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RESEARCH PAPER

Antibiofilm, Antioxidant and Quorum Quenching Activities of Propolis Samples from Southwest Anatolia

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Abstract

In this study, antibiofilm, antioxidant and quorum quenching activities of the ethanol extracts of propolis samples collected from Muğla district were investigated. Antimicrobial activity was determined using the well diffusion and broth tube dilution methods, antibiofilm activity with microplate biofilm method, and antioxidant activity with DPPH radical scavenging, β-carotene linoleic acid and ferric thiocyanate methods. To determine the antimicrobial activity of the extracts, *Listeria monocytogenes* ATCC 7944, *Streptococcus mutans* CNCTC 8/77, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Salmonella typhimurium* ATCC 14028 and *Candida albicans* ATCC 10239 strains were used. Minimum inhibitory concentrations (MIC) against microorganisms were determined from 1 to >100 mg/ml. The lowest MIC value was found as 1 mg/ml for AP6 propolis extract against *Salmonella typhimurium.* According to the antibiofilm activity results, highest biofilm were detected at concentrations of MIC as 82.60% for AP1 against *S. mutans*, 67.45% for AP2 against *L. monocytogenes*, 73.02% for AP3 against *S. mutans*, 64.05% for AP4 against *L. monocytogenes*, 70.58% for AP5 against *S. typhimurium*; 93.43% for AP6 against *S. typhimurium* and 72.43% for AP7 against *S. mutans*. AP7 extract had the highest antioxidant activity with an IC_{50} value of 3.94 mg/ml for DPPH radical scavenging method and with 91.10% reduction rate for β-carotene linoleic acid method. AP1 extract had the highest reduction percentage rate of 51.77% in the ferric thiocyanate method.

Introduction

Propolis is a natural resin, collected mainly by the honey bee, *Apis mellifera*, which has been shown to have many biological activities including antioxidant and antimicrobial effects, both conferred by phenolic compounds, especially flavonoids (Gonçalves, Santos, & Srebernich, 2011; Talla et al., 2017; Tamfu et al., 2020). More than 150 components such as polyphenols, phenolic aldehydes, sesquiterpene quinines, coumarins, amino acids, steroids and inorganic components have been identified in propolis samples (Marcucci, 1995; Anjum et al., 2019). Propolis has long been used in oriental folk medicine for curing infections (Cheng & Wong, 1996; Blicharska & Seidel, 2019) and in European ethno-pharmacology as an antiseptic and anti-inflammatory agent for healing wounds and burns (Ghisalberti, 1979; Rojczyk, Klama-Baryla, Labus, Wilemska-Kucharzewska, & Kucharzewska, 2020). Propolis exhibits antimicrobial, antioxidant, anti-inflammatory, anaesthetic and other properties (Bankova, de Castro, & Marcucci, 2000).

The purposes of the study were to determine antibiofilm, antioxidant and quorum quenching activities of propolis samples.

Materials and Methods

Propolis samples and preparation of alcohol extracts

Propolis samples were collected from seven different areas; Marmaris (Osmaniye: AP1; Merkez: AP5); Fethiye (Yanıklar: AP2; Uzunyurt: AP3); Datça (AP4); Milas (AP6); and Bodrum (Gümüşlük: AP7) in Muğla located in Southwest Anatolia. Each sample was cut into small pieces after cooling at -20°C and extracted with 96%

ethanol (1:10 w/v) at 37°C 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°C until test experiments (Blonska et al., 2004).

Antibiofilm Activity

Antibiofilm activities in MIC, MIC/2, MIC/4 and MIC/8 concentrations for propolis extracts were determined on polystyrene flat-bottomed microtitre plates as described by Merritt, Kadouri and O'Toole, (2005).

Antioxidant Methods

Determination of DPPH Radical Scavenging Activity

Antioxidant activity of the propolis extracts were determined based on its ability to react with the stable 1,1-diphenyl-2-picryl hydrazyl (DPPH) free radical (Burits & Bucar, 2000) Percentage of inhibition and the concentration of sample required for 50% scavenging of the DPPH free radical (IC_{50}) were determined. BHT and ascorbic acid were used as a positive control.

β-carotene Linoleic Acid Methods

β-Carotene-linoleic acid test system was used to assay lipid-peroxidation inhibitory activity (Dapkevicius, Venskutonis, van Beek, & Linssen, 1998).

Ferric Thiocyanate (FTC) Method

A screw-cap vial containing a mixture of 4 mg of sample in 4 mL of 99.5% ethanol, 4.1 mL of 2.51% linoleic acid in 99.5% ethanol, 8.0 mL of 0.02 M phosphate buffer (pH 7.0) and 3.9 mL of water (final concentration 0.02%, w/v) was placed in an oven at 40°C in the dark (Mitsuda, Yuasumoto, & Iwami, 1996). To 0.1 mL of this mixture in a test tube, 9.7 mL of 75% (v/v) ethanol, 0.1 mL 30% ammonium thiocyanate and finally, 0.1 mL of 2×10^{-2} M ferrous chloride in 3.5% hydrochloric acid was added to the reaction mixture. Three minutes after the addition of ferrous chloride, the absorbance was measured at 500 nm. This step was repeated every 24 h until the control reached its maximum absorbance value.

Quorum Quenching Activities Methods

The bacterial strains employed in the study were *Chromobacterium violaceum* CV026 for anti-quorum sensing, *C. violaceum* CV12472 for violacein inhibition effects of the propolis extracts. Anti-quorum sensing experiments were carried out according to the methods of Koh and Tam (2011), while the violacein inhibition experiments were performed as described by Choo, Rukayadi, & Hwang, (2006).

Results

Prior to investigation of biofilm inhibitory potential of test samples, MIC values of propolis samples were determined on the selected microorganisms and biofilm inhibition assay was performed at MIC and sub-MIC concentrations. The anti-biofilm activity results are given in Table 1 as percentage inhibition values. The antibiofilm activity results showed that the highest biofilm inhibition were observed at MIC concentrations. The antioxidant potential of propolis samples were evaluated using three different methods: DPPH radical scavenging assay, β-carotene-linoleic acid assay and Ferric thiocynate method and the results presented on Figure 2-4. Prior to quorum quenching activity determination, the MIC values of the propolis extracts were determined against *C. violaceum* CV 12472 and CV026 and presented in Table 2. The MIC and sub-MIC concentrations were then used for the determination of percentage violacein inhibition of samples (Table 3, Figure 1). The MIC values of the propolis extracts against *C. violaceum* CV026 biomