SUSCEPTIBILITY OF ORAL PATHOGENIC BACTERIA AND FUNGI TO BRAZILIAN GREEN PROPOLIS EXTRACT

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Abstract

The aim of this study was to investigate the antimicrobial activity of ethanolic extract and fractions of Brazilian green propolis (BGP) collected by bees from *Baccharis dracunculifolia* against 16 oral pathogenic microorganisms. BGP was examinated by Reversed-Phase High-Performance Liquid Chromatography (RPHPLC) and its absorption spectra was assessed using UV-Spectrophotometer. Identification of flavonoids and other chemical constituents were carried out using authentic standards. Antimicrobial activity was evaluated by agar diffusion and dilution method. The results indicate that all microorganisms tested were susceptible to BGP. None of the essayed fractions (Coumaric acid, Kaempferol, Pinobanskin-3-acetate, Chrysin, Galangin, Kaempferide, and Artepillin C) was more active than the extract, suggesting a synergistic effect of propolis constituents for the antimicrobial activity.

Key words: Green Brazilian propolis, oral pathogens, antimicrobial activity.

Introduction

Propolis is a natural resinous hive product used by the bees to protect the hive against the invasion of microorganisms and is considered to be a natural antibiotic[1]. It has also been used extensively in folk medicine by the Brazilian population for several years. Many beekeepers, pharmacists and local laboratories in Brazil produce a great variety of propolis derivatives for medicinal use. Beekeepers commonly chew raw propolis to treat mouth and upper digestive track infections. Available literature indicates that few antimicrobial studies have been carried out using Brazilian propolis and there are only a few reports documenting the chemical constituents and their biological activities[2,3]. The aim of this study was to evaluate the antibacterial and antifungal activity of Brazilian green propolis extract and fractions against *Candida* spp., Gram positive and Gram negative oral pathogenic bacteria.

Material and Methods

Crude Brazilian green propolis was obtained from an apicultural region of Minas Gerais State, Brazil by PharmaNectar®(REF. LOT. SBN97). 100g of crude propolis was kept in a freezer for 24 h and powdered in a blender. After dissolving, ethanolic extracts of Brazilian green propolis (BGP) was filtered through Sigma nº1 filter paper. The filtered extract was concentrated under vacuum to furnish 62g of a crude extract. Analysis of flavonoids from ethanolic extracts of bud and unexpanded leaf exudates and ethanolic extracts of propolis was performed by RPHPLC[2] with a chromatograph equipped with a YMC PACK ODS-A column (RP-18, column size 4.6 x 250 mm; particle size 5μm) and Photodiode Array Detector (SPD- M10A, Shimazu Co., Japan). The column was eluted by using a linear gradient of water (solvent A) and methanol (solvent B) starting with 30% B (0-15 min), and increasing to 90% B (15-75min), held at 90% B (75-95 min) and decreasing to 30% B (95-105 min) with a solvent flow rate of 1 mL/min and detection with a diode array detector. Chromatograms were recorded at 268nm. The ethanolic extract of propolis was measured the absorption spectra, using UV-Spectrophotometer.

Identification of flavonoids and other chemical constituents were carried out using authentic standards purchased from Extrasythese Co. (France). The authentic standard of 3,5-dipremyl-4-hydroxycinnamic acid (artepillin C) was donated from Hayashibara Biochemical Laboratory (Okayama, Japan). The authentic standard of Pinobanksin and Pinobanksin-3-acetate were donated from Dr. E. Wollenweber (Institue für Botanik, Technishe Hochschule Darmstadt, Germany). The minimal inhibitory concentration (MIC) was defined as the lowest concentration of propolis in which no bacterial growth was detected. Determination of MICs by the agar dilution method was performed, following the serial concentrations of BGP and different fractions were achieved (%v/v) in plates containing Brucella agar (Oxoid), as follows: 0.1, 0.2, 0.4, 0.8, 1.75, 3.5, 7.0 and 14.0. Each antimicrobial test also included plates containing the culture medium plus ethanol, in order to obtain a control of the solvent antibacterial effect[4,5]. The antimicrobial and antifungal susceptibility test for *Streptococcus mutans* (ATCC 70069), *Strepetococcus sanguis* (ATCC 10557), *Lactobacillus casei* (ATCC 393), *Tanerella forsythensis* (ATCC 700191), *Bacteroides fragilis* (ATCC 25285), *Staphylococcus aureus* (ATCC 12692), *Fusobacterium necrophorum* (ATCC 25286), *Actinobacillus actinomycetemcomitans* (ATCC 33384), *Porphyromonas gingivalis* (ATCC 33277), *Fusobacterium nucleatum* (ATCC 23726), *C. albicans* (ATCC 18804), *C. tropicalis* (ATCC 750), *C. glabrata* (ATCC 2001), *C. parapsilosis* (ATCC 22019), *C. krusei* (ATCC 2340) and *C. guilliermondii* (ATCC 201935) were studied with reference microdilution method following the NCCLS M27-AZ Standards by using RPMI 1640 medium (Sigma-USA) with L-glutamine and phenol red and without sodium bicarbonate[6, 7]. Yeast suspension were inoculated into microplate wells which contained 1/64-1/8000 dilution of BGP and fractions solutions. Microplates were evaluated after incubation at 37ºC for 48 h. Sterile blank disks (CECON - São Paulo - Brazil) were soaked in 20 μl of the BGP solution, 20μl of each component Coumaric acid, kaempferol, Pinobanskin-3-acetate, chrysin, galangin, kaempferide, and artepillin C, and applied to the agar surface previously seeded with the microorganism.

