

Inhibitory Effect of Bursa Propolis on Dental Caries Formation in Rats Inoculated with *Streptococcus sobrinus*

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Abstract: The effect of propolis on the growth of *Lactobacillus casei* RSKK 591, *Streptococcus mutans* NCTC 10449 and *Streptococcus sobrinus* DSN *sobrinus* 20742 was investigated in vitro. Bursa propolis had the most inhibitory effect on *S. sobrinus*. The effect of Bursa propolis on rats inoculated with *S. sobrinus* was also studied. In rats inoculated with the bacteria and given propolis, the severity of sulcal enamel and superficial dentine lesions was significantly less than that in the control group, but colony forming unit numbers and the caries scores in other levels were not different. Weight gains, and the food and water consumption of the rats were nearly the same, apart from a decrease in weight gain in the control group in the first week, and a decrease in water intake in the propolis group after the second week. The results of this study suggest that propolis is effective in controlling dental caries in the rat model.

Key Words: Propolis, rat, dental caries, cariostatic agents

Bursa Propolisinin Diş Çürüğü Oluşumuna Etkisinin Sıçan Dişlerinde İncelenmesi

Özet: Altı propolis numunesinin *Lactobacillus casei* RSKK 591, *Streptococcus mutans* NCTC 10449 ve *Streptococcus sobrinus* DSN *sobrinus* 20742 üzerine etkinliği değerlendirilmiş, en yüksek inhibitör etkiyi Bursa propolisi 100 µg ile *Streptococcus sobrinus* üzerine göstermiştir. Bunun üzerine, karyojenik diyetle beslenen, streptomisine dirençli *Streptococcus sobrinus* ile inoküle edilen sıçanlara içme suyuyla %1 EEP (Bursa) veya %1 etanol verilmiştir. Hayvanlar feda edildikten sonra sol yarım mandibulaları plak florasındaki *Streptococcus sobrinus* düzeyini belirlemek, sağ yarım mandibulaları ise çürük tayini için kullanılmıştır. Deney grubunda sulkuslarda mine ve yüzeyel dentinde izlenen çürüklerin şiddeti kontrol grubuna göre daha düşük bulunurken, gruplar arasında diğer düzeylerdeki çürük oluşumu ve *Streptococcus sobrinus*'un koloni oluşturan birim sayısı açısından istatistiksel fark bulunmamıştır. Gruplardaki ortalama yem ve su tüketimleri ve hayvanların ağırlık artışları benzer bulunmuştur. Çalışmanın sonuçları propolisin çürük profilaksisinde alternatif bir ajan olabileceğini göstermektedir.

Anahtar Sözcükler: Sıçan, diş çürüğü, propolis, diş çürüğünü önleyici ajanlar

Introduction

Mutans streptococci participate in dental plaque formation by glucosyl-transferases (GTase), which synthesize water insoluble glucans from sucrose (Kidd and Joyston-Bechal, 1997; Gibbons and van Houte, 1975). This group of bacteria, formerly classified as *Streptococcus mutans*, is now separated into 7 different species: *S. mutans*, *S. sobrinus*, *S. cricetus*, *S. rattus*, *S. downei*, *S. ferrus*, and *S. macacae*. *S. sobrinus* is known to possess cariogenic properties in vitro. It can produce acid in large amounts and has the capacity to adhere to enamel and other surfaces, and has been found to be more cariogenic than *S. mutans* in rats given a mild cariogenic diet (de Soet et al., 1991).

Propolis, the resinous hive product collected by bees, is known to possess antibacterial, anti-inflammatory, and anesthetic activities (Hay and Greig, 1990; Ikeno et al., 1991). It has been used as a traditional remedy since ancient times in many countries (Velikova et al., 2001). The constituents of propolis vary widely due to climate, season, location and year, and its chemical formula is not stable (Caillas, 1978; Ghisalberti, 1979; Cheng and Wong, 1996). The most important pharmacologically active constituents in propolis are flavanoids (flavones, flavonols, flavonones), phenolics, and aromatics. Flavanoids are thought to account for much of the biologic activity in propolis. The active components of propolis showing an antimicrobial effect

include pinocembrin, galangin, caffeic acid, and ferrulic acid (Grange and Davey, 1990).

In this study, our aim was to study the effect of different propolis samples on 3 cariogenic bacteria, together with the inhibitory effect of propolis on dental caries formation and plaque microbiology in rats. Weight gains, and the food and water consumptions of the rats during the experiment were also evaluated.

