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Chemical composition effects onto antimicrobial and antioxidant activities of propolis collected from different regions of Turkey

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Abstract - Chrysin, apigenin, flavonoids, flavanones, naringenin, ethyl oleate, 3-4-dimethoxy-cinnamic acid and 9-octadecenoic acid were the predominant components of propolis samples collected from different regions of Turkey. The extracts of P3 from Denizli-Başkarcı, P5 from Denizli and P7 fromTekirdağ had effective antibacterial activities on Gram-negatives. Chrysin, which has antibacterial activity, was found to be high concentration. The extracts of P3, P2B from Aydın and P6 from Konya had much more effective antibacterial activities on Gram-positives. The total antioxidant activity increased with the increasing amount of extracts added to linoleic acid emulsion. All doses of propolis ethanol extract displayed antioxidant activity.

Key words: Turkish propolis, GC-MS, chemical composition, antimicrobial and antioxidant activity.

INTRODUCTION

Pharmacological activities of propolis such as antibacterial, antifungal, anti-trypanosomal, and antiviral effects have been ascribed to ethanolic extracts of propolis (Kujumgiev *et al*., 1999; Kartal *et al*., 2003; Prytzyk *et al*., 2003). In propolis samples from Turkey (Bursa, Trabzon, Gumushane and Erzurum cities) flavanones, terpenoids, flavones, ketones, alcohols, aromatic acids, aliphatic acids and their esters, and derivatives are the main compound groups (Sorkun *et al.,* 2001).

Propolis ethanol extracts can scavenge 1,1-diphenly-2-picrylhydrazyl (DPPH) and hydroxyl radicals (Kumazawa *et al*., 2004; Wang *et al*., 2004). Flavonoids have been reported to show an antioxidative effect and those are known to inhibit lipid peroxidation in model systems (Ferrali *et al*., 1997; Brown *et al*., 1998; Sugihara *et al*., 1999). Numerous researchers have been reported the biological activities of propolis collected in Europe but only a few reports can be found about Turkish propolis. Therefore, complete chemical analysis of ethanol extract of propolis sample has to be examined for the complete characterisation of pharmacological activity of propolis from different regions of Turkey. However, such studies have been carried out rarely so far (Keskin *et al*., 2001; Kolankaya *et al*., 2002; Kartal *et al*., 2003; Yıldırım *et al*., 2004, Aliyazicioglu *et al*., 2005; Gunduz *et al.* 2005, Sonmez *et al.* 2005, Şahinler and Kaftanoğlu, 2005; Silici and Kutluca, 2005; Uzel *et al*., 2005). In this study, antimicrobial and antioxidant activities of propolis samples from different regions of Turkey were examined and these effects were investigated depending to the chemical composition of ethanol extract of propolis samples.

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MATERIALS AND METHODS

Propolis samples and preparation of alcohol extracts. Eight samples of propolis were collected from Denizli (P1, P5), Denizli-Sarayköy (P2A), Aydın (P2B), Denizli-Başkarcı (P3), Aydın (P4), Konya (P6) and Tekirdağ (P7) cities in spring 2004. Each sample was cut into small pieces after cooling at –20 ºC, and extracted with 96% ethanol (1:10 w/v) at 37 ºC for 5 days. The ethyl alcohol extracts were then filtered through a Whatman No. 1 filter paper and evaporated to dryness under vacuum. The samples were kept at -20 ºC for the sample preparation to examine in GC-MS and antimicrobial activity experiments (Blonska *et al*. 2004).

Characterisation of the chemical compounds in propolis extracts by GC-MS. About 5 mg of propolis dry residual powder 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 analysed by GC-MS. A GC 6890N from Hewlett-Packard (Palo Alto, CA, USA) coupled with mass detector (MS5973, Hewlett-Packard) was used for the analysis of extracted propolis samples.

Experimental condition of GC-MS system was as follows: DB5 MS column (30 m x 0.25 mm and 0.25 μm of film thickness) was used and flow rate of mobile phase (He) was set at 0.7 ml/min. In the gas chromatography part, temperature was kept for 1 minl at 50 °C then increased to 150 °C with 10 °C/min heating ramp. After this period, temperature was kept at 150 °C for 2 min. Finally, temperature was increased to 280 °C with 20 °C/min heating ramp and then kept at 280 °C for 30 min. Organic compounds into the propolis samples were identified using standard Willey and Nist Libraries available in the data acquisitions system of GS-MS, if the comparison scores were obtained higher than 95%. The peaks of the compounds have been identified on the basis of their retention times on the GC-MS chromatograms. For the quantification of the compounds in the ethanol extract, no internal and external standard was used. Only percent reports of the compounds in the samples were used. In this case, the relative error could not be higher than 5%.

