.2	256, 274(sh), 370	[M-H] [−] —301	Quercetin		+	+		
28	27.2	310	[M-H] [−] —345 MS/MS—330 (30), 301 (80), 271 (100)	7-Methoxy-3-hydroxy-2,2-dimethyl-8-prenyl chromane-6-propenoic acid		+	+	+	
29	28.2	266, 350	[M-H] [−] —447	Kaempferol hexoside					+
30	28.9	258, 270(sh), 370	[M-H] [−] —315 MS/MS—300	Isorhamnetin		+	+		
31	30.1	264, 370	[M-H] [−] —285	Kaempferol			+		
32	30.4	264, 370	[M-H] [−] —299 MS/MS—284	Kaempferide		+	+	+	
33	32.8	310	[M-H] [−] —231 MS/MS—187	4-Hydroxy-3-prenyl—cinnamic acid (drupanin)	+	+	+		

Table 1 continued

Comp. code	RT (min)	UV/vis (nm)	MS m/z (%)	Proposed characterization	Extracts				
					A	B	C	D	E
34	34.3	260, 273(sh), 368	[M-H] [−] —315 MS/MS—300	Rhamnetin			+		
35	33.6	310	[M-H] [−] —229 MS/MS—185	2,2-Dimethyl chromene-6-propenoic acid	+	+			
36	35.0	310	[M-H] [−] —313 MS/MS—269	2,2-Dimethyl-8-hexyl chromene-6-propenoic acid	+	+			
37	35.4	325	[M-H] [−] —393 MS/MS—349 (100), 145 (100)	2-Methoxy-6-prenyl-4-(phenylpropionyloxy)-cinnamic acid			+		
38	35.8	265, 366	[M-H] [−] —313 MS/MS—298	Kaempferol-dimethyl ether			+	+	+
39	37.5	310	[M-H] [−] —315 MS/MS—271	3-Hydroxy-2,2-dimethyl-8-prenyl chromane-6-propenoic acid	+	+	+	+	
40	38.2	315	[M-H] [−] —299 MS/MS—255	4-Hydroxy-3,5-diprenyl cinnamic acid (artepillin C)	+	+	+	+	
41	39.7	313	[M-H] [−] —317 MS/MS—231 (100), 187 (60)	3-Prenyl-4-hydroxy-5-pentoxo cinnamic acid	+				
42	40.1	320	[[M-H] [−] —363 MS/MS—319 (30), 187 (100)	3-Prenyl-4-dihydrocinnamoyloxy-cinnamic acid (baccharin)	+	+	+	+	

Compounds **27**, **30**, **31**, **32**, **34** and **38** are flavonol aglycones with characteristic UV/vis spectra and MS molecular ions. In addition, the fragmentation of the four latter compounds generated a characteristic loss of a methyl radical. Isorhamnetin (**30**) exhibited base peak at m/z 300 (Table 1), suggesting loss of a methyl radical at position 3' of the B-ring (Carazzone et al. 2013). Kaempferide (**32**) was detected in most extracts, unlike other flavonols (**18**, **27**, **29**, **30**, **31** and **34**). This flavonol is the most abundant in leaves of *Baccharis dracunculifolia* (Paula et al. 2017) and the most frequently flavonoid reported for Brazilian green propolis (Simões et al. 2004).

Prenylated phenylpropanoids, such as artemillin C, drupanin and baccharin, flavonoids such as kaempferide (Berretta et al. 2017), caffeoylquinic acids and triterpenes are relevant constituents of Brazilian green propolis (Fernandes-Silva et al. 2013). Overall, the chemical profile of the brown sample analyzed (Table 1) fits well the composition of “alecrim-do-campo” propolis, although the sample analyzed lacks the characteristic deep green color of propolis from Minas Gerais. Several papers have reported propolis from southern Brazil with composition similar to the typical green propolis (Coelho et al. 2017). In fact, populations of *B. dracunculifolia* are more abundant in central and southeast Brazil, but their range extends to the south of the country (Ferronato et al. 2007). However, other plants probably contribute as resin sources of Itaparã propolis. This could account for the occurrence of compounds **1**, **4**, **18**, **23** and **30**, so far unreported for green propolis.