Materials and Methods

Propolis: Propolis samples were collected from hives located in 4 different regions (Bursa, İzmir, Ankara, and Marmaris) in Turkey. Two other samples from Brazil and Japan were kindly given as gifts. The samples were kept in a deep freezer at -20°C for a few days. The hardened propolis was ground by a grinder (Bianchi, 1995) and 15 g of ground propolis was dissolved in 50 ml of 96% ethanol. This mixture was preserved for a few days in a bottle corked tightly and kept in an incubator at 30°C . After dissolving, it was filtered twice with Whatman No. 4 and No. 1 filter papers. The alcohol evaporated during the extraction process was completed to 50 ml by adding alcohol. This solution was called ethanol extract of propolis (EEP) and was kept at $+4^{\circ}\text{C}$ until use (Sorkun et al., 1996). Later, this stock solution was diluted by adding appropriate amounts of distilled water.

Organisms: *Lactobacillus casei* RSKK 591, *S. mutans* NCTC 10449, and *S. sobrinus* DSN *sobrinus* 20742 were used. These were grown for 24 h in tryptic soy broth (Difco Lab., Detroit, MI, USA) containing 8% w/v sucrose.

Antimicrobial Activity: Ten microliters of the cultures containing approximately 10^8 cells of the known bacteria were inoculated onto Mueller-Hinton agar plates (Difco Lab.) by a sterile loop. Sterile paper disks (6 mm in diameter), permeated with a propolis solution of all 6 brands (the concentration of propolis in the paper disks was 100 μg) were placed onto agar plates containing 1 of the bacteria mentioned, and were incubated for 48 h in a desiccator containing 5-10% CO_2 . Duplicate experiments were performed. Disks containing ethanol were used as controls, and the inhibition zones were measured after 48 h (Kujumgiev et al., 1993; Diğrak and Yılmaz, 1995).

Propolis Components: According to the results of the mentioned antimicrobial activity test, the extract of Bursa propolis was analyzed by gas chromatography-

mass spectrophotometry (GC-MS). For GC-MS analysis, a GC 5890, from Hewlett-Packard (Palo Alto, CA, USA) coupled with a mass detector (MS 5972, Hewlett-Packard), was used. The experimental conditions of the system were as follows: an HP 1 column (25 m x 0.2 mm and 0.02 μm of film thickness) was used and the flow rate of mobile phase (He) was set at 1.0 ml/min. In the GC part, temperature was kept for 1 min at 50°C , and then increased to 200°C with $15^{\circ}\text{C}/\text{min}$ heating ramp. After this, temperature was kept at 200°C for 5 min. Finally, temperature was increased to 280°C with $25^{\circ}\text{C}/\text{min}$ heating ramp and then kept at 280°C for 10 min. 1 microliter of EEP extract was injected into the system to screen the sample and identify the compounds present (Sorkun et al., 2001). The compounds were identified by a computer search using the Wiley Library (HP commercial library) and mass spectra patterns. In some cases, when identical spectra were not found, only the structural type of the corresponding component was proposed on the basis of its mass-spectral fragmentation (Velikova et al., 2000).

Rat Caries Model: The effect of propolis on the development of dental caries in rats was examined using 26 three-week-old weanling Wistar albino rats of both sexes and similar body weights (Tanzer and Slee, 1983; Havenaar et al., 1984). They were fed a cariogenic diet (56% sucrose was added to their basal diet) and were given bidistilled deionized water containing 200 $\mu\text{g}/\text{ml}$ ampicillin for 4 days at the beginning of the experiment in order to suppress the endogenous flora. The animals were divided randomly into 2 groups. Inoculation with *S. sobrinus* DSN *sobrinus* 20742 resistant to 500 $\mu\text{g}/\text{ml}$ streptomycin was started on day 7 of the experiment for the experimental group, after checking the eradication of the endogenous flora. The 24-h culture of *S. sobrinus* in brain-heart infusion broth (Difco) containing approximately 10^8 cells was injected directly into the oral cavity of the rats (0.2 ml/rat) once a day for 7 days. In addition, the remaining bacterial suspension was added to their drinking water (3% solution) for 7 days. The level of infection was then checked by an oral swab on mitis-salivarius agar plates containing 200 $\mu\text{g}/\text{ml}$ streptomycin (MSAS) to confirm the implantation of the bacteria. Inoculation was repeated once a week during the experiment for colonization to continue (Skartveit et al., 1991). The rats were divided into 2 groups: the experimental group was given water containing 1% EEP from Bursa (final concentration of propolis 1 mg/ml), and