Positive and negative controls of the discs containing 30μg of tetracycline, Nystatin 30mg, and 20μl of Ethanol 93,2ºC were used. After 48 hours of incubation at 37ºC, the diameters of the inhibition zones were measured and compared. The results of the diameters of the inhibition zones were reported as Means $\pm$ Standard Deviation (M±SD). The inhibitory ability of the various propolis solutions tested on the oral pathogenic bacteria and fungi was compared with nonparametric Kruskal-Wallis test. Differences of the level $p<0.05$ were considered to be significant.

Results

Nº	**Compounds**	**Results** (mg/g)
1	Coumaric acid	3.56
2	Cinnamic acid	1.66
3	Quercetin	1.38
4	Kaempferol	1.77
5	Isorhamnetin	0.91
6	Sakuranetin	5.57
7	Pinobanskin-3-acetate	13.92
8	Chrysin	3.51
9	Galangin	9.75
10	Kaempferide	11.60
11	Artepillin C (3,5-diprenyl-4-hydroxycinnamic acid)	82.96

Table 1 - Chemical constituents of propolis sample BGP (SBN97). HPLC test.

Microorganisms	MIC (μg/mL)	MBC (μg/mL)	Inhibition zones (M±SD =mm)
C. albicans	20-50	100-300	16.3±0.52
C. tropicalis	20-50	100-300	12.3±0.08
C. glabrata	20-50	100-300	15.6±0.50
C. krusei	20-50	100-400	28.3±0.15
C. parapsilosis	20-50	100-400	18.6±0.08
C. guilliermondii	20-50	100-400	12.6±0.57
S. mutans	25-50	200-400	18.3±1.15
S. sobrinus	25-50	200-400	28.6±0.57
P. intermedia	20-50	200-400	17.5±2.50
T. forsythensis	30-60	300- 500	14.0±0.00
B. fragilis	25-50	300-500	15.3±1.15
S. aureus	25-50	200-400	16.3±2.08
P. gingivalis	30-50	200-400	14.0±0.00
F. nucleatum	30-60	200-400	15.2±0.26
F. necrophorum	30-60	200-400	17.3±0.57
A. actinomycetemcomitans	30-60	200-400	14.6±0.57

Table 2. Minimum Inhibitory Concentration (MIC); Minimum Bactericidal Concentration (MBC), Means and Standard Deviation (M±SD) of diameter inhibition zones obtained in agar diffusion test using Brazilian Green Propolis Extract (BGP) against *Candida* spp., Gram positive and Gram negative oral pathogenic bacteria. (Tests in triplicate).