Determination of anti-mycoplasma activity

The antibacterial activity of extracts A–E is shown in Table 2. Extracts A–C showed bacteriostatic activity against all mycoplasma strains tested. The lowest MIC value obtained was 3.9 µg/mL. This MIC value was obtained with extract A against *M. bovis*, *M. genitalium*, *M. hominis*, *M. hyorinis* and *M. pneumoniae*. The same MIC value was obtained with extracts B (*M. bovis*, *M. hominis*, *M. penetrans* and *M. pneumoniae*), C (*M. bovis*, *M. genitalium*, *M. hominis*, *M. penetrans* and *M. pneumoniae*), D (*M. gallisepticum* and *M. hyorhinis*) and E (*M. hominis*). Some extracts had higher MIC values, depending on the organisms tested. MIC value 7.8 was obtained with extracts A and B (*M. gallisepticum*), C (*M. hominis*), D (*M. hominis*, *M. penetrans* and *M. pneumoniae*) and E (*M. genitalium* and *M. hyorinis*). MIC value 250 µg/mL was obtained with extracts A (*M. penetrans*), B (*M. genitalium*), D (*M. bovis*) and E (*M. penetrans* and *M. pneumoniae*). The highest MIC value (1000 µg/mL) corresponded to extract E (*M. bovis*; Table 2). The most sensitive strains among the strains tested were *M. hominis* and *M. hyorhinis*. The 2 µg/mL solution of enrofloxacin, used as positive control, was active against all tested mycoplasma strains.

The values of MICs obtained in most tests of the present work (3.9 and 7.8 µg/mL) are much lower than results reported for extracts of “alecrim-do-campo” propolis against several pathogenic bacteria. Aqueous and hydroalcoholic extracts of propolis of the Brazilian stingless bee *Melipona quadrifasciata* were tested against different bacterial species (dos Santos et al. 2017). MIC values varying between 250 and 1000 µg/mL were obtained with

Table 2 Minimum inhibitory concentration (MIC, $\mu\text{g/mL}$) of *n*-hexane (A); *n*-hexane–ethyl acetate (B); ethyl acetate (C); ethanol (D) and aqueous (E) extracts of a brown propolis sample from Itapar (Southern Brazil) against strains of *Mycoplasma*

Strains/control	A	B	C	D	E
<i>M. bovis</i>	3.9	3.9	3.9	250	1000
<i>M. gallisepticum</i>	7.8	7.8	3.9	3.9	15.6
<i>M. genitalium</i>	3.9	250	3.9	15.6	7.8
<i>M. hominis</i>	3.9	3.9	7.8	7.8	3.9
<i>M. hyorhina</i>	3.9	7.8	–	3.9	7.8
<i>M. penetrans</i>	250	3.9	3.9	7.8	250
<i>M. pneumoniae</i>	3.9	3.9	3.9	7.8	250

–: not-tested

aqueous extract and hydro-alcoholic propolis extracts of *M. quadrifasciata* against *M. mycoides* subsp. *capri*, *M. genitalium*, *M. capricolum*, *M. pneumoniae* strain FH, *M. pneumoniae* strain 129 and *M. hominis*. MIC values of aqueous extract and hydro-alcoholic extracts against *M. genitalium* were 1000 and 250 $\mu\text{g/mL}$, respectively, and against *M. hominis*, 250 and 500 $\mu\text{g/mL}$, respectively. These MIC values are higher than those shown in Table 2. A possible explanation for the differences of MIC values between this study and works by other authors is that the procedures of fractionation of propolis may lead to higher or lower concentration of the antimicrobial chemical substances in each fraction. Furthermore, different extraction procedures lead to extraction of different compounds, and thus distinct antibacterial activity.

