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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

Table 1
Chemical compositions percent of ethanol extract of propolis samples collected different areas

Compounds	TKA	TAR	TMU	TTB	BP
Aromatic alcohols					
Benzyl alcohols	0.12	0.27	0.10	0.25	—
Phenyl ethanol	—	0.23	0.04	0.28	0.01
2-napthalenemethanol	0.72	0.73	—	0.43	0.30
5-azulenemethanol	—	—	—	0.10	—
1- napthalenemethanol	0.72	0.73	—	0.43	0.30
2-pherothrenol	—	—	0.28	—	—
Aromatic acids					
Benzoic acid	0.08	1.23	0.28	1.04	0.11
Benzenepropanoic acid	0.03	0.07	—	0.21	0.18
4-pentenoic acid, 5-phenyl	2.34	—	—	—	—
Caffeic acid	2.36	0.16	0.60	0.14	—
2-Propenoic Acid, 3-phenyl	—	0.31	3.79	0.67	0.04
2-propenoic acid, 3-(4-methoxyphenyl)	—	0.04	—	0.02	—
1-phenanthrenecarboxylic acid	—	—	—	0.16	—
Aromatic aldehydes					
Benzaldehyde	—	0.05	0.15	—	—
Cinnamic acid and its esters					
Cinnamyl cinnamate	0.46	—	74.97	0.7	—
Benzyl cinnamate	0.24	—	2.60	1.09	—
Benzyl benzoate	—	—	0.36	0.11	—
Cinnamic acids	—	—	—	—	—
1-3-hydroxy-4-methoxycinnamic acid	—	—	1.90	0.40	—
Naphthalene					
1-naphthalene	0.39	—	—	0.22	0.31
Fatty acids					
Lauric acid	—	—	—	0.33	—
Myristic acid	—	0.21	—	0.13	—
Palmitic acid	0.38	0.33	0.35	—	0.42
Oleic acid	1.00	0.77	—	0.63	—
Stearic acid	0.13	—	—	0.11	—
Linoleic acid	—	0.33	—	0.27	—
Linear hydrocarbons and their acids					
Cyclohexadecane	0.26	0.32	0.94	0.85	0.80
Hexadecane	—	0.23	—	—	—
Nonadecane	0.60	—	—	—	0.24
Octadecane	—	—	0.50	0.40	0.70
Octadecanoic acid	0.30	—	0.30	—	0.20
Flavanone					
2-propen-1-one	4.36	8.20	5.40	1.80	3.20
4H-1-Benzopyran-4-one	7.10	6.05	—	8.70	0.75
Danthron	2.14	3.19	—	1.91	0.40
Naringenin	7.00	7.42	2.40	1.90	0.40
4′,5-dihydroxy-7-methoxyflavanone	0.26	—	—	0.79	—
Chrysin	8.05	1.61	—	2.03	—
3,4′,7-trimethoxy flavanone	0.26	—	—	0.17	—
Ferruginol	—	0.16	—	—	—
Thunbergol	—	0.56	—	—	—
Flavonones					
Pinobanksin and its derivatives	1.50	6.70	3.10	5.70	0.54
Quercetin and its derivatives	3.60	2.40	3.40	3.10	1.80
Galangine and its derivatives	2.90	1.30	2.10	0.80	0.60
Apigenin and its derivatives	1.40	0.85	0.35	—	—

anaerobic jar with gas generating kit (90% N_2, 5% CO_2 and 5% H_2) for 48–96 h. The number of colonies was counted and MBC was taken as the concentration at which a 99.9% reduction in CFU of the original inoculum occurred. The MBC was defined as the lowest concentration of EEP where no growth was recorded. For the time-kill assay an inoculum

of approximately 10^5 CFU in supplemented Brucella broth containing the EEP at MBC was prepared. After incubation at 37 °C for 0, 2, 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 48, 52 h aliquots of 0.1 ml of each culture were inoculated onto the surface of supplemented Brucella agar. After incubation for 48–96 h at 37 °C in an anaerobic jar the CFU was determined. Time-kill curves were plotted having time against the percentage of relative viable count. All assays were performed in duplicate.