Microorganisms	BGP	CA	KOL	PIN	CHR	GAL	KDE	ART	NYS	TET	ET
C. albicans	16.3±0.52	7.0±0.0	6.0±0.0	6.0±0.0	8.0±0.0	9.0±0.0	7.0±0.	8.0±0.0	21.0±3.60		7.0±0.00
C. tropicalis	12.3±0.08	6.0±0.0	7.0±0.0	0.0±0.0	8.0±0.0	0.0±0.0	9.0±0.0	9.0±0.0	20.3±1.52		8.0±0.00
C. glabrata	15.6±0.50	7.0±0.0	7.0±0.0	8.0±0.0	8.0±0.0	9.0±0.0	6.0±0.0	7.0±0.0	22.0±2.64		8.0±0.00
C. krusei	28.3±0.15	8.0±0.0	7.0±0.0	0.0±0.0	8.0±0.0	0.0±0.0	9.0±0.0	9.0±0.0	20.3±0.50		9.0±0.00
C. parapsilosis	18.6±0.08	7.0±0.0	0.0±0.0	7.0±0.0	8.0±0.0	8.0±0.0	7.0±0.0	7.0±0.0	27.0±1.00		8.00±0.00
C. guilliermondii	12.6±0.57	7.0±0.0	8.0±0.0	8.0±0.0	7.0±0.0	9.0±0.0	8.0±0.0	8.0±0.0	26.0±1.15		9.00±0.00
S. mutans	18.3±1.15	7.0±0.0	7.0±0.0	8.0±0.0	8.0±0.0	9.0±0.0	6.0±0.0	7.0±0.0		22.0±1.50	6.0±0.00
S.sobrinus	28.6±0.57	9.0±0.0	7.0±0.0	7.0±0.0	8.0±0.0	8.0±0.0	7.0±0.0	6.0±0.0		26.4±1.22	9.0±0.00
P. intermedia	17.5±2.50	8.0±0.0	8.0±0.0	8.0±0.0	7.0±0.0	9.0±0.0	8.0±0.0	8.0±0.0		23.1±1.30	9.0±0.00
T. forsythensis	14.2±1.00	9.0±0.0	0.0±0.0	0.0±0.0	7.0±0.0	8.0±0.0	8.0±0.0	7.0±0.0		20.2±1.00	7.0±0.0
B. fragilis	15.3±1.15	6.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	7.0±0.0	7.0±0.0	7.0±0.0		18.0±0.50	7.0±0.00
S. aureus	16.3±2.08	6.0±0.0	7.0±0.0	7.0±0.0	8.0±0.0	8.0±0.0	7.0±0.0	6.0±0.0		21.3±1.64	8.0±0.00
P. gingivalis	14.0±0.0	0.0±0.0	8.0±0.0	8.3±1.5	9.0±0.6	8.5±0.5	7.0±0.0	8.0±0.0		18.0±0.0	7.0±0.0
F. nucleatum	15.9±1.15	7.0±0.0	6.0±0.0	0.0±0.0	0.0±0.0	8.0±1.1	9.6±0.5	7.3±1.5		15.6±1.57	7.0±0.0
F. necrophorum	17.3±0.57	7.0±0.0	0.0±0.0	0.0±0.0	7.0±0.0	7.0±0.0	7.0±0.0	8.0±0.0		18.6±2.15	8.00±0.00
A. actinomycetemcomitans	14.6±0.57	6.0±0.0	6.0±0.0	6.0±0.0	7.0±0.0	6.0±0.0	7.0±0.0	6.0±0.0		16.5±0.50	6.0±0.00

Table 3. Mean and Standard Deviation (M±SD) (mm) diameter of inhibition zones for *Candida* spp., Gram-positive and Gram-negative oral bacteria to Brazilian Green Propolis Extract and its HPLC fractions (mean of three experiments). Legend: BGP= Brazilian Green Propolis Extract; Nys= Nystatin; TET = Tetracycline, ET= Ethanolic Alcohol; CA= coumaric acid; KOL= Kaempferol; PIN= Pinocembrin; CHR= Chrisyn; GAL= Galangin; KDE= Kaempferide; ART= Artepillin C.

Discussion

In this study it was verified the antimicrobial activity of a sample of green propolis originated from *Bacharis dracucunlifolia* ("alecrim") against 16 pathogenic microorganisms of the oral cavity (table 1). Microorganisms associated with oral mucosa diseases, dental caries, periapical abscess and periodontitis were selected for this study. More than 200 different components have been identified in propolis solutions and phenolic compounds seem to be associated with biological activities, including propolis antimicrobial properties[8,9,10,11]. In this work it was verified the antimicrobial susceptibility of specific components of ethanolic fractions of BGP. Kruskal-Wallis test indicated no significant differences in the susceptibility profile of isolated compounds (tables 1 and 2) in comparison to total BGP ($P< 0.05$).

None of the assayed fractions was more active than BGP, suggesting that the antibacterial activity is probably caused by the synergistic effects of different compounds, corroborating previously reported results[12,13] . In yeast, propolis biological mechanism of action appears to be associated with cell wall and plasm membrane disruption observed through electron microscopy[14]. On the other hand, propolis mechanism of action on bacteria is complex and a simple analogy to the mode of action of classic antibiotics can not be made[13,15,16]. Although the antimicrobial properties of propolis have been subject of many investigations, it is difficult to compare different studies, since composition of propolis may vary geographically and several methods of study are used in different laboratories[17]. However, in this study both Gram positive and Gram negative bacteria were sensitive to BGP and to isolated compounds differently of other studies[17,18,19]. This finding shows that Brazilian green propolis has significant antimicrobial potential against bacteria and yeast, but the effect will be specie dependent[16].