Screening of antimicrobial activity of propolis samples. *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 35218, *Morganella morganii* (clinical isolate), *Klebsiella pneumoniae* ATCC 27736, *Proteus vulgaris* RSKK 96026, *Salmonella enteritidis* RSKK 171, *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 12598, *Listeria monocytogenes* Li6, *Bacillus subtilis* ATCC 6633 and *Candida albicans* (clinical isolate) were used for antimicrobial activity test. Antimicrobial activity of propolis samples was determined by the disc diffusion method (Kartal *et al*., 2003). The antimicrobial screening was performed using Nutrient agar and Yeast Extract Peptone Dextrose (YEPD) agar for the yeast. The culture suspensions were prepared and adjusted against 0.4 Mc Farland turbidity standard tubes. All propolis samples were grated before extraction performed with 96% EtOH to obtain 0.1 mg/ml extract concentration (w/v). Empty sterilised discs of 6 mm (Schleicher and Schuell, No. 2668, Germany) were each impregnated with 20 ml of samples and placed on agar plates. The plates were incubated at 37 °C for 24 h for bacteria, and 48 h for *C. albicans*. Inhibition zones formed on the medium were evaluated in mm. The solvent control (ethyl alcohol) did not show any antimicrobial activity. Reference discs used for control are as follows: ketoconazole (50 mg), ampicillin (10 mg), streptomycin (10 mg), penicillin (10 U), chloramphenicol (15 mg) and gentamicin (10 mg). All tests were done in duplicate and the inhibition zones were compared with those of reference discs.

Antioxidant activities.
Chemicals. β-carotene, linoleic acid, 1,1-diphenly-2-picrylhydrazyl (DPPH), buthylated hydroxytoluene (BHT), buthylated hydroxyanisol (BHA) and α-tocopherol were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Pyrocatechole, Tween-20, Folin-Ciocalteau's phenol reagent (FCR), sodium carbonate, ethanol, chloroform and the other chemicals and reagents were purchased from Merck (Darmstat, Germany). All other unlabeled chemicals and reagents were analytical grade.

DPPH assay. The hydrogen atom or electron donation abilities of the corresponding extracts and some pure compounds were measured from the bleaching of the purple-coloured ethanol solution of DPPH. This spectrophotometric assay uses the stable radical DPPH as a reagent (Cuendet *et al*., 1997; Burits and Bucar, 2000). Different concentrations of the propolis extracts (200 ml) in ethanol were added into 5 ml of a 0.004% (w/v) ethanol solution of DPPH. After 30 min incubation period at room temperature, the absorbance was measured against a blank at 517 nm. Inhibition of free radical by DPPH in percent (I %) was calculated using the following expression:

$$I\ \% = (A_{blank} - A_{sample} / A_{blank}) \times 100$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. Extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotted inhibition percentage against extract concentration. Synthetic antioxidant reagent BHA was used as a positive control and all tests were carried out in triplicate.

Determination of total antioxidant activity. Heat-induced oxidation of an aqueous emulsion system of β-carotene and linoleic acid was used as the antioxidant activity test model (Pratt, 1992). One millilitre of β-carotene (0.2 mg/ml) was dissolved in chloroform and added into an Erlenmeyer flask containing linoleic acid (0.02 ml) and Tween 20 (0.2 ml). The mixture was than dosed with 0.2 ml of the corresponding flavonoid or standard solution in ethanol at a concentration of 10^{-3} M. Distilled water (50 ml), saturated with oxygen for 15 min, and was added into the flask. The resulting mixture was then shaken and kept for 2 h at 50 °C. The absorbance of the samples was measured at 470 nm, immediately after prepared (t = 0 min) and at the end of the experiment (t = 120 min). Antioxidant activity of the propolis extracts was calculated as percent inhibition of oxidation versus control sample without propolis extract added, using the following equation:

$$\text{Antioxidant activity } \% = 100 \times [1-(A_s^0 - A_s^{120}) / (A_c^0 - A_c^{120})]$$

where A_s^0 is the absorbance of the sample at 0 min, A_s^{120} is the absorbance of sample at 120 min, A_c^0 is the absorbance of control sample at 0 min, and A_c^{120} is the absorbance of control sample at 120 min.

