Effect of Mouthrinse Containing Propolis on Oral Microorganisms and Human Gingival Fibroblasts

Fatih Özana, Zeynep Sümerb, Zübeyde Akın Pocatc, Kürşat Erd, Ülkü Özane, Orhan Değerf

ABSTRACT

Objectives: The aim of this study was to compare the effects of four different mouthrinse containing propolis solutions and mouthrinse containing 0.2% chlorhexidine (CHX) on oral microorganisms and human gingival fibroblasts.

Methods: Four different solutions of propolis were prepared and propylene glycol and alcohol were used as solvents for each propolis sample. Mouthrinse containing propolis was prepared at four different concentrations as 10%, 5%, 2.5% and 1%. Besides, CHX was used as control group. The antibacterial effects of five solutions on oral microorganisms were tested and their cytotoxic effects on human gingival fibroblasts were evaluated by agar diffusion test.

Results: At this concentrations effectiveness of mouthrinse containing propolis samples on oral microorganisms were not found as effective as CHX. On the contrary, samples found less cytotoxic on human gingival fibroblasts than CHX.

Conclusions: Standardized preparations of propolis can be used as a mouthrinse at appropriate concentrations. To obtain a standardized chemical composition, advanced researches are needed. (Eur J Dent 2007;1:195-201)

Key Words: Mouthrinse; Propolis; Chlorhexidine; Antibacterial activity; Cell culture.

INTRODUCTION

Mouthrinses are widely used as adjuncts to oral hygiene and in the delivery of active agents to the teeth and gums. The ability of these rinses to influence plaque formation and to alter the course of gingival inflammation has been extensively studied and was reviewed by Kornman.1

Natural products have been used for folk medicine purposes throughout the world for thousands of years. Many of them have demonstrable pharmacological properties, such as antimicrobial, anti-inflammatory and cytostatic, among others2 and more recently propolis has been recognized as useful for human and veterinary medicine.

Propolis, a substance made by the honeybee,
is a potent antimicrobial and anti-inflammatory agent. Honeybees collect the resin from cracks in the bark of trees and leaf buds. This resin is masticated, salivary enzymes are added and the partially digested material is mixed with bee wax and used by bees to seal holes in their honeycombs, smooth out the internal walls and protect the entrance against intruders.3 In general, propolis is composed of 50% resin and vegetable balsam, 30% wax, 10% essential and aromatic oils, 5% pollen and 5% various other substances, including organic debris depending on the place and time of collection.4,5 The constituents of propolis vary widely due to climate, season, location and year, and its chemical formula is not stable.6-8

The most important pharmacologically active constituents in propolis are flavonoids (flavones, flavonols, falavonones) phenolics, and aromatics.9 Flavonoids are well-known plant compounds that have antioxidant, antibacterial, antifungal, antiviral, and anti-inflammatory properties. As an anti-inflammatory agent, propolis is shown to inhibit synthesis of prostaglandins, activate the thymus gland, aid the immune system by promoting phagocytic activity, stimulate cellular immunity, and augment healing effects on epithelial tissues.10-12 Additionally, propolis contains elements, such as iron and zinc that are important for the synthesis of collagen.9,13

MATERIALS AND METHODS

Preparation of propolis containing mouthrinse

Propolis sample was produced by honeybees (Apis mellifera L.) in the region of Yomra, Trabzon, Turkey rich in Picea orientalis, Fagus orientalis, Castanes sativa, Rhodddendron ponticum, Rhododendron luteum, Rubus caucasicus.21 Propolis was provided by Trabzon Agricultural Development Cooperative. Hand collected propolis were kept desiccated and in the dark up to their processing.

Mouthrinse containing propolis was prepared at four different concentrations: (1) 10% w/v propolis, 25% v/v of 70% ethanol, 10% v/v propylene glycol and deionized water; (2) 5% w/v propolis, 25% v/v of 70% ethanol, 10% v/v propylene glycol and deionized water; (3) 2.5% w/v propolis, 25% v/v of 70% ethanol, 10% v/v propylene glycol and deionized water; (4) 1% w/v of 70% ethanol, 10% v/v propylene glycol and deionized water. Mouthrinse containing 0.2% chlorhexidine (CHX) was used as a control group.

