SUPPLEMENTARY MATERIAL

The effect of seasons on Brazilian red propolis and its botanical origin: chemical composition and antibacterial activity

Bruno Bueno-Silva[a,b*], Alexandre Marsola[b], Masaharu Ikegaki[c], Severino M. Alencar[d], Pedro L. Rosalen[b]

[a] Dental Research Division, Guarulhos University, Guarulhos, São Paulo, Brazil - Zipcode: 07023-070

[b]Piracicaba Dental School, State University of Campinas – UNICAMP, Piracicaba – Sao Paulo – Brazil – Zipcode: 13414-903

[c] Faculty of Pharmaceutical Sciences, Federal University of Alfenas; Alfenas – Minas Gerais – Brazil - Zipcode37130-000

[d] College of Agriculture "Luiz de Queiroz" (ESALQ/USP), University of São Paulo, Piracicaba – São Paulo – Brazil – Zipcode:13418-900

[*] Corresponding author at: Dental Research Division, Guarulhos University, Guarulhos, São Paulo, Brazil. Praça Teresa Cristina, 229 - Centro - Guarulhos - SP – Brasil - Zipcode: 07023-070

Email address: bruno.silva@prof.ung.br

Abstract

The aim of this study was to evaluate the effect of seasons on the chemical composition and antibacterial activity of Brazilian red propolis (BRP) and its plant source. BRP was collected from Maceio, Alagoas state, Northeast of Brazil, during one year. Chemical composition was determined by physico-chemical analyses and HPLC while antimicrobial activity was assessed against *Streptococcus mutans*, *Streptococcus sobrinus*, *Staphylococcus aureus* and *Actinomyces naeslundii* by determining the minimal inhibitory and bactericidal concentrations (MIC and MBC, respectively). The comparative chemical profiles varied quantitatively according to the collection period. Formononetin was the most abundant compound in both propolis and resin while isoliquiritigenin, (3S)-neovestitol, (3S)-vestitol are suggested as responsible for antimicrobial activity of Brazilian red propolis. MIC varied from 15.6-125 µg/mL; whereas MBC varied from 31.2-500 µg/mL. Therefore, season in which propolis and its botanical source are collected indeed influences their chemical compositions, resulting in variations in their antibacterial activity.

Keywords: Brazilian red propolis, seasonal effect, antimicrobial, phenolic compounds, *Apis mellifera*

1. Experimental

1.1. Sample preparations

1.1.1. Harvest of propolis and resin extracted from Dalbergia ecastophyllum

Brazilian red propolis (type 13) and resin of *Dalbergia ecastophyllum (L.) Taub* were collected from the mangrove region over the course of one year in Marechal Deodoro, a city in the vicinity of Maceio, Alagoas, in northeastern Brazil; this region has a wet tropical climate (SL 09.40 and WL 35.41). Taxonomic analysis of the plant (*Dalbergia ecastophyllum*) was carried out in the department of Biological Science at the College of Agriculture "Luiz de Queiroz" (University of São Paulo), Piracicaba, São Paulo, Brazil. A voucher specimen (reference number ESA 96543) was deposited in the herbarium of Biological Science department of the same college. Harvesting was performed every two months, during one year. Three samples from three different hives (beehouse) were collected in each period of collection. Also, the same box was used all year around and plant material was precisely collected from same site in every period of collection.

1.1.2. Propolis extraction

Red propolis was ground to a fine powder, and 2 g (dry weight) was mixed with 25 mL of 80 % (v/v) ethanol and shaken at 70°C for 30 min. After extraction, the mixture was centrifuged, and the supernatant was evaporated under low pressure to produce the ethanolic extract of propolis (EEP), which was prepared at 2 % (w/v) with ethanol 80 % (v/v) (Silva, Rosalen, Cury, Ikegaki, Souza, Esteves and Alencar 2008). The EEP was then used for the chemical and biological assays.