As far as we are concern this is the first documented paper that reports the antimicrobial activity of isolated compounds of Brazilian green propolis extract against pathogenic oral bacteria and yeast. The MIC and the MBC results showed significant differences of BGP on the microorganisms of the same group, e.g., MIC values for *Candida* spp. ranged from 20 to 50 mg/ml. Similarly, for microaerophilic Gram positive bacteria, MIC values ranged from 25 to 50mg/ml could be detected. The same profile was observed for MBC. The results of diffusion in agar have not shown significant statistical differences between less pathogenic species (*S.sanguis*/ *C. glabrata*) and more aggressive microorganisms (*A. actinomycetemcomitans* / *C. albicans*).

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References

1. Shainler N, Kaftanoglu O. Natural product propolis: chemical composition. Nat Prod Res 2005;19:183-188.

2. Park YK, Alencar SM, Aguiar CL. Botanical origin and chemical composition of Brazilian propolis. J Agric Food Chem 2002;50: 2502- 2506.

3. Orsi RO, Sforcin JM, Funari SR, et al. Effects of Brazilian and Bulgarian propolis on bactericidal activity of macrophages against *Salmonella typhimurium*. Int Immunopharmacol 2005;5:359- 368.

4. National Committee for Clinical Laboratory Standards, Development of In Vitro Susceptibility Testing Criteria and Quality Control Parameters; Approved Guideline-Second Edition. M23-T3 NCCLS, Wayne, PA; 2001.

5. Popova M, Silici S, Kaftnoglu O, et al. Antibacterial activity of Turkish propolis and its qualitative and quantitative chemical composition. Phytomed 2005;12:221- 228.

6. National Committee for Clinical Laboratory Standards, Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard-Fifth Edition: M7-A5 NCCLS, Wayne, PA; 2001.

7. Sonmez S, Kirilmaz L, Yucesoy M, et al. The effect of bee propolis on oral pathogens and human gingival fibroblasts. J Ethonopharmacol 2005;102:371-376.

8. Marcucci MC. Propolis: chemical composition, biological properties and therapeutic activity. Apidologie 1995;26:83-99.

9. Santos FA, Bastos EMA, Uzeda M, et al. Antibacterial activity of Brazilian propolis and fractions against oral anaerobic bacteria. J Ethnopharmacol 2002;80:1-7.

10. Bankova V. Recent trends and important developments in propolis research. Evidence- Based Complementary Altern Med 2005;2:29-32.

11. Duarte S, Roasalen PL, Hayacibara MF, et al. The influence a novel propolis on mutans streptococci biofilms and caries development in rats. Arch Oral Biol 2006;51:15-22.

12. Kujumgiev A, Tsvetkova I, Serkedjieva YU. Antibacterial, antifungal and antiviral activity of propolis of different geographic origin. J Ethnopharmacol 1999;64:235- 240.

13. Cushnie TPT, Lamb AJ, Antimicrobial activity of flavonoids. Int J Antimicrob Agents 2005;26:343-356.

14. Aga H, Shibuya T, Sugimoto T, et al. Isolation and identification of antimicrobial compounds in Brazilian propolis. Biosci Biotechnol Biochem 1994;58:945- 946.

15. Takaisi-Kikuni NB, Schilcher H. Electron microscopic and microcalorimetric investigations of the possible mechanism of the antibacterial action of a defined propolis provenance. Planta Med 1994;60:222-227.

16. Stepanovíc S, Antíc N, Dakíc I, et al. In vitro antimicrobial activity of propolis and synergism between propolis and antimicrobial drugs. Microbiol Res 2003;158:353-357.

17. Drago L, Mombelli B, De Vecchi E, et al. In vitro antimicrobial activity of propolis dry extract. J Chemother 2000;12:390-395.

18. Mirzoerva OK, Grishanin RN, Calder PC. Antimicrobial action of propolis and some of its components: the effects on growth, membrane potential and motility of bacteria. Microbiol Res 1997;152:239-246.

19. Sforcin JM, Fernandes A Jr, Lopes CA, et al. Seasonal effect on Brazilian propolis antibacterial activity. J Ethnopharmacol 2000;73:243-249.