Bacterial strains

A number of 50 subjects treated at the Cumhuriyet University School of Dentistry were scraped the entire length of the dorsum of tongue, buccal surface, tooth surface, and dental plaques with a sterile brush by an oral surgeon. Bacteria strains, isolated from clinical specimens of patients, were used: Staphylococcus spp (n=15), Streptococcus spp (n=15), Escherichia coli (n=10) and six standard strains (Candida albicans ATCC 27853, C. albicans ATCC 76615, E. coli ATCC 25922, E. coli ATCC 11230, Staphylococcus aureus ATCC 29213, S. aureus ATCC 658).
Determination of disinfectant efficacy

For investigation all isolates were incubated in blood agar at 37°C for 18 h, and before using all strains were suspended with brain heart broth to 0.5 McFarland turbidity standards and diluted to yield a final inoculums 10^6 CFU (colony forming unit) in 2 µl as described in National Committee for Clinical Laboratory Standards (NCCLS, 1997). 100 µl from each bacterial suspension were transferred to micro plates.

Serial concentrations of propolis (20%, 10%, 5%, 2.5%) and CHX were used directly. 100 µl from all solution were transferred to wells. At 1st, 3rd, 5th, 10th and 20th minutes samples were transferred to brain heart agar and blood agar by using an iron inoculum’s replicator which can transfer 2µl liquid. And all samples were incubated at 37°C for over night.

Gingival fibroblast cell culture

 Cultures of fibroblasts were established from gingival biopsies obtained from healthy individual. The biopsies were stored at 4 h at 4ºC at in hank’s salt solution containing penicillin/streptomycin and amphotericin (all from Biochrom KG, Berlin, Germany) prior to amplification. The gingival tissue samples were cut into 1–2 mm³ pieces, and then washed twice with hank’s salt solution. Thereafter, the cut biopsies were placed into tissue culture flasks (25 cm²). The explants were incubated with culture medium consisting of Dulbecco’s Modified Eagles Medium (DMEM, Sigma, St. Louis, MO, USA), 10 mm HEPES, glucose (4.5 g/L), NaHCO₃ (3.7 g/L), penicillin (100 U/mL), streptomycin (100 mg/mL), and amphotericin (2.5 mg/mL) (all from Biochrom KG, Berlin, Germany), supplemented with 10% heat inactivated fetal calf serum (FCS) (PAN Systems, Aidenbach, Germany). Cells were grown at 37°C in a humidiﬁed atmosphere of 10% CO₂ in air. Culture medium was renewed twice per week until cells reached confluency. For subcultivation, cells were detached from the culture flasks with 0.25% Trypsin/EDTA Solution (Sigma) for 3–5 min. Cells used for the experiments proliferated in logarithmic phase between the 7th and 12th passages. Cell morphology was visualized with phase contrast microscopy (Nikon, Eclipse, TS 100).

Agar diffusion method

The agar diffusion tests were performed according to International Standards (International Standard ISO 10993–5, 1999). Briefly, the cultures were harvested using 0.25% trypsin solution (Gibco, Germany). Stock cultures were seeded in 35 mm diameter of cell culture petri dishes (Nunc, Wiesbaden, Germany) at a density of 1 x 10⁴ cells per petri dish and sub-cultured once a week. After the formation of confluent cell layer, the medium was removed and replaced with complete medium containing 1.5% agarose (FMC BioProducts, Rockland, ME, USA). After solidifying the agarose, the cells were stained with a vital dye (Neutral Red; Sigma). During the experimental procedures, the cells were protected from light to prevent cell damage elicited by photo-activation of the stain. Experimental solutions were applied by using sterile round Whatman papers with a diameter of 6 mm. For the each solution, four replicate dishes and four additional dishes containing positive and negative control materials were prepared. As negative control, DMEM was used, while absolute phenol was used as positive control. After an exposition period of 24 h at 37°C, the cell responses were evaluated by inverted microscope observation. In this study, cell lysis was scored as follows: 0=no cell lysis detectable; 1=less than 20% cell lysis; 2=20% to 40% cell lysis; 3=>40% to <60% cell lysis; 4=60% to 80% cell lysis; 5=more than 80% cell lysis. For each sample, one score was given and the median score value for all parallels from each samples was calculated for the lysis zone. Cytotoxicity was then classified as follows: 0-0.5=non cytotoxic; 0.6-1.9=mildly cytotoxic; 2.0-3.9=moderately cytotoxic; 4.0-5.0=markedly cytotoxic. The median (instead of the mean) was calculated to describe the central tendency of the scores, because the results were expressed as an index in a ranking scale.