1.1.3. Dalbergia ecastophyllum resin extraction

Resin was extracted from branch bark with a knife (Lecron) immediately after the branch was harvested. A 20-mg sample (dry weight) of the resin was then mixed with 1 mL of 80 % (v/v) ethanol to prepare the ethanolic extract of resin (EER) (Silva, Rosalen, Cury, Ikegaki, Souza, Esteves and Alencar 2008). The EER was used for both chemical and biological analyses.

1.2. Chemical assays

1.2.1. UV-VIS spectra

UV-VIS spectra of the EEP and EER samples (25 µL of each extract plus 30 mL of 96 % ethanol) were recorded as these solutions were scanned at 200-500 nm using a UV-spectrophotometer (UVMini 1240, Shimadzu Co.(Silva, Rosalen, Cury, Ikegaki, Souza, Esteves and Alencar 2008)

1.2.2. Reversed Phase–High Performance Thin Layer Chromatography (RP-HPTLC)

Samples of EEP (6 µL) and EER (6 µL) were applied to the lower edge of pre-coated silica gel plates

(RP-18 $F_{254}S$ - Merck Co), followed by the application of a mobile phase composed of ethanol-water (55:45, v/v). The chromatograms were observed under UV light at 366 nm, prior to and after development with the anisaldehyde reagent (4-methoxy-benzaldehyde, acetic acid, sulfuric acid/1.0:48.5:0.5), followed by incubation at 100°C for 5 min (Alencar et al. 2007).

1.2.3. Reversed-Phase High Performance Liquid Chromatography (RP-HPLC)

EEP and EER were performed using an RP-HPLC instrument equipped with a Shimadzu ODS-A column (RP-18, column size 4.6 x 250 mm; particle size 5 μm) and a photodiode array detector (SPD-M10AVp, Shimadzu Co.). The EEP and EER were filtered (0.22 μm diameter - Millipore) before each 20-μL sample was injected in the HPLC system. The column was eluted using a linear gradient of water (solvent A) and methanol (solvent B), starting with 40 % and increasing to the concentration to 60 % (45 min). The concentration was then held at 90 % (45-75 min) before decreasing to 30 % for solvent B (75-85 min), using a solvent flow rate of 1 mL/min and a diode array detector. Chromatograms were recorded at 260 nm, as described by Alencar, Oldoni, Castro, Cabral, Costa-Neto, Cury, Rosalen and Ikegaki (2007). The following authentic standards of phenolic acids and flavonoids (Extrasynthese Co.) were examined: *p*-coumaric, ferulic acid, cinnamic acid, gallic acid, quercetin, kaempferol, kaempferide, apigenin, isorhamnetin, rhamnetin, sakuranetin, isosakuranetin, vestitol, nesovestitol, liquiritigenin, isoliquiritigenin, hesperidin, hesperetin, biochanin A, pinocembrin, chrysin, acacetin, galangin, myricetin, tectochrysin, formononetin and artepillin C. The detection limits (LD) of ferulic acid, liquiritigenin, quercetin, vestitol, neovestitol, isoliquiritigenin, formononetin and biochanin A were 0.012, 0.011, 0.008, 0.010, 0.011, 0.018, 0.008 and 0.009 μg/g, respectively, whereas their quantification limits (LQ) were 0.036, 0.034, 0.024, 0.031, 0.032, 0.055, 0.026 and 0.029 μg/g, respectively.

1.2.4. Stereochemical analysis

Optical rotation values were determined using a polarimeter model 192 (Perkin-Elmer). CD measurements were carried out Jasco J-600 spectrometer (Jasco, Tokyo, Japan).

1.3. Biological assays

The microorganisms used in this study were *Streptococcus mutans* UA159, *Streptococcus sobrinus* 6715, *Staphylococcus aureus* ATCC 25923 and *Actinomyces naeslundii* ATCC 12104.