2.6. Statistical analysis

All the statistical analyses were performed by the SPSS 11.0 (SPSS Inc., Chicago, IL, USA) statistical package. The data obtained from inhibition of microorganisms related to the propolis types were compared by Covariance analysis test. *P*-values less than or equal to 0.05 were evaluated as statistically significant.

3. Results

In the present study, chemical compositions of ethanol extract of propolis samples collected from different areas were given in Table 1.

The yields of dry propolis extracts in the studied propolis samples were found to be; 17.5% (w/v) for Rize (TAR), 9.4% (w/v) for Kazan (TKA), 13.6% (w/v) for Mugla (TMU), 5.8% (w/v) for Tahtakopru (TTB), 4.6% (w/v) for Brazil (BP) using 96% ethanol as solvent (Table 2). The best yield of soluble content was found the propolis collected from Rize region. Different propolis samples collected from different areas showed different solubility in ethanol even if the same amount of propolis samples were tried to be dissolved in the same volume of ethanol.

The MICs of propolis samples ranged from 4 to 512 μg/ml (Table 3). The control sample (96% aqueous ethanol, v/v) did not effect the growth of bacteria (data not shown). Values of MIC observed for Kazan EEP sample were significantly lower than those observed for the other EEP samples ($P<0.001$). By using covariance analysis, it was shown that, different types of propolis significantly affected the MIC values statistically ($P=0.004$). EEP sample from Kazan was the most active one against anaerobic oral bacteria, followed by Rize, Mugla, Tahtakopru-Bursa, and Brazil. The MIC and MBC values of the propolis samples were given in Table 3. It has been observed that EEP samples were more effective against Gram positive anaerobic bacteria than Gram negative anaerobic bacteria ($P<0.05$). Death was observed within 4 h of incubation for *P. anaerobius* and *P. micros* and *L. acidophilus* and *A. naeslundii*, while 8 h for *P. oralis* and *P. melaninogenica* and *P. gingivalis*, 12 h for *F. nucleatum*, 16 h for *V. parvula*. After these periods no viable cells (CFU) were detected.

4. Discussion

The resinous hive product has been used as a remedy for treatment of many diseases in folk medicine since ancient times [1,17]. Many recent studies showed that sub-inhibitory concentration of EEP was used to value its action on some important virulence factors like lipase and coagulase enzymes, and biofilm formation in *Staphylococcus aureus* [18] and mutant streptococci [19]. Besides its antimicrobial activity, propolis is considered safe in low doses [1]. Six propolis solutions were evaluated for their cytotoxicity and they were found to be safe for gingival fibroblasts [20].

Initially, it was studied to determine the organic compound compositions before the determination of MICs and MBCs of EEP samples. For this purpose, the GC–MS system was used after the derivation of some organic compounds in the EEP samples. In many cases, it is not possible to predict the antibacterial activity of the propolis by measuring the composition. Organic and inorganic compositions of propolis have been reported highly different depending on the region where bees collect the samples [21]. Also, composition of propolis could be changed dramatically in the same region with a few distances of the changing plant diversity and limited bees travel distance from the propolis collected field to the propolis deposited places [14]. However, some compounds give the synergic effect to the other compound activities in the propolis samples. For this reason, it is not possible to report the exact microbial activity of very well defined composition of propolis theoretically. After testing the propolis, some more information could be obtained about real antimicrobial activity of ethanol extract of propolis. It has been concluded that the percent compositions of the four propolis samples collected from different areas of Turkey were found to have similar contents of flavonoids.

