SUPPLEMENTARY MATERIAL

Microbiological control and antibacterial action of a propolis-containing mouthwash and control of dental plaque in humans

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Abstract: Propolis is a bee product with several biological properties. The aim of this study was investigated a propolis-containing mouthwash, its organoleptic properties, microbial contamination and its antibacterial action in vitro. This mouthwash was assessed in vivo to control dental plaque in humans. The presence of microorganisms was analyzed, and the minimum inhibitory concentration against *Streptococcus mutans* was determined. A comparative study was done in vivo using propolis, chlorhexidine, and propolis plus chlorhexidine in lower concentrations for 14 days. Dental plaque was analyzed by the Patient Hygiene Performance (PHP) index. The odontological product was yellow, cloudy, free of microbial contamination, and exerted an inhibitory action in vitro. Individuals who used a propolis-containing mouthwash for 14 consecutive days in combination or not to chlorhexidine showed a similar PHP index to those who used chlorhexidine. The product exerted an antibacterial action in vitro and in vivo, exhibiting a positive action in the control of dental plaque.

Keywords: propolis; chlorhexidine; odontological product; antibacterial action

Experimental

Propolis sample and chlorhexidine

Propolis was collected in the Beekeeping Section, UNESP, Campus of Botucatu. Ethanolic extracts of propolis (30%) were prepared (30 g of propolis, completing the volume to 100 mL with 70% ethanol), in the absence of bright light, at room temperature, with moderate shaking. After a week, extracts were filtered and the dry weight of the extracts was calculated (130 mg/mL).

Chlorhexidine 0.12% was purchased from a local drugstore in Botucatu, SP, Brazil.

Preparation of a propolis-containing mouthwash and its organoleptic properties

Different concentrations of propolis (0.4, 1.3, 2.6 and 6.5%, in a final volume of 7.5 mL) and CHX (7.5 mL, only at 0.1%) totaling 15 mL were combined and put in an amber glass flask, named A, B, C and D, respectively. Organoleptic tests were performed to check the color, smell, flavor and pellets of the manufactured product within 120 days.

All combinations exhibited a pleasant odor of propolis. The combination A was tasteless, combinations B and C tasted only propolis and combination D had a bitter taste. The combination A was slightly cloudy with no precipitates (Fig. S1A). The combinations B and C showed the same characteristics (yellow/cloudy) and few precipitates (Fig. S1B and Fig. S1C, respectively). On the other hand, the combination D was cloudy with a strong precipitate formation (Fig. S1D).

Microbiological control of the product

Most probable number (MPN) of thermotolerant coliforms, mesophilic bacteria count, mold and yeast, the presence of *Shigella*, *Staphylococcus aureus*, *Salmonella* and *Pseudomonas aeruginosa* were investigated according to a Brazilian Resolution (RDC 24/11) of the National Agency of Sanitary Surveillance (ANVISA) with regard to the quality control of specific drugs, searching and identifying microbial contaminants (Brasil, 2011).

The microbiological quality of the product was assessed using 2.6% propolis and 0.12% CHX, showing acceptable sensorial properties in the organoleptic analysis. The microbiological control of propolis and CHX alone was investigated as well.

The various preparations of propolis and CHX were assayed at 0, 10, 20, 30, 60, 90 and 120 days after preparation for a possible microbial contamination. For such analyses, 10 mL of the samples were added into 90 mL of sterile peptone buffered water and homogenized during 2 min. From the initial dilution (10^{-1}), decimal dilutions (10^{-2} and 10^{-3}) were prepared in saline.

Minimum inhibitory concentration and minimum bactericidal concentration

Streptococcus mutans strains were previously cultured (37 ºC for 24 h) in brain-heart infusion (BHI – Merck, Darmstadt, Germany) broth. After, bacteria were standardized at 0.5 MacFarland scale in sterile saline solution. The sensitivity assays were performed by broth microdilution to determine the MIC values. Various concentrations of propolis (20-10000 µg/ mL) and CHX (1-600 µg/mL) totaling 100 µL were incubated alone in 96-wells microplates containing BHI plus Tween 80 (0.5%). A control of propolis solvent (70% ethanol) was also included, in the same concentrations found in propolis (0.011 – 5.5%). To determine the MIC of the combinations propolis/CHX, the concentrations were used according to Table S1.

