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

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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.

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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 others[2] 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]

The medicinal use of propolis was nearly forgotten in modern era due to the discovery and effective use of antibiotics. Nowadays, however, since several pathogens are developing resistance to potent antibiotics, and the latter causing side effects in humans, there is an increased need to search and screen for new antimicrobial agents is growing.

Chlorhexidine (CHX), a biguanide antimicrobial has a significant history of safe and efficacious use for oral health applications.[14] The in vitro antimicrobial spectrum of CHX is well-documented in the literature. CHX is effective against a wide variety of bacteria, including gram-positives, gram-negatives, aerobes, and anaerobes.[15] It is effective against bacteria commonly found in the oral cavity[16] and against organisms associated with diseases of the oral cavity.[17] The effects of CHX are based on its unique properties that include broad spectrum antimicrobial activity at low concentrations that persists over time. Clinical studies demonstrate significant improvements following CHX treatments on several indices of oral health.[14,18] Clinical studies indicate the effects of CHX on bacteria found in the saliva, tongue and subgingival regions.[18-20]

The aim of this study was to compare the disinfectant effects of mouthrinse containing propolis and mouthrinse containing CHX on oral microorganisms with dose-response and time-response and their cytotoxic effects on human gingival fibroblasts.

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 37ºC for 18 h, and before using all strains were suspended with brain hearth broth to 0.5 McFarland turbidity standards and diluted to yield a final inoculums 10^4 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 37ºC 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 mm^3 pieces, and then washed twice with hank's salt solution. Thereafter, the cut biopsies were placed into tissue culture flasks (25 cm^2). 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), $NaHCO_3$ (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 37ºC in a humidified atmosphere of 10% CO_2 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 $l0^6$ 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 of 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.(n=15)	Streptococcus spp. (n=15)	E.coli (n=10)	E.coli (ATCC 25922)	E.coli(ATCC 11230)	Staphylococcus aureus (ATCC 29213)	Staphylococcus aureus (ATCC6538)	Candida albicans (ATCC 27853)	Candida albicans (ATCC 76615)
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	-	-	-	-	-	-	-	-	-
	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 al[32] and Bonhevi et al[33] 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 Schilcher[34] 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 al[25] 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 al[38] 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 on 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

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.

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