Determination of total phenolic compounds. Total phenolic constituents of the aforesaid extracts of propolis were determined by the literature methods involving the Folin–Ciocalteau reagent and pyrocatechol as standard Slinkard and Singleton (1977). Extract solution (0.1 ml), containing 0.2 mg extract was transferred into a volumetric flask, 46 ml distilled water and 1 ml Folin-Ciocalteau reagent were added, and then flask was shaken thoroughly. After 3 min shaken time, 3 ml of 2% Na_2CO_3 solution was added and the mixture was hold for 2 h with intermittent shaking. Finally, absorbance was measured at 760 nm. The same procedure was repeated for all standard pyrocatechol solutions and a standard curve was obtained by the equation given below:

Absorbance = 0.00247 x total phenols [pyrocatechol equivalent (μg)] – 0.00054

Statistical analysis: The data were analysed and treatments compared using the one-way ANOVA with 95% confidence limits (SPSS 9.0 statistical analysis system program). A level of $p < 0.05$ was used as the criterion for statistical significance.

TABLE 1 – Chemical composition of ethanol extract of propolis samples determined by GC-MS*

Compound	P1	P2A	P2B	P3	P4	P5	P6	P7
Aromatic alcohol	0.10	0.20	0.08	0.17	0.20	0.33	–	0.25
Aromatic acid	0.17	0.08	0.33	0.25	0.17	0.39	–	0.88
Aromatic diol	0.09	0.13	0.17	–	0.08	–	0.26	–
Diphenyl amine	0.18	0.10	0.22	0.06	0.15	0.13	0.22	0.18
Alcoholic terpenes	–	–	–	–	–	–	–	–
Aromatic esters	–	–	–	0.08	0.15	0.05	0.15	–
3-4-Dimethoxy-cinnamic acid	1.29	1.09	0.74	0.89	1.09	0.81	1.19	0.52
Flavanones	3.72	1.40	4.70	5.01	3.84	2.67	4.52	3.33
Cinnamic acid	–	–	0.67	1.77	1.70	0.84	–	0.84
Flavonoids	7.51	6.03	16.30	16.45	7.46	5.10	12.07	4.57
Naringenin	2.21	3.46	1.20	7.06	1.19	2.30	4.04	8.40
Apigenin	8.01	7.56	10.93	11.28	7.29	11.50	7.75	14.02
5-Hidroxy-7-methoxy flavonone	0.84	0.62	2.05	1.94	1.59	1.23	2.24	0.73
Chrysin	14.9	12.66	12.62	17.59	13.07	18.57	13.28	22.18
Vitamine E	0.07	0.36	–	0.09	0.17	–	0.24	–
2-Napthalene methanol	0.23	0.07	0.21	0.31	0.08	0.13	0.18	0.18
2-Propenoic acid-phenyl	0.12	0.07	0.93	0.25	–	0.24	–	0.58
Phenyl ethyl alcohol	0.77	2.69	–	–	0.57	0.55	2.16	–
Hexadeconoic acid ethyl ester	0.76	0.67	0.70	0.80	0.67	0.57	0.75	0.44
Ethyl oleate	1.15	0.62	2.05	1.95	1.59	1.23	2.23	1.26
9-Octadecenoic acid	1.84	0.63	2.47	1.87	1.25	1.41	2.41	1.33

P1: Denizli, P2A: Denizli-Sarayköy, P2B: Aydın, P3: Denizli-Başkarcı, P4: Aydın, P5: Denizli, P6: Konya, P7: Tekirdağ.
* Chemical composition was determined as per cent of each chemical species out of total soluble organic compounds in ethyl alcohol. All results are given with 5 parallel experiments with 95% confidence interval and with maximum 0.02 standard deviations.

RESULTS AND DISCUSSION

Chemical composition and antimicrobial activity of propolis extracts

In the present study, the antimicrobial and antioxidant activities and chemical compounds of ethanol extract of propolis collected from different regions of Turkey in spring 2004 were determined. Analyses of compounds in propolis samples were done by GC-MS and the results are given in Table 1.

Chrysin, apigenin, flavonoids, flavanones, naringenin, ethyl oleate, 3-4-dimethoxy-cinnamic acid and 9-octadecenoic acid were the predominant compounds in propolis samples. Kosalec *et al.* (2004) has been reported that the high variability of flavanone will be affected the biological activity of propolis. Park *et al.* (1998) were determined that all ethanolic extracts of propolis from various regions in Brazil contained pinocembrin and galangin. Bankova *et al*. (2002) studied the chemical composition of ten propolis samples from Bulgaria, Italy and Switzerland and concluded that they contained mainly pinocembrin, pinobanksin, chrysin, galangin, prenyl esters of caffeic and ferrulic acids. Kujumgiev *et al*. (1993) found flavonoids and phenolic acid esters as main constituents in Bulgarian propolis samples. Some of the main components in the propolis samples collected Bulgaria, Italy and Switzerland were found to be the same, but Turkish propolis samples had some different organic compounds also. Uzel *et al.* (2005) reported that the main compounds of four Anatolian propolis samples (Bartın, Bursa-Orhangazi, Trabzon and Ankara-Mamak) were flavonoids such as pinocembrin, pinostropin, isalpinin, pinobanksin, quercetin, naringenin, galangine and chrysin. Sorkun *et al.* (2001) found flavanones, terpenoids, flavones, ketones, alcohols, aromatic acids, aliphatic acids and their esters, and derivatives as main constituents in Bursa, Trabzon-Caglayan, Gumushane-Sogutagil and Erzurum-Askale propolis samples.