RESULTS

Effect of mouthrinse containing propolis on oral microorganisms

Evaluations revealed significant effects of CHX on all tested microorganisms at 1st, 3rd, 5th, 10th and 20th minutes. All microorganisms were susceptible to CHX at 1st. In comparison to the mouthrinse containing propolis, CHX showed significantly strong antimicrobial activity. In this study, we
evaluated that *Streptococcus spp* and *Candida albicans* are susceptible to low concentrations of propolis. *Staphylococcus spp* and *E. coli* are more resistant. All results were showed on Table 1. As a result we found that 10% propolis solution was effective on *Candida albicans* ATCC 27853, *C. albicans* ATCC 76615, *E. coli* ATCC 25922, *E. coli* ATCC 11230, *Staphylococcus aureus* ATCC 29213, *S. aureus* at 1st minute.

Cytotoxicity of mouthrinse containing propolis on gingival cells

Cytotoxic effect of mouthrinse containing propolis and CHX were investigated using the agar diffusion test for 24 h. At no point in time, cytotoxic reactions were detected in any of the four replicates with mouthrinse 5%, 2.5% and 1.25%. (non cytotoxic). There was no zone of decolonization around the samples. Even the cells directly under this concentration of mouthrinse, which could be examined by removing the materials from the agar overlay, did not show any signs of cell injury and were similar to negative controls. Concentration of mouthrinse containing propolis at %10 was ranked mildly cytotoxic. CHX was showed moderately cytotoxic. On the overall, lysis index score was 5 (markedly cytotoxic) in positive control group and 0 (non cytotoxic) in negative control group.

Table 1. Resistance of oral microorganism to propolis.

| Propolis concentration % | Staphylococcus spp. | Streptococcus spp. | E. coli | E. coli (ATCC 25922) | Staphylococcus aureus | Staphylococcus aureus | Candida albicans | Candida albicans |
|--------------------------|---------------------|------------------|--------|---------------------|----------------------|----------------------|----------------|----------------|
|                          |                     |                  |        |                     |                      |                      |                |                |
| 1st minute               |                     |                  |        |                     |                      |                      |                |                |
| 10                       | -                   | -                | 2+     | +                   | -                    | -                    | -              | -              |
| 5                        | 1+                  | -                | 2+     | +                   | +                    | +                    | -              | -              |
| 2.5                      | 2+                  | -                | 2+     | +                   | +                    | +                    | +              | -              |
| 1.25                     | 5+                  | -                | 3+     | +                   | +                    | +                    | +              | -              |
| 3rd minute               |                     |                  |        |                     |                      |                      |                |                |
| 10                       | -                   | -                | 2+     | +                   | -                    | -                    | -              | -              |
| 5                        | 1+                  | -                | 2+     | +                   | +                    | +                    | -              | -              |
| 2.5                      | 2+                  | -                | 2+     | +                   | +                    | +                    | +              | -              |
| 1.25                     | 5+                  | -                | 3+     | +                   | +                    | +                    | +              | -              |
| 5th minute               |                     |                  |        |                     |                      |                      |                |                |
| 10                       | -                   | -                | 2+     | +                   | -                    | -                    | -              | -              |
| 5                        | 1+                  | -                | 2+     | +                   | +                    | +                    | -              | -              |
| 2.5                      | 2+                  | -                | 2+     | +                   | +                    | +                    | +              | -              |
| 1.25                     | 5+                  | -                | 3+     | +                   | +                    | +                    | +              | -              |
| 10th minute              |                     |                  |        |                     |                      |                      |                |                |
| 10                       | -                   | -                | -      | +                   | -                    | -                    | -              | -              |
| 5                        | 1+                  | -                | 2+     | +                   | +                    | +                    | -              | -              |
| 2.5                      | 2+                  | -                | 2+     | +                   | +                    | +                    | +              | -              |
| 1.25                     | 5+                  | -                | 3+     | +                   | +                    | +                    | +              | -              |
| 20th minute              |                     |                  |        |                     |                      |                      |                |                |
| 10                       | -                   | -                | -      | +                   | -                    | -                    | -              | -              |
| 5                        | 1+                  | -                | -      | +                   | -                    | -                    | -              | -              |
| 2.5                      | 2+                  | -                | 2+     | +                   | +                    | +                    | +              | -              |
| 1.25                     | 5+                  | -                | 3+     | +                   | +                    | +                    | +              | -              |