the control group was given water containing 1% ethanol, both after inoculation with *S. sobrinus*. All the rats were given the same diet and their respective drinking water ad libitum for 6 weeks. The rats were weighed once a week, and their food and water consumption rates were recorded together with their physical status (Bowen et al., 1983; Grenby and Colley, 1983). The rats were sacrificed by an excess ketamine-xylazine combination given intraperitoneally. The mandibles were dissected, the right half mandibles were fixed with 10% neutral formalin solution for caries scoring, and the left half mandibles were kept in 5 ml of phosphate-buffered saline solution (PBS) for bacteriological sampling after being cleaned of flesh, and the number of colony forming units (CFU) was calculated (Mundorff et al., 1984).

Microbial Analysis of Plaque: Suspensions of plaque in PBS were diluted and plated onto MSAS for *S. sobrinus* counts. The culture plates were incubated at 37 °C for 48 h in a desiccator containing 5-10% CO₂. The identities of recoverants were confirmed by an automatized system using well established biochemical, physiological, and morphological techniques, and CFU numbers were calculated for both groups.

Caries Scoring: The right half mandibles were put in 0.06% murexide (ammonium purpurate) in 70% ethanol for 18-20 h in darkness, rinsed and air-dried. Then they were scored for smooth surface caries using the Keyes (Keyes, 1958b) scoring system in a double-blind manner by the same examiner using a dissecting microscope (Keyes, 1958a; Firestone and Navia, 1986; Shaw, 1986).

After hemisectioning in a medial-distal direction with a diamond disk, they were also scored for sulcal and approximal caries. Both the number and severity of the lesions were evaluated. The lesions were grouped as enamel (E), superficial dentine (D_s), moderate dentine (D_m), and profound dentine (D_x) (Keyes, 1958b; Firestone and Navia, 1986).

The weight gains of the rats in both groups were compared with Student's t-test. Statistical analysis for CFU numbers was carried out using the Mann-Whitney U test. Statistical comparisons of the data for caries scores were carried out using the Mann-Whitney U test and Fischer's exact x² test. The Mann-Whitney U test was used when the values were as distant as possible. Values of P < 0.05 were considered significant for all analyses mentioned.

Results

The antimicrobial activity of propolis samples against 3 of the cariogenic bacteria is shown in Table 1. The most inhibitory effect observed was of propolis obtained from Bursa on *S. sobrinus* with a concentration of 100 µg. The Brazilian and Japanese samples did not show any inhibitory effect on the bacteria tested.

The analysis of Bursa propolis by GC-MS is shown in Figure 1, and the mass chromatogram of GC-MS is summarized in Table 2. Both of these show that the propolis specimen obtained from Bursa contains aromatics, flavonoids (especially pinocembrin), and terpenoids.

Table 1. Antimicrobial activity of 6 different extracts of propolis against 3 cariogenic bacteria.

Propolis (µg/ disk)	Zone of inhibition, mm								
	<i>S. mutans</i>			<i>S. sobrinus</i>			<i>L. casei</i>		
	100	50	25	100	50	25	100	50	25
İzmir	8.5	-	-	-	-	-	-	-	-
Bursa	13.75	11	-	15.25	-	-	8.5	-	-
Brazilian	-	-	-	-	-	-	-	-	-
Beytepe	9.75	-	-	10	-	-	-	-	-
Marmaris	8.375	-	-	8.75	-	-	-	-	-
Japanese	-	-	-	-	-	-	-	-	-

(Values represent the mean values obtained from duplicate experiments)