Table 2
Geographical origins and other properties of propolis samples

Phyto-geographical region	Sample location	Collection year	Symbol	Solubility in ethanol (% w/v)	Yield (% w/v)
Irano-Turanian	Ankara-Kazan	2003	TKA	4.20	9.45
European-Siberian	Rize-Anzer	2003	TAR	7.00	17.50
Mediterranean	Mugla	2003	TMU	8.50	13.60
Mediterranean	Bursa-Tahtakopru	2003	TTB	3.80	5.80
Mediterranean	Brazil-Paranagreen	2001	BP	2.80	4.60

Table 3
Minimum inhibitory concentrations and minimum bactericidal concentration of ethanolic extracts of five different propolis samples

Microorganisms	TKA		TAR		TMU		TTB		BP		TOTAL (Mean+SD)	
	MIC (μg/ml)	MBC (μg/ml)	MIC (μg/ml)	MBC (μg/ml)	MIC (μg/ml)	MBC (μg/ml)	MIC (μg/ml)	MBC (μg/ml)	MIC (μg/ml)	MBC (μg/ml)	MIC (μg/ml)	MBC (μg/ml)
P.anaerobius ATCC 27337	8	16	16	16	16	32	32	32	32	64	20.8+10.1	32.0+18.4
P. micros ATCC 33270	8	8	8	8	16	16	32	32	16	32	16.0+9.2	19.2+11.4
P. oralis ATCC 33269	128	256	256	512	256	512	256	512	256	512	230.4+54.0	460.8+107.9
P.melaninogenica ATCC 25845	128	128	256	256	128	256	256	256	256	512	204.8+66.0	381.6+132.1
P. gingivalis ATCC 33277	64	128	128	256	256	512	512	512	512	512	294.4+198.2	384.0+170.6
F. nucleatum ATCC 10953	128	256	128	256	256	512	256	256	512	512	256.0+147.8	358.4+132.1
V. parvula ATCC 10790	256	512	512	512	512	512	512	512	512	512	460.8+107.9	512.0+0.0
L.acidophilus ATCC 4356	4	8	16	16	32	64	64	128	128	128	48.8+46.8	68.8+54.8
A.naeslundii ATCC 12104	16	16	16	16	32	32	64	128	128	256	51.2+44.4	89.6+98.1
Total (Mean+SD)	80.2+82.8	147.5+164.4	148.4+164.0	205.3+200.7	167.1+163.2	272.0+231.3	220.4+185.9	263.1+196.8	261.3+199.1	337.8+208.9	175.9+171.8	245.1+207.4

However, different individual compounds were detected while total flavonoids percents of Brazilian propolis sample were found to be considerably lower than the four propolis samples collected from different areas of Turkey. These differences could be resulted from different constituents of the propolis samples. If the content of the hydrophilic compounds in the propolis samples were high, the amount of solubility of the propolis samples would be increased. In other words, flavonoids constituents of the propolis samples could be high for the high soluble propolis sample.

According to Grange et al., propolis is more active against Gram positive bacteria than Gram negative [22]. Similarly, we found that MIC values of Gram positive anaerobic bacteria were lower than MIC values of Gram negative anaerobic bacteria ($P<0.05$). In this present study, it was determined that Turkish propolis samples particularly the one from Kazan (the most active) were highly effective against anaerobic oral pathogens. The antimicrobial activity of propolis samples was ordered in descending order as follows: Rize, Mugla, Tahtakopru, and Brazil. There was a significant difference between MIC values of EEP samples of five different areas ($P = 0.04$).

Since increasing resistance to antibiotics may lead to the failure of therapy of oral cavity diseases [23,24], we investigated the antibacterial activity of the natural propolis product. All tested anaerobic bacterial reference strains were determined susceptible to this bee product and the MIC values ranged from 4 to 512 μg/ml regardless of the ethanolic extract of propolis origin in which propolis was collected. Differences in MIC profiles obtained with the ethanolic extracts of propolis could be explained by the diverse plant origins of propolis. Time-kill assays showed that Gram positive anaerobic bacteria seem to be more sensitive to EEP than Gram negative anaerobic bacteria.

Propolis ethanolic solutions have been widely used commercially on the market as toothpaste, mouth wash etc. However, it is still an unofficial drug in pharmacy [25]. A step further should be given to verify if a dose sufficient to kill the target microorganisms can be reached within the oral cavity, without causing major local or systemic adverse effects. Even though we studied on nine bacterial strains and more studies with bigger numbers are needed, taking into consideration the increasing resistance in anaerobic bacteria the effective antimicrobial activity of propolis give hope in the treatment of oral cavity diseases.

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