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In vitro antimicrobial activity of propolis samples from different geographical origins against certain oral pathogens

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Abstract

Propolis is an agent having antimicrobial properties, however, its composition can vary depending on the area where it is collected. In the present study, the antimicrobial activity of five propolis samples, collected from four different regions in Turkey and from Brazil, against nine anaerobic strains was evaluated. Ethanol extracts of propolis (EEP) were prepared from propolis samples and we determined minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of EEP on the growth of test microorganisms by using agar dilution method. All strains were susceptible and MIC values ranged from 4 to 512 μg/ml for propolis activity. Propolis from Kazan-Ankara showed most effective MIC values to the studied microorganisms. MBC values of Kazan-Ankara EEP samples were ranged from 8 to 512 μg/ml. Death was observed within 4 h of incubation for *Peptostreptococcus anaerobius* and *micros* and *Lactobacillus acidophilus* and *Actinomyces naeslundii*, while 8 h for *Prevotella oralis* and *Prevotella melaninogenica* and *Porphyromonas gingivalis*, 12 h for *Fusobacterium nucleatum*, 16 h for *Veillonella parvula*. It was shown that propolis samples were more effective against Gram positive anaerobic bacteria than Gram negative ones. The organic chemical compositions of EEPs were determined by high-resolution gas chromatography coupled to mass spectrometry (GC–MS). The main compounds of EEPs were flavonoids such as pinobanksin, quercetin, naringenin, galangine, chrysin and aromatic acids such as cafeic acid. Because of increased antimicrobial resistance, propolis may be kept in mind in the treatment of oral cavity diseases.

Keywords: Antimicrobial activity; Propolis; Anaerobic bacteria; Oral cavity

1. Introduction

Propolis, known as bee glue, is a sticky substance having colors from dark-brown to yellow with respect to its origin. It is a resinous and waxy substance collected from the buds and bark of trees by bees [1]. Progressive studies have shown that propolis has antimicrobial, anti-inflammatory, hepatoprotective, anti-oxidative effects and stimulates immune system along with many biological ways [2–8]. Propolis has been used by human since ancient times and as a folk medicine because of its antimicrobial properties [1,9]. Propolis may act against a wide range of bacteria, fungi, yeasts, viruses and invading larvae. The constituents of propolis vary depending on the area from where it is collected. The most important active constituents of propolis are aromatic acids, phenolic compounds, especially flavonoids (flavones, flavonols, and flavonones) and phenolic acids. The antimicrobial properties of this mixture of natural substances are mainly attributed to the flavonones pinocembrin, to the flavonols galangin and to the caffeic acid phenethyl ester [9]. Some prenylated *p*-coumaric acids were shown to possess antibacterial activity by Aga et al. [10] while Bankova et al. [11] reported the

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antibacterial activity of volatile compounds and diterpenic acids in Brazilian propolis. The last studies have demonstrated that inhibitory effect of propolis on bacteria depends on synergism of many compounds [12].

Although propolis has been shown variable activity against different bacteria and there are many products containing propolis on the world market such as ethanol extracts, toothpastes and mouth rinses, very few studies have been made for the antibacterial activity of propolis on anaerobes from oral cavity [13]. There has been an only limited study on antibacterial activity of Turkish propolis; in addition, there was no report on in vitro antimicrobial activity of Turkish propolis against anaerobic oral bacteria. The aim of this study is to examine the composition of propolis, to assess the performance of in vitro minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC) and time kill assay from ethanol extract of five different propolis samples collected from four regions (Kazan, Rize, Mugla, Tahtakopru) of Turkey and one of Brazil against nine anaerobic oral bacteria.