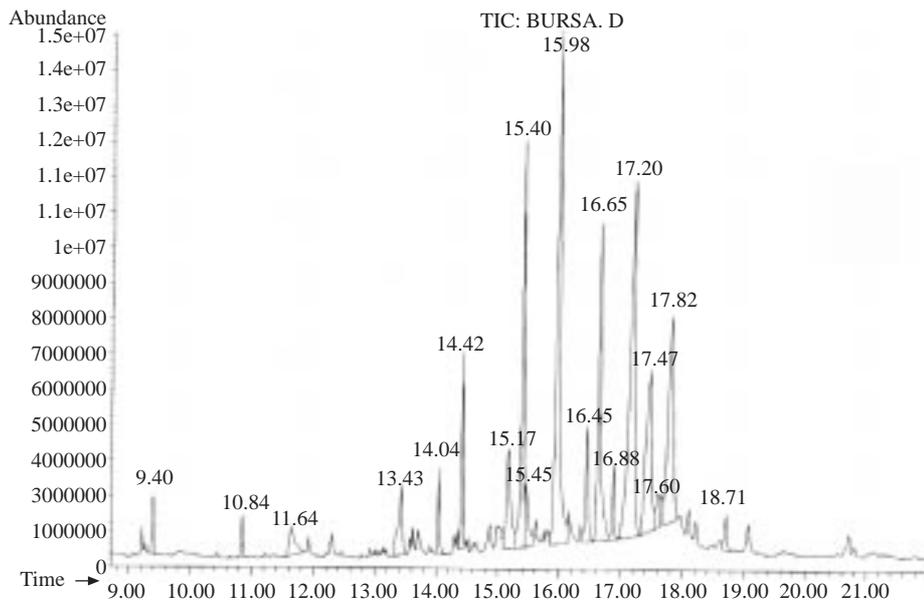


Figure 1. Mass chromatogram of propolis sample obtained from Bursa propolis.

Table 2. Mass chromatogram table of GC-MS obtained from Bursa propolis.

Peak number	Retention time	Concentration %	Substance
1	9.40	1.13	Terpenoid
2	10.84	0.79	Terpenoid
3	11.64	1.59	Aromatic aldehyde
4	13.43	2.75	Aliphatic acid
5	14.04	1.41	Aromatic alcohol
6	14.42	3.03	Aromatic acid (Ferulic acid)
7	15.17	4.51	Aromatic acid
8	15.40	8.76	Flavonone
9	15.45	2.32	Aromatic aldehyde
10	15.98	18.90	Flavonone (Pinoembrin)
11	16.45	2.99	Aromatic alcohol
12	16.65	9.52	Flavonone
13	16.88	1.54	Aromatic
14	17.20	18.38	Aromatic alcohol
15	17.47	9.12	Flavonone
16	17.60	1.10	Flavonone
17	17.82	11.20	Aromatic ketone
18	18.71	0.95	Aromatic hydrocarbon

The weight gains of the rats in both groups are shown in Table 3 and Figure 2. The initial body weights and the weight gains of the rats in both groups throughout the experiment were statistically the same, apart from the difference in the first week ($P < 0.05$). In the first week, a small decrease in approximate weight gain was observed in the control group (Figure 2). The weight gains within the groups themselves were also evaluated using a paired t-test, and the weight gains of the rats within each group were found to be significant between weeks 0 and 1, 1 and 6, and 0 and 6 (Table 4).

Figure 3 shows the approximate weekly food and water consumption of the rats, and these seem to be parallel throughout the experiment except for a decrease in water consumption in the propolis group after the second week.

Microbiology: No statistical difference was found between the *S. sobrinus* counts in plaque obtained from the left half mandibles ($U = 56, P = 0.247$).

Caries scores: Smooth surface caries scores by number and severity of lesions are shown in Table 5. No statistical differences were found between the propolis and control groups. Sulcal caries scores, also by number and severity, are shown in Table 6. The severity of sulcal enamel ($U = 15.00, P = 0.00$), and sulcal superficial dentine lesions ($U = 38.500, P = 0.030$) was significantly

Table 3. Weight gains of the rats throughout the study.

Week	Group	N	Ave.	St.dev.	T	P
0	Cont.	13	34.4615	7.9856	1.815	0.083
	Exp.	12	39.7500	6.4155		
1	Cont.	13	41.3077	9.0405	2.602	0.016*
	Exp.	12	50.3750	8.3261		
2	Cont.	13	50.9231	12.8385	0.948	0.353
	Exp.	12	55.6250	11.8765		
3	Cont.	13	56.9615	13.9201	0.647	0.524
	Exp.	12	60.2917	11.5708		
4	Cont.	13	62.3846	14.0002	0.742	0.465
	Exp.	12	66.4583	13.3765		
5	Cont.	13	74.8077	16.3765	0.631	0.534
	Exp.	12	78.7917	15.0763		
6	Cont.	13	80.1923	18.8597	0.608	0.549
	Exp.	12	84.3750	15.1179		

(*P < 0.05, Student's t-test) (Cont. = control group, Exp. = experimental group)