- : susceptible  +: resistant

* Since CHX showed strong antimicrobial activity against all microorganisms even at 1st minute, it was not added to table.
DISCUSSION

Propolis has been extensively studied for its biological properties, mainly antimicrobial activity.22-28 Some authors found propolis samples active only against gram-positive bacteria and some fungi.4,9 Additionally, others found also weak activity against gram-negative bacteria.5,29 Our experimental solution had significant effect on gram-positive strains than on gram-negative strain. Also, we can say that experimental solutions showed enough effect on gram-negative strains and on Candida strains.

Mechanisms of activity of propolis against microorganisms are still controversial. Some components present in propolis extracts like flavonoids (quercetin, galangin, pinocembrin) and caffeic acid, benzoic acid, cinnamic acid, probably act on the microbial membrane or cell wall site, causing functional and structural damages.9,30,31 According to Amoros et al32 and Bonhevi et al33 its activity against microorganisms is more related to the synergistic effect of flavonoids [and other phenolics] than to the individual compounds. These findings are in agreement with those of Takaisikikuni and Schilcher34 who observed that the antibacterial action against Strep. agalactiae was complex, involving several mechanisms such as the formation of pseudomulticellular streptococci; disorganization of the cytoplasm, the cytoplasmatic membrane, and the cell wall; partial bacteriolysis; and inhibition of protein synthesis. They concluded that a simple analogy could not be made with the mode of action of any classic antibiotics. There are no reports dealing with bacterial resistance to constituents of propolis and these properties of propolis may influence the success of antibiotic therapy in the oral cavity.35

Propolis has mucoprotective properties, as has been described for oral and gastric mucosa.36 The mucosal interfaces of the human body are colonized by microbial flora indigenous to these locations. A well-known example is the human mouth that harbors a diverse and significant numbers of microorganisms.37 Oral microorganisms are found in the saliva as non-adhering populations and as plaque, a microbial biofilm, adherent to the surfaces of the tooth and tongue. Clinical researches have examined the association between these microorganisms and specific oral conditions such as dental caries, periodontal disease and oral malodor. Koo et al35 stated that mouthrinse containing propolis showed significant reduction of dental plaque compared to the placebo and also significant inhibition of insoluble polysaccharide formation. Muray et al38 found that a mouthrinse containing 10% propolis had no significant effect on dental plaque regrowth although a slight reduction (14%) was observed. On the other hand, studies showed that propolis prevented caries development.39,40

Propolis is relatively non-toxic and studies have exhibited a no-effect level in a mice study of 1400 mg/kg weight/day leading the authors to propose that a safe dose in humans would be 1.4 mg/kg weight/day, or approximately 70 mg/day.41 Our experimental propolis solutions showed significant activity on Candida strains; so it can be useful for preventing candidial infections.

The development of new therapies for treatment of oral cavity diseases is of great importance since the systemic and local administration of antimicrobials brings about several problems. Some of these problems are: selection of multiresistant microorganisms, interbacterial transfer of resistance determinants and unpleasant side effects. A relatively large number of chemical agents, which are mostly synthetic compounds, have been used for many purposes, control of dental plaque, elimination of oral pathogens, against malodor, etc. The experimental mouthrinse solutions showed significant inhibitory activity against oral microorganisms not as effective as CHX; but was found less cytotoxic on human gingival fibroblasts.