When the abovementioned plate was prepared, an inoculum (100 µL) of standardized bacterial suspensions containing approximately 1.5 x 10^6 CFU/mL was included in each test. The microplates were incubated (37 ºC for 24 h) and the results were recorded after adding the indicator dye redox resazurin 0.01% (50 µL). MIC was defined as the lowest concentration of the agents that inhibited the growth of microorganisms as indicated by resazurin with no color change (Osaka and Hefty, 2013).

To determine the MBC, an aliquot of each well containing the bacteria incubated with the MIC of the variables or with concentrations higher than the MIC was spread on BHI agar medium and incubated for 24 h for 37 ºC. MBC was defined as the lowest concentration of the agents that resulted in no visible growth on the agar, confirming the absence of viable bacteria.

For the identification of a synergistic or antagonistic effect in the combinations, a Fractional Inhibitory Concentration Index (FICI) was calculated by determining the fractional inhibitory concentration (FIC) of each combination of the variables, defined by the relationship between the MIC of the substance used in combination and the MIC of the same substance alone. FICI is the sum of FICs (Chung et al., 2011). A classification for interaction between the variables was based on the index: FICI $\leq$ 0.5 indicated a synergistic effect; 0.5 < FICI < 4 meant no effect and FICI $\geq$ 4 suggested an antagonistic effect (Odds, 2003).

In vivo assays – subjects

For the *in vivo* assay, forty non-smoking, medication-free individuals between the ages of 20 and 40 years, including both male and female participants, were randomly chosen. A clinical evaluation was carried out and the exclusion criteria were: those who had tartar, infectious foci, residual roots, infection in the dental pulp, and those who were using dental braces or retainer plates. This study also necessitated exclusion of individuals who were missing one or more of the teeth selected for evaluation.

This research was approved by the Ethics Committee of the School of Medicine, UNESP (CEP 4045-2011) and an informed consent was signed by all blood donors.

Experimental groups and mouthwashes

Subjects were divided into four double-blind groups (n = 10), as follows: Group 1: placebo mouthwash (sterile distilled water); Group 2: mouthwash containing propolis 2.6%; Group 3: mouthwash with CHX 0.12%; Group 4: mouthwash with 0.06% CHX combined with propolis 1.3%. All participants received a kit containing the instructions, 14 flasks (15 mL) with the mouthwash, placebo toothpaste, toothbrush and dental floss.

An initial assessment of dental plaque was recorded. Teeth were stained with 2% basic fuchsine using a cotton bud. Surfaces with dental plaque were stained and analyzed according to the PHP index. After assessment of their oral health, dental plaques were removed and participants commenced the experiment under similar conditions, using the mouthwash the same night (Zanatta and Rösing, 2007). The rinses were performed every night for 1 min after the last brushing for 14 consecutive nights.

The second evaluation of the trial took place after the 14 days. Plaques were stained as before and the PHP index of each individual was recorded. The teeth were also examined for possible evidence of staining by the mouthwash used. Finally, the subjects' teeth were cleaned professionally by a dentist.

Patient hygiene performance index

This index is specific to determine dental plaque in the vestibular surfaces of the upper right first molar and upper right central incisor, in the lingual surface of the first lower left molar, in

the labial surface of the lower left central incisor, and in the lingual surface of the lower right first molar (Podshadley and Haley, 1968).

Each tooth surface was assigned a score of zero (no plaque) or 1 (plaque present), and the cumulative score of each tooth was recorded (a number between zero and five for the sum of all surfaces). Scores were tallied for all teeth and divided by the number of sides analyzed for each subject. After obtaining the individual scores, the total score of each group was divided by the number of individuals of the group.