Comparing data of Tables 1 and 2, it is possible to obtain directions to suggest which, among the 42 constituents characterized, probably have higher activity against the mycoplasma strains tested. In this regard, data of Table 2 suggest that the less polar extracts A–C tended to exert higher activity (as denoted by their lower MIC values) than the more polar methanol (D) and aqueous (E) extracts. Picking up two contrasting examples from Table 2, mean values of MIC were 4.5 $\mu\text{g/mL}$ for extract C (ethyl acetate) and 219.3 $\mu\text{g/mL}$ for extract E (aqueous). The combined compositions of extracts A–C comprise prenylated phenylpropanoids (28, 33, 39, 40, 41 and 42), flavonols (27, 30, 31, 32 and 38), caffeoylquinic acids (12–15, 19–22) and the diterpene 23. Quite distinct chemical profile characterizes extracts D and E. Constituents found in D and E were benzoic (4) and cinnamic acids (1, 3), caffeoylquinic acids (12–17, 20–22, 24–26), in addition to quinic acid (2) (Table 1). Extract E, the one with lowest anti-mycoplasma activity, contained virtually only non-prenylated quinic, benzoic and cinnamic acids, as well as caffeoyl and feruloylquinic acids (Table 1). From these observations, it seems reasonable to suggest that prenylated

phenylpropanoids, e.g. artemillin C (40) and baccharin (42), in addition to flavonoids, e.g. quercetin (27), kaempferol (31), kaempferide (32), rhamnetin (34) and kaempferodimethyl ether exerted anti-mycoplasma activity stronger than quinic acid and non prenylated benzoic and cinnamic acids (e.g. methyl gallate; 4, and hydroxy caffeic acid; 3, respectively).

Prenylated phenylpropanoids have been established as highly antimicrobial (Jorge et al. 2008), as well as flavonoids (Cushnie and Lamb 2005). The former are characteristic of Brazilian “alecrim-do-campo” propolis, while flavonoids are major constituents of temperate poplar propolis, a recognized source of antibacterial substances (Popova et al. 2005). However, caffeoylquinic acids, predominant in the extracts D and E, are also known to possess antibacterial activity. Lou et al. (2011) determined the mechanism of action of chlorogenic acid (caffeoylquinic acid): it bounds to and disrupt the outer membrane of bacteria, causing the exhaust of cytoplasm macromolecules. Mycoplasmas are cells with no cell walls. Thus, they are simpler than gram positive and gram negative bacteria. Possibly, this explains the lower activity of the more polar extracts D and E, in which predominate substances chemically akin to chlorogenic acid.

MIC values regarding alcohol extracts of “alecrim-do-campo” propolis from Paran against *Staphylococcus aureus* varied in the range 710–2,850 $\mu\text{g/mL}$ (Pamplona-Zomenhan et al. 2011). MIC values of propolis from the state of So Paulo against *S. aureus* and *Kocuria rhizophila* were 100–300 $\mu\text{g/mL}$ (Jorge et al. 2008). A comparison of these results with values on Table 2 suggests that the activity of propolis of “alecrim-do-campo” seems to be higher against mycoplasmas than against cell wall-bearing bacteria.

Conclusion

Although Itapar is in the south of Brazil, the chemical profile of the propolis analyzed corresponds to “alecrim-do-campo” propolis. However, some constituents detected have not been reported previously in this propolis type. Thus, plants other than *B. dracunculifolia* probably contribute also as resin sources for Itapar propolis.

Itapar propolis is highly active toward most strains tested, as shown by the tests using extracts rich in prenylated phenylpropanoids and flavonoids, whereas extracts containing mainly chlorogenic acids exerted lower activity. The results indicate that Itapar propolis may turn out a promising resource for the development of antibacterial therapeutic products, including drugs against mycoplasma pathogenic strains.

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