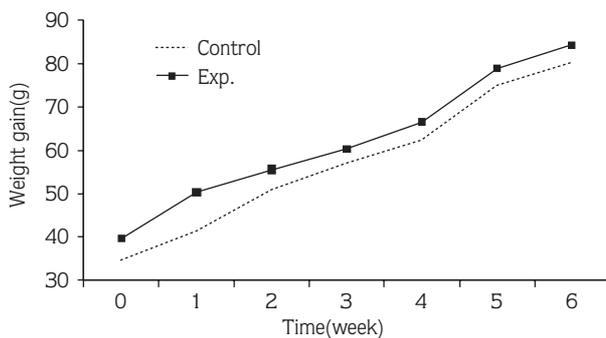


Figure 2. Weight gain of the rats throughout the study (the lines represent the mean weight gains of the rats in each group over the 6-week experimental period).

Table 4. Weight gains of the rats within each group according to weeks.

Group	Week	Average	Sd	T	P
Control Group	0-1	6.8462	3.2621	7.567	0.00*
	1-6	38.8846	11.1638	12.558	
	0-6	45.73308	11.7644	14.016	
Exp. Group	0-1	10.6250	2.7726	13.275	0.00*
	1-6	34.0000	8.3312	14.137	
	0-6	44.6250	9.8075	15.762	

(*P < 0.05, paired t-test) (Sd = standard deviation)

lower in the propolis group ($P < 0.05$ was considered significant). The severity of sulcal moderate and profound dentine lesions, and the numbers of sulcal enamel, superficial, moderate and profound dentine lesions, were not statistically different between the control and propolis groups according to Fisher's exact χ^2 test results (Table 6).

Discussion

In our study, 1 Brazilian, 1 Japanese and 4 Turkish propolis samples were tested for antimicrobial activity against cariogenic bacteria, and the greatest inhibitory effect was found to be of Bursa propolis against *S. sobrinus* (Table 1). In rats given water containing propolis, the total *S. sobrinus* counts in dental plaque did

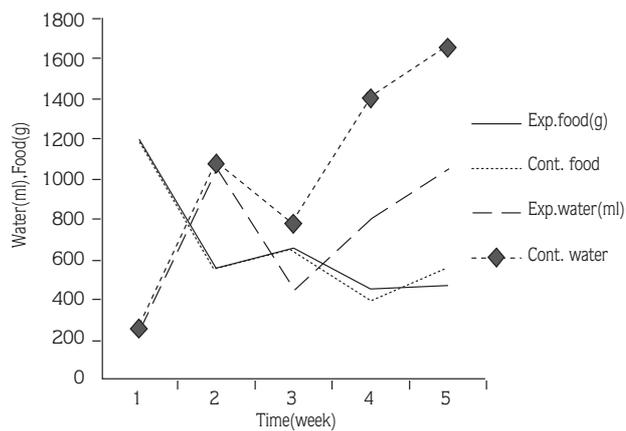


Figure 3. Mean weekly water and food intake over the 6-week experimental period. (Exp. = propolis group, cont. = control group, the values are mean food and water intake values of the rats within the groups in grams and milliliters according to weeks).

not change, while the severity of enamel and superficial dentine lesions was markedly decreased compared with the control group. No toxic effects of propolis were observed in rats under the conditions studied. These results are in agreement with other authors who state that propolis could be an effective agent for controlling dental caries (Ikeno et al., 1991; Diğrak and Yılmaz, 1995; Koo et al., 2000).

The average food and water consumption rates were also evaluated in our study (Fig. 2), and a decrease in water consumption for the propolis group from the graph after the second week was observed. We assume that the rats in the experimental group disliked the taste or smell of EEP and that they kept their body volumes and osmolarity constant by concentrating their urine by a

mechanism revealed earlier (Tanzer, 1976). Therefore, it was not possible for us to observe a dramatic loss of weight in the experimental group during the study.

A number of agents were studied in the hope that an alternative agent could be found for dental caries prophylaxis. Nakamura et al. (1985) reported that mutastein had an inhibitory effect on dental caries. From their data, mutastein caused a 34% suppression of caries development by *S. sobrinus* infection in rats. In another study, Zdanowicz et al. (1989) found that adding 50 ppm barium and 10 ppm fluoride to the drinking water of rats significantly reduced caries severity scores. They also found that these 2 effects were additive, but operated by separate mechanisms. Skartveitz et al. (1991) studied the effect of topical TiF_4 on rats with equimolar solutions of neutral and acidified NaF and found significantly reduced caries scores for total, buccal + lingual, and sulcal values.