The antimicrobial activities of EEP and EER were assessed by their minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) against the microorganisms tested. The methodology described by Koo et al. (2000) was modified into a microtechnique, in which 190 μL of Brain Heart Infusion (BHI) broth with inoculum (1-2 x 10^5 ufc/mL) and 10 μL of EEP, EER or control solution were dispensed onto a microplaque. To determine the minimal inhibitory concentration (MIC), the concentrations of the extracts tested was varied from 7.8 to 1000 μg/mL, and bacterial growth was assessed by adding 0.01 % resazurin stain (Aldrich). Ethanol (4 %) was used as the control vehicle and clorexidine (0.12 %) as a

positive control. MIC values were defined as the lowest concentration of a given extract that could inhibit bacterial growth. To determine minimal bactericidal concentration (MBC) values, an aliquot (30 μL) of each incubated tubes with concentrations higher than MIC was cultured on BHI agar supplemented with 5% defibrinated sheep blood for 18-24 hours, at 37 ºC, with 10 % CO_2. MBC was determined as the lowest concentration that allowed no visible bacterial growth in agar (Koo et al., 2000). Five separate experiments were performed in triplicate for each sample collected in each period of collection.

2. Figures

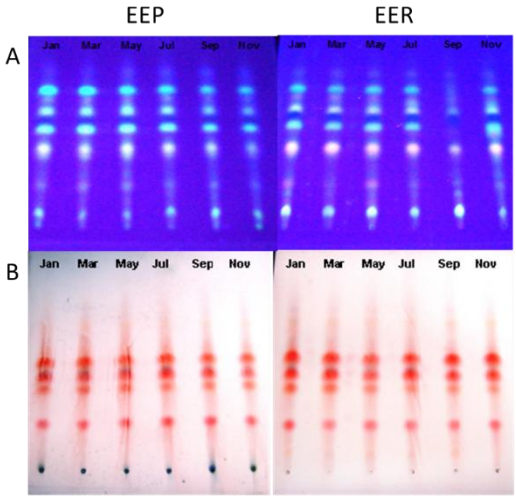

Figure S1: RP-HPTLC of ethanolic extracts of red propolis (EEP) and resin of *Dalbergia ecastophyllum* (EER) developed at a UV light of 366 nm (A) using anisaldehyde reagent (B).