2. Materials and methods

2.1. Propolis samples and preparation of ethanol extracts of propolis

Geographical origin and some other properties of four different Anatolian and one Brazilian propolis samples were selected during site surveys according to the criteria such as clean surrounding, and free of pesticides. Ethanol extracts of propolis (EEP) was prepared as Kilic et al. [14]. Concentrated solution called EEP (obtained diluting the original EEP solution in 1:10, w/v) was evaporated to dryness. About 5 mg of residue was mixed with 75 μl of dry pyridine and 50 μl bis (trimethylsilyl) trifluoroacetamide (BSTFA), heated at 80 °C for 20 min, and then the final supernatant was analyzed by gas chromatography coupled to mass spectrometry (GC–MS).

2.2. GC–MS analysis

GC–MS analysis was performed as Sorkun et al. [15]. Organic compound composition of EEP samples was measured by using peak area of target compound and sum of peak areas as a percent in the chromatogram of propolis samples. And organic compounds of the propolis samples were identified by using standard Willey and Nist Libraries available in the data acquisition system of GC–MS if the comparison scores were obtained higher than 90%.

2.3. Bacterial strains

The type strains used were obtained from the American Type Culture Collection (ATCC), (Rockville, MD): *Peptostreptococcus anaerobius* (ATCC 27337), *Peptostreptococcus micros* (ATCC 33270), *Prevotella oralis* (ATCC 33269), *Prevotella melaninogenica* (ATCC 25845), *Porphyromonas gingivalis* (ATCC 33277), *Fusobacterium nucleatum* (ATCC 10953) *Veillonella parvula* (ATCC 10790), *Lactobacillus acidophilus* (ATCC 4356), *Actinomyces naeslundii* (ATCC 12104). All strains were cryopreserved at −86 °C. For each experiment, the bacteria were inoculated into 5% blood brain heart infusion agar, supplemented with menadione (1 μg/mL) and hemin (5 μg/mL) and incubated under anaerobic conditions at 37 °C (an anaerobic jar with gas generating kit) for 48–96 h.

2.4. Determination of the MIC

The agar dilution method was used as recommended by the National Committee for Clinical Laboratory Standards (NCCLS) [16]. Serial two-fold dilutions of EEP were prepared in Brucella agar, which was supplemented with 5% sheep blood, menadione (1 μg/ml) and hemin (5 μg/ml) by the manufacturer. Agar dilutions ranged from 0.5 to 1024 μg/ml. Two controls were used: (1) agar plates containing no EEP (2) agar plates containing ethanol at 1% final concentration. Each antimicrobial test was also re-performed with plates containing the culture medium plus ethanol as solvent control. The inoculums were prepared by picking three to five colonies of the test organism and inoculating them into 5 ml of enriched thioglycolate broth supplemented with vitamin K(1 μg/ml), hemin (5 μg/ml) and $NaHCO_3$ (1 mg/ml). The broth cultures were incubated over-night at 37 °C and used to prepare an organism suspension in prereduced Brucella broth (Difco) equivalent in density to a 0.5 McFarland standard. Each plate was then inoculated with a multipoint inoculating device (Steers replicator), which delivered a final inoculum of approximately 10^5 CFU per spot. The inoculum size was verified by plating serial dilutions of the inoculum and performing colony counts. The plates were incubated at 37 °C in an anaerobic jar with gas generating kit (90% N_2, 5% CO_2 and 5% H_2) for 48 h. All experiments were performed in duplicate while the MICs of EEP were determined. *Bacteroides fragilis* ATCC 25285 was used as quality-control organism recommended by NCCLS [16].

2.5. Determination of minimum bactericidal concentration of EEP and time-kill assay

Determination of minimum bactericidal concentration (MBC) of EEP for the nine reference strains of anaerobic bacteria was performed by macro dilution broth method as described by the NCCLS [16]. Serial two-fold dilutions of EEP were prepared in macro dilution tubes with concentrations ranging from 1–2048 μg/mL. A final inoculum of approximately 10^5 CFU in supplemented Brucella broth was inoculated into tubes of containing EEP dilutions and incubated for 48 h. After incubation, 0.1 ml of diluted cultures were inoculated onto the surface of supplemented Brucella agar and all plates were incubated at 37 °C in an