In a study by Ikeno et al. (1991), dental caries in 2 rat groups given propolis either at the same time, or after the inoculation of *S. sobrinus*, were inhibited by 56.2 and 62.2%, respectively. In our study, according to the severity of dental caries lesions, the severity of lesions in enamel and superficial dentine in the sulci was significantly lower in the propolis group than in the control group. We could also have expected also a dramatic suppression in other levels, but it is known that *S. sobrinus* has its real cariogenic effect on the surface and subsurface area of the tooth. In deeper areas, lactobacilli are responsible for the progression of dental caries. Propolis can not diffuse thoroughly to those areas, and its effect on lactobacilli is very limited. Furthermore, our experiment was not long enough to see many profound lesions.

Table 5. Smooth surface caries scores by number and severity (n = number, s = severity, Exp. = propolis group).

	Score	Control (n)	Control (n%)	Exp. (n)	Exp. (n%)	P
Buccal + lingual (n)	0,1	9	69.2	11	91.7	0.186
	2	4	30.8	1	8.3	
Buccal + lingual (s)	0	7	53.8	8	66.7	0.404
	1,2	6	46.2	4	33.3	
Proximal (n)	0	10	76.9	9	75.0	0.637
	1,2	3	23.1	3	25.0	
Proximal (s)	1	9	69.2	8	66.7	0.613
	2	4	30.8	4	33.3	

Table 6. Sulcal caries scores by number and severity (n = number, s = severity, Exp. = propolis group, E = enamel, Ds = superficial dentine, Dm = moderate dentine, Dx = profound dentine).

	Score	Control (n)	Control (n%)	Exp. (n)	Exp. (n%)	P
E (n)	0,1,2	7	53.8	7	58.3	0.570
	3,4,5	6	46.2	5	41.7	
Ds (n)	0,1	7	53.8	8	66.7	0.404
	2,3,5	6	46.2	4	33.3	
Dm (n)	0,1	11	84.6	9	75.0	0.459
	2,3	2	15.4	4	25.0	
Dm (s)	1	9	69.2	9	75.0	0.550
	2	4	30.8	4	25.0	
Dx (n)	0	11	84.6	9	75.0	0.459
	1	2	15.4	3	25.0	
Dx (s)	0	12	92.3	9	75.0	0.265
	1	1	7.7	3	25.0	

(The values represent Fisher's exact x2 test results, E(s) (U = 15.00, P = 0.00) and Ds(s) (U = 38.500, P = 0.00) values were lower in the experimental group than in the control group according to Mann-Whitney U test results)

The anticariogenic effect of propolis is due not only to its antimicrobial effect on some cariogenic bacteria, but also to its ability to inhibit glucosyltransferase activity and extracellular polysaccharide synthesis. In a study on propolis, cinnamic acid almost completely inhibited glucosyltransferase activity and was reported to be the probable active agent against dental caries (Ikeno, 1991). Cinnamic acid could not be identified clearly in our sample, but we suppose that it could be somewhere between retention times 13.43 and 14.04, where aromatics are found (Figure 1).

The chemical composition of propolis samples showed that the most important pharmacologically active components in propolis are flavonoids, and various phenolics and aromatics. Of these, antimicrobially effective components include pinocembrin, galangin, caffeic acid and ferulic acid. Velikova et al. (2000) isolated and identified pinocembrin, pinobanksin and its acetate, prenyl esters of caffeic and ferulic acids, from 2 Bulgarian, 1 Greek, 5 Turkish and 2 Algerian samples that all display the typical pattern of "poplar type" propolis. Of these samples, the Turkish ones contained diterpenic acids that have been sparsely isolated before, and pimaric, isopimaric, abietic and dihydroabietic acid.

These chemical differences, however, did not result in a significant change in the biologic activity of the samples. The Turkish samples were effective against *S. aureus* and *C. albicans*. Sorkun et al. (2001) isolated and identified flavonoids, aromatic acid and esters, ketones and terpenoids from samples collected near the city of Bursa. They stated that the flavonoid content of propolis samples collected from the Bursa region is high. In our study, terpenoids, aromatics, and flavonoids were detected in Bursa sample used for detecting dental caries formation in rats. Flavonoid content (especially pinocembrin) was markedly high, as expected. Ferrulic acid was also evidently high in the sample (Table 2). The antimicrobial activity attributed to pinocembrin and ferrulic acid is confirmed in our study.

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