One problem associated with the medical preparation and use of propolis is its heterogeneous chemical composition. The concentration of the various constituents largely depends on factors like geographic origin, plant sources, and proper collection and handling techniques. New studies, using advanced researches are needed to solve this problem. If a standard chemical composition can be obtained, standard effects can be obtained.

CONCLUSIONS

Based on our results, we suggest that the administration of propolis at appropriate concentrations might be effective on oral microorganisms and non-cytotoxic to gingival fibroblasts. In addition, according to previous
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studies, propolis prevents dental caries and periodontal disease, since it demonstrated significant antimicrobial activity against the microorganisms involved in such diseases. These results give hope to us that propolis, a natural product, can be used for oral rehabilitation of patients for various purposes.

REFERENCES

1. Kornman KS. The role of supragingival plaque in the prevention and treatment of periodontal diseases. A review of current concepts. J Periodontal Res 1986;21(suppl):522.
2. Wu-Yuan CD, Green L, Birch WX. In vitro screening of Chinese medicinal toothpastes: their effects on growth and plaque formation on mutans streptococci. Caries Res 1990;24:198-202.
3. Molan P. Why honey is effective as a medicine. Part 2. The scientific explanation of its effects. Bee World 2001;82:22-40.
4. Neiva Moreno MI, Isla MI, Cudmani NG, Vattuone MA, Sampietro AR. Screening of antibacterial activity of Amaicha del Valle (Tucuman, Argentina) propolis. J Ethnopharmacol 1999;68:97-102.
5. Sforcin JM, Fernandes A, Lopes CA, Bankova V, Funari SR. Seasonal effect on Brazilian propolis antibacterial activity. J Ethnopharmacol 2000;73:243-249.
6. Ghisalberti EL. Propolis: a review. Bee World 1979;60:59-84.
7. Markham KR, Mitchell KA, Wilkins AL, Daldy JA, Lu Y. HPLC and GC-MS identification of the major organic constituents in New Zealand propolis. Phytochemistry 1996;42:205-211.
8. Cheng PC, Wong G. Honey bee propolis: prospects in medicine. Bee World 1966;77:8-15.
9. Marcucci MC. Propolis: chemical composition, biological properties and therapeutic activity. Apidologie 1995;26:83-99.
10. Wade C, Friedrich JA. Propolis power plus: the health-promoting properties of the amazing bee energizer. New Canaan, CT: Keats, 1996.
11. Koo H, Gomes BP, Rosalen PL, Embrosano GM, Park YK, Cury JA. In vitro antimicrobial activity of propolis and Arnica Montana against oral pathogens. Arch Oral Biol 2000;45:141-148.
12. Madarova L. Antibacterial properties of propolis. Ceskoslovenska Stomatologie 1980;80:304-307.
13. Scheller S, Ilewiix L, Lucial M, Skrobidiurska D, Matuga W. Biological properties and clinical application of Propolis IX. Investigation of the influence of EEP on dental pulp regeneration. Arzcinw Forsch 1978;28:289-291.
14. Eley BM. Antibacterial agents in the control of supragingival plaque—a review. Br Dent J 1999;27:286-296.
15. Emilson CG. Susceptibility of various microorganisms to chlorhexidine. Scand J Dent Res 1977;85:255-265.
16. Hennessy TD. Antibacterial properties of hibitane. J Clin Periodontol 1977;4:36-48.
17. Newman MG, Hulem C, Colgate J, Anselmo C. Antibacterial susceptibility of plaque bacteria. J Dent Res 1979;58:1722-1732.
18. Loesche WJ, Kazor C. Microbiology and treatment of halitosis. Periodontol 2000 2002;28:256-279.
19. Roldan S, Winkel EG, Herrera D, Sanz M, Van Winelkoff AJ. The effects of a new mouthrinse containing chlorhexidine, cetylpyridinium chloride and zinc lactate on the micro flora of oral halitosis patients: a dual-centre, double-blind placebo-controlled study. J Clin Periodontol 2003;30:427-434.