The disc diffusion method was used to determine the inhibition zones of the different ethyl alcohol extracts from eight propolis samples. Six Gram-negative, four Gram-positive bacterial strains and one yeast strain were used (Table 2).

Pseudomonas aeruginosa, Klebsiella pneumoniae, Proteus vulgaris, Salmonella enteritidis, as Gram-negative bacteria, were sensitive to EEP samples of P3, P5 and P7. Chrysin, a typical poplar flavonoid, has antibacterial activity (Popova *et al*., 2005) and the high concentration in these samples could explain the activity of our samples against Gram-negative bacteria. All samples were found to be more effective on tested Gram-positive bacteria; also showed anticandidal activity against to the tested *Candida albicans.* Some authors reported that propolis was active against Gram-positive bacteria and some fungi, but showed a limited effect against Gram-negative bacteria (Grange and Davey, 1990; Kujumgiev *et al*., 1999; Nieva *et al*., 1999; Sforcin *et al*., 2000; Salomao *et al*., 2004), while Castaldo and Capasso (2002) showed that propolis samples *in vitro* had antimicrobial activity mainly against Gram-positive (*Staphylococcus* spp. and *Streptococcus* spp.) and Gram-negative bacteria (*Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris* and *Pseudomonas aeruginosa*). Many researchers have reported that some foodborne bacteria and fungi may be inhibited by propolis extracts (Grange and Davey, 1990; Marcucci, 1995; Özcan, 2000). Antimicrobial activity of propolis attributes mainly on the presence of compounds such as flavonoids or phenolic and benzoic acids and

TABLE 2 – Antimicrobial activities of ethanol extract of Turkish propolis obtained from different regions

Tested microorganisms	Antimicrobial activities of propolis extracts								Standard antibiotic (content/disc)	Inhibitory zone (mm)
	P1	P2A	P2B	P3	P4	P5	P6	P7		
Pseudomonas aeruginosa ATCC 27853	-[a]	-	-	+	-	+	-	++	Gentamicin (10 μg)	16
Escherichia coli ATCC 35218	-	-	-	-	-	-	-	-	Penicillin (10 U)	19
Morganella morganii	-	-	-	-	-	-	-	-	Chloramphenicol (30 μg)	15
Klebsiella pneumoniae ATCC 27736	-	-	-	+	-	+	-	+	Streptomycin (15 μg)	11
Proteus vulgaris RSKK 96026	-	-	-	+	-	+	-	+	Ampicillin (10 μg)	10
Salmonella enteritidis RSKK 171	-	-	-	++	-	+	-	+	Ampicillin (10 μg)	13
Staphylococcus aureus ATCC 25923	+	+	++	++	++	+	++	+	Ampicillin (10 μg)	9
Staphylococcus aureus ATCC 12598	++	+	++	++	+	+	++	+	Ampicillin (10 μg)	8
Listeria monocytogenes Li6	++	++	++	+++	+++	++	++	++	Ampicillin (10 μg)	10
Bacillus subtilis ATCC 6633	++	++	+++	+++	++	++	+++	++	Ampicillin (10 μg)	15
Candida albicans	+	+	++	++	++	+	++	+	Ketoconazole (50 μg)	16

P1: Denizli, P2A: Denizli-Sarayköy, P2B: Aydın, P3: Denizli-Başkarcı, P4: Aydın, P5: Denizli, P6: Konya, P7: Tekirdağ.
[a] – = no inhibition, + = ≤ 8 mm, ++ = >8 to < 12 mm, +++ = ≥ 12 mm.

their esters (Kujumgiev *et al*., 1993; Park *et al*., 1997; Marcucci *et al*., 2001; Kosalec *et al*., 2003; 2004) and the mechanism of this activity depends on synergism among some flavonoids, phenolic acids and other compounds in propolis (Burdock, 1998; Pepeljnjak and Kosalec, 2004). Koo *et al*. (2000) and Duarte *et al*. (2006) found that fatty acid (oleic, palmitic, linoleic and stearic) in propolis effected on the microbial composition of dental plaque and oral pathogens.