Statistical analysis

Prior to the development of this research, the sample (n) of experimental groups was calculated by Prof. Lidia Raquel de Carvalho (Department of Biostatistics, UNESP). Analysis of variance and Tukey test were used for multiple comparisons. The significance level was 0.05.

Table S1. Concentration of propolis and chlorhexidine (µg/mL) used to analyse the minimum inhibitory concentration of the combinations.

Combination	Propolis (µg/mL)	Chlorhexidine (µg/mL)
1	1325.00	2.50
2	663.00	2.50
3	663.00	1.25
4	1325.00	1.25
5	1325.00	0.83
6	663.00	0.83
7	2650.00	5.00
8	2650.00	2.50
9	2650.00	1.25
10	2000.00	5.00
11	2000.00	2.50
12	2000.00	1.25
13	1800.00	5.00
14	1800.00	2.50
15	1800.00	1.25

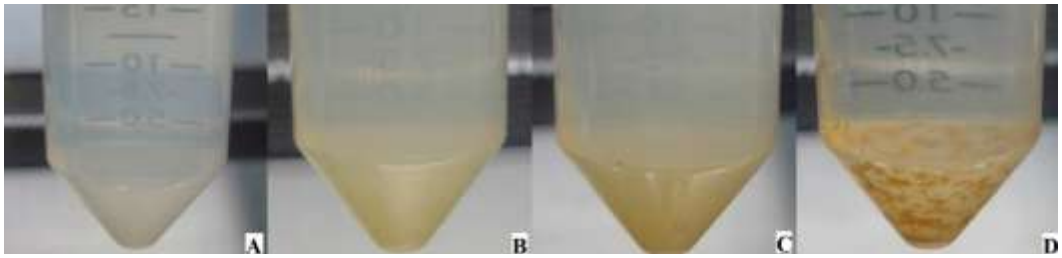

Figure S1. Color and precipitate formation of the combinations A, B, C and D after 120 days.

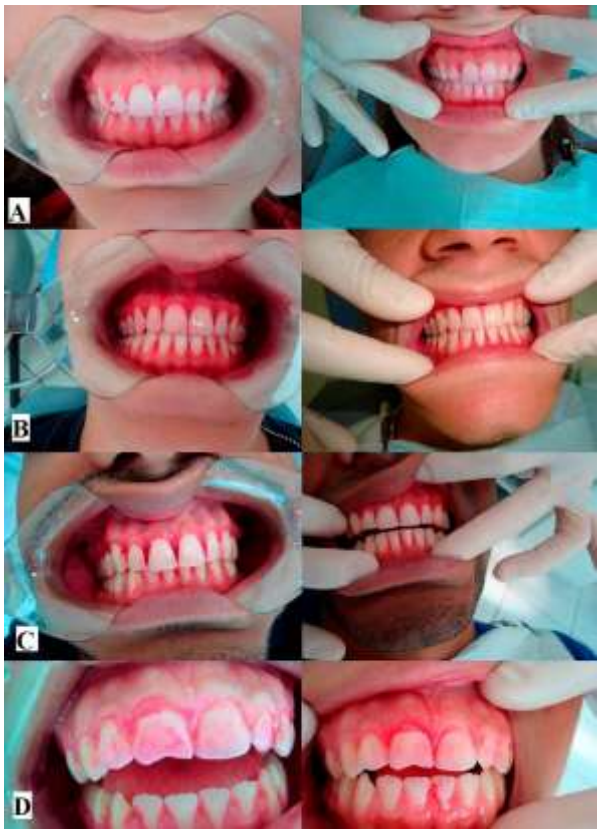

Figure S2. Evaluation of dental plaque and teeth surfaces before (left side) and after 14 days (right side) using a mouthwash containing (A) distilled water (control), (B) propolis, (C) CHX and (D) P/CHX.

References

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