20. Sekino S, Ramberg P, Uzel NG, Socransky S, Lindhe J. Effect of various chlorhexidine regimens on salivary bacteria and de novo plaque formation. J Clin Periodontol 2004;31:609-614.
21. Davis PH. Flora of Turkey and East Aegean Islands, Edinburgh University Press, Edinburgh, Bols, 1985:1-9.
22. Grange JM, Davey RW. Antibacterial properties of propolis [bee glue]. J Royal Soc Med 1990;83:159-160.
23. Bonhevi JS, Coll FV, Jrdja RE. The composition, active components and bacteriostatic activity of propolis in dietetics. J Am Oil Chem Soc 1994;71:529-532.
24. Sönmez, S, Kirilmaz L, Yucesoy M, Yücel B, Yılmaz B. The effect of bee propolis on oral pathogens and human gingival fibroblasts. J Ethnopharmacol 2005;102:371-376.
25. Koo H, Cury JA, Rosalen PL, Ambrosano GM, Ikegaki M, Park YK. Effect of a mouthrinse containing selected propolis on 3-day dental plaque accumulation and polysaccharide formation. Caries Res 2002;36:445-448.
26. Park YK, Koo MH, Abreu JAS, Ikegaki M, Cury JA, Rosalen PL. Antimicrobial activity of propolis on oral microorganisms. Current Microbiol 1998;36:24-28.
27. Kujumgiev A, Bankova V, Ignatove A, Popov S. Antibacterial activity of propolis, some of its components and their analogs. Pharmazie 1993;48:785-786.
28. de Castro SL, Higashi KO. Effect of different formulations of propolis on mice infected with Trypanosoma cruzi. J Ethnopharmacol 1995;46:55-58.
29. Dobrowolski JW, Vohora SB, Sharma K, Shah SA, Naqvi SA, Dandiya PC. Antibacterial, antifungal, antiinflammatory and anti-ypterycic studies on propolis bee product. J Ethnopharmacol 1991:35:77-82.
30. Cook NC, Samma S. Flavonoids: chemistry, metabolism, cardioprotective effects and dietary sources. Nutr Biochem 1996;7:66-76.
31. Mirzoeva 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.
32. Amaros M, Lurton E, Boustie J, Girre L, Sauvager F, Cormier M. Comparison of the anti-herpes simplex virus activities of propolis and 3-methyl-but-2-enyl caffeate. J Nat Prod 1994;57:644-647.
33. Bonhevi JS, Coll FV, Jrdia RE. The composition, active components and bacteriostatic activity of propolis in dietetics. J Am Oil Chem Soc 1994;71:529-532.
34. Takaisikikuni NB, Schilcher H. Electron-microscopic and micro calorimetric investigations of the possible mechanism of the antibacterial action of a defined propolis provenance. Planta Med 1994;60:222-227.
35. Andres MT, Chung WO, Roberts MC, Fierro JF. Antimicrobial susceptibilities of Porphyromonas gingivalis, Prevotella intermedia and Prevotella nigrescens spp. isolated in Spain. Antimicrob Agents and Chemother 1998;42:3022-3023.
36. Liu CF, Lin CC, Lin MH, Lin YS, Lin SC. Cytoprotection by propolis ethanol extract of acute absolute ethanol-induced gastric mucosal lesions. Am J Chin Med 2002;30:245-254.
37. Socranskys SS, Haffajee AD. Dental biofilms: difficult therapeutic targets. Periodontol 2000 2002;28:12-55.
38. Murray MC, Worthington HV, Blinkhorn AS. A study to investigate the effect of a propolis-containing mouthrinse on the inhibition of de novo plaque formation. J Clin Periodontol 1997;24:796-798.
39. Hayacibara MF, Koo H, Rosalen PL, Duarte S, Franco EM, Bowen WH, Ikegaki M, Cury JA. In vitro and in vivo effects of isolated fractions of Brazilian propolis on caries development. J Ethnopharmacol 2005;101:110-115.
40. Ikeno K, Ikeno T, Miyazawa C. Effects of propolis on dental caries in rats. Caries Res 1991;25:347-351.
41. Burdock GA. Review of the biological properties and toxicity of propolis. Food Chem Toxicol 1998;36:343-367.