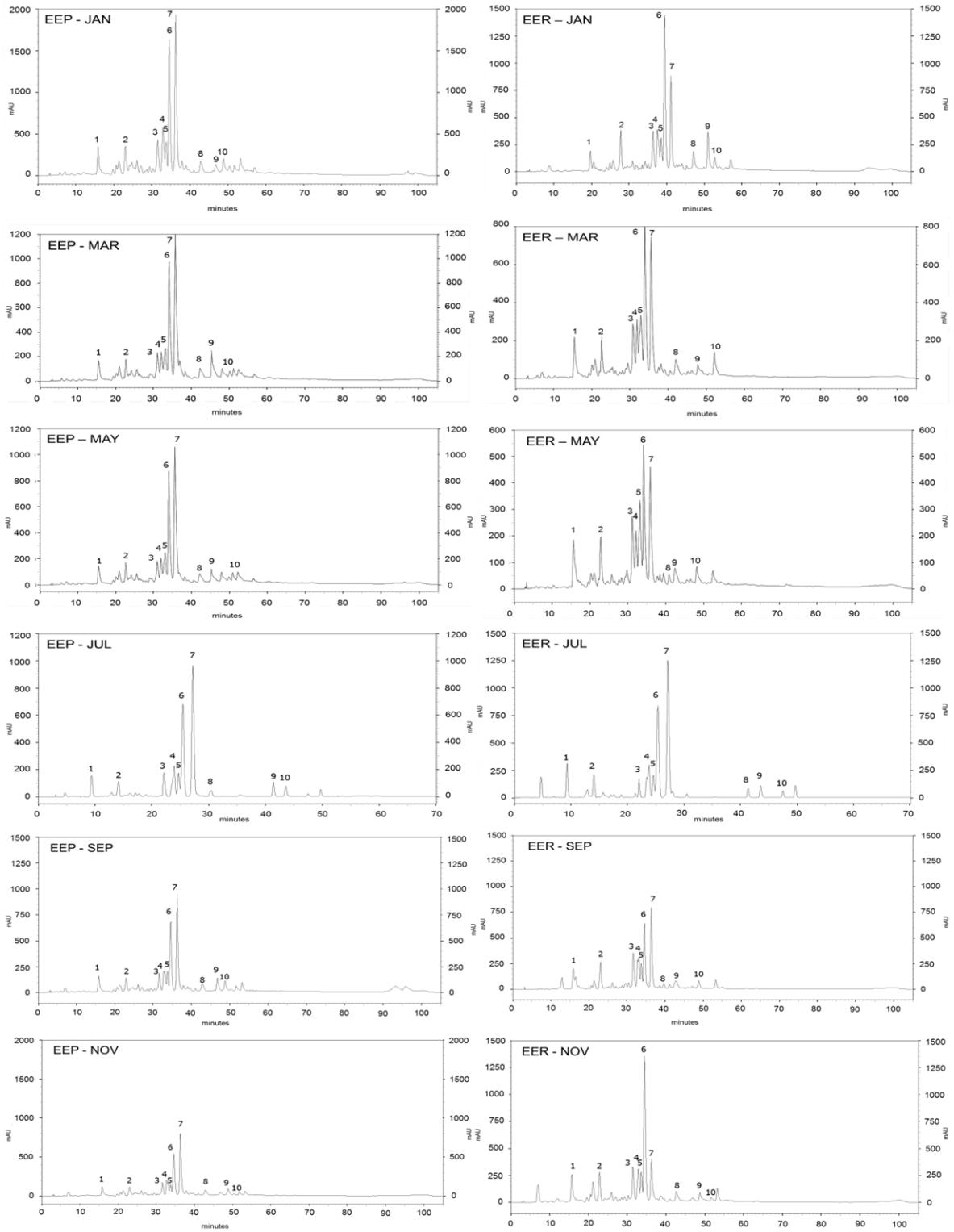

Figure S2: HPLC chromatograms of ethanolic extracts of red propolis (EEP) and resin of *Dalbergia ecastophyllum* (EER) collected from January to November. 1 Ferulic acid; 2. Liquiritigenin; 3 quercetin; 4, vestitol; 5 neovestitol; 6 isoliquiritigenin; 7, formononetin, 8 biochanin A ; 9, UV λ 257 nm; 10, UV λ 280 nm; 11 UV λ 256 nm.

3. Table

Table S1: Quantification of EEP and EER compounds (μg.g^{-1}) through percentage of relative area calculated by HPLC analysis. Results were presented as Means and Standard Deviation of three samples injections.