In present study, while antimicrobial screening clearly indicated that P3, P2B, and P6 samples of propolis had much more powerful antibacterial activity for Gram-positive bacteria, P3, P5 and P7 samples of propolis had much more powerful antibacterial activity for Gram-negative bacteria when compared with the others. However, these propolis samples not found of different significantly in their activity against test microorganisms ($F = 1.015$, $df = 12$, $p > 0.05$). Compared to reference discs, *S. aureus* ATCC 12598, *B. subtilis* and *L. monocytogenes* were found that low concentrations of these propolis were more effectively than reference discs.

Antioxidant activity of ethanolic propolis extracts

Tri complementary test systems, namely free radical scavenging, beta-carotene/linoleic acid system and the amount of total phenolic compound are used. Free radical scavenging capacities of the extracts, measured by DPPH assay, IC_{50} are ranging from 34.03 to 46.00 μg/ml. All the propolis extract studied showed free radical scavenging activity. The highest activity was shown by samples P2A from Sarayköy-Denizli (IC_{50}= 34.03 μg/ml propolis extract was necessary to obtain 50% of DPPH degradation). It is interesting to note that the inhibition of P2A extract was almost similar to that of BHA, which is a synthetic antioxidant. In addition, it was shown that free radical scavenging activities of other extracts were close to each other and the inhibition values were high.

The existing of ethanol extract of propolis can hinder the extent of β-carotene bleaching by neutralising the linoleate free radicals formed in the system. The ethanol extracts of propolis and BHA at 20, 40, 80 and 160 ppm concentration as measured by bleaching of β-carotene. It can be seen that P2A extract exhibited 62, 75, 82 and 92% antioxidant activity at 20, 40, 80 and 160 ppm concentrations, respectively. It was shown that in all samples the colour of β-carotene was not changed as the extract amount increased, i.e. antioxidant activity was increased.

Phenols are very important plant constituents because of their scavenging ability due to their hydroxyl groups (Hatano *et al*., 1989). According to the recent reports, a highly positive relationship between total phenols and antioxidant activity was found in many plant species (Vinson *et al*., 1998; Oktay *et al*., 2003). Pyrocatechol equivalents of phenols (81.62 μg) were detected in 200 μg P2A, while other samples are ranging from 53.67 μg (P4) to 68.17 μg (P3). This shows that there is a directly relationship between phenolic compounds and antioxidant activity, and the other aromatic alcohols increases the antioxidant activity of phenolic species. However, vitamin E content of P2A and P6 is high compared to the other propolis sample extracts. Vitamin E content of propolis extract seems to be effective on the antioxidant activity of propolis extracts.

The phenolic compounds may contribute directly to the antioxidative action (Duh *et al*., 1999). In addition, it was reported that phenolic compounds were associated with antioxidant activity and play an important role in stabilising lipid peroxidation (Yen *et al*., 1993).

In conclusion, antioxidative capacity of propolis samples obtained from four different regions in Turkey by means of DPPH free radical scavenging and total antioxidant capacity as well as amounts of their phenolic compounds was observed to exert similar inhibition to the synthetic antioxidant BHA. Particularly from the view point of free radical scavenging, P2A propolis ethanolic extract (IC_{50}: 34.03 μg/ml) was shown to have an inhibition as good as BHA (IC_{50}: 33.02 μg/ml). Besides, we found out that the chemical contents and antioxidant capacity of 8 different propolis samples were depending on where they were obtained. According to the data, the sample coded P2A was found to be rich in aromatic alcohol, aromatic diol, 3,4-dimethoxy cinnamic acid, chrysin, phenyl ethyl alcohol and α-tocopherol. It may also be said that, therefore, P2A could have the best antioxidant capacity due to its richer phenyl ethyl alcohol and α-tocopherol amount. Choi *et al.* (2005) were showed that strong antioxidant and antimicrobial activity of propolis from several regions of Korea were related with total polyphenol

and flavonoid contents. However, Isla *et al.* (2001) reported that the correlation between flavonoid contents and antioxidant activity is significant, but other factors would be involved. In conclusion, Anatolian propolis samples could be evaluated as an approach to cancer treatment and alternative for synthetic food preservatives, depending on their high inhibitory capacity on the radicalic reactions in living organisms. The propolis samples had much more effective antibacterial activities on Gram-positive bacteria. However, among Gram-negative bacteria, only *P. aeruginosa, K. pneumoniae, P. vulgaris* and *S. enteritidis* were sensitive against the extracts of P3, P5 and P7.

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