Compounds (μg.g^{-1}) / Month	EEP						EER					
	Jan	**Mar**	**May**	**Jul**	**Sep**	**Nov**	**Jan**	**Mar**	**May**	**Jul**	**Sep**	**Nov**
1- Ferulic acid	14.30^{a} (± 0.16)	12.20^{b} (± 0.13)	12.43^{b} (± 0.33)	16.00^{c,g} (± 0.41)	13.07^{b} (± 0.37)	14.90^{a,c} (± 0.41)	10.36^{d} (± 0.20)	20.60^{e} (± 0.24)	24.16^{f} (± 0.16)	25.00^{f} (± 0.41)	16.76^{g} (± 0.25)	17.94^{h} (± 0.41)
2- Liquiritigenin	15.88^{a} (± 0.45)	11.94^{b} (± 0.29)	13.30^{c} (± 0.12)	15.82^{a} (± 0.15)	13.96^{c} (± 0.13)	15.52^{a} (± 0.16)	21.32^{d} (± 0.16)	19.32^{e} (± 0.26)	21.92^{d,f} (± 0.08)	22.68^{f} (± 0.16)	25.56^{g} (± 0.04)	21.70^{d,f} (± 0.82)
3- Quercetin	19.08^{a} (± 0.07)	18.00^{a,c} (± 0.41)	15.98^{b} (± 0.25)	19.14^{a} (± 0.33)	17.87^{c} (± 0.21)	21.22^{d} (± 0.20)	21.16^{d} (± 0.29)	25.76^{e} (± 0.29)	29.68^{f} (± 0.25)	15.28^{b} (± 0.03)	33.64^{g} (± 0.18)	25.17^{d} (± 0.85)
4- (3S)-Vestitol	25.64^{a} (± 0.28)	14.95^{b} (± 0.29)	15.03^{b} (± 0.20)	15.21^{b} (± 0.50)	13.30^{c} (± 0.08)	17.56^{e} (± 0.22)	29.04^{f} (± 0.80)	22.76^{g} (± 0.20)	21.68^{g} (± 0.21)	25.80^{a} (± 0.57)	17.68^{e} (± 0.27)	25.52^{a} (± 1.22)
5- (3S)-Neovestitol	15.60^{a} (± 0.04)	24.71^{b} (± 1.05)	25.44^{b} (± 0.90)	31.24^{c} (± 1.23)	16.69^{a,g} (± 0.16)	15.01^{a} (± 0.41)	16.92^{a,g} (± 0.52)	27.84^{d} (± 0.96)	44.20^{e} (± 0.82)	22.20^{f} (± 0.46)	23.88^{b,f} (± 0.65)	18.68^{g} (± 0.48)
6- Isoliquiritigenin	61.51^{a,e} (± 0.83)	74.44^{b} (± 2.43)	74.84^{c} (± 2.86)	83.71^{d} (± 2.16)	61.42^{e} (± 0.06)	64.71^{e} (± 0.36)	80.08^{f} (± 2.07)	77.72^{f} (±1.69)	60.60^{a,e} (± 1.67)	59.68^{a,e} (± 1.27)	55.80^{a} (± 0.16)	96.17^{g} (± 2.23)
7- Formononetin	78.66^{a,d,f} (± 1.50)	88.31^{a,f} (± 2.36)	110.71^{b} (± 8.59)	114.52^{b} (± 1.85)	93.86^{a} (± 5.72)	112.78^{b} (± 9.07)	58.32^{c} (± 1.56)	64.36^{c,g} (± 2.29)	49.52^{c,h} (± 4.08)	73.56^{c,f} (± 1.80)	77.40^{a,g,f} (± 1.05)	33.98^{h} (± 4.50)
8- Biochanin A	10.77^{a,c,d} (± 0.53)	11.51^{a,c,d} (± 2.49)	7.98^{a,b,d} (± 0.82)	2.36^{b} (± 0.17)	8.97^{a,d} (± 1.23)	9.64^{a,d} (± 0.90)	15.96^{c} (± 3.27)	13.96^{c,d} (± 2.51)	5.96^{a,b} (± 0.98)	7.08^{a,b} (± 0.65)	4.16^{b} (± 0.75)	11.24^{a,c,d} (± 1.61)
9- Not identified	8.08^{a,c} (± 1.90)	23.11^{b} (± 1.41)	11.53^{a,c} (± 1.20)	12.35^{a,c} (± 2.59)	13.97^{a,c} (± 2.45)	12.96^{a,c} (± 2.43)	24.76^{b} (± 2.75)	7.12^{a,c} (± 1.67)	14.68^{a} (± 2.67)	9.88^{a,c} (± 1.62)	15.20^{c} (± 2.74)	9.16^{a,c} (± 2.49)
10- Not identified	10.46^{a,b,c,d} (± 0.33)	8.56^{a,b,c,d} (± 1.64)	8.45^{a,b,c,d} (± 1.05)	10.94^{a,b,c,d} (± 2.90)	12.60^{a,c,d} (± 1.25)	8.40^{a,b,c,d} (± 2.05)	8.96^{a,b,c,d} (± 0.82)	13.40^{c,d} (± 1.88)	13.56^{c,d} (± 1.81)	6.68^{a,b} (± 0.83)	9.28^{a,b,c,d} (± 1.95)	5.00^{b} (± 0.82)

Statistical analysis performed through ANOVA followed by Tukey Test. Different letters means statistical significance between same compounds from different months of collection ($p \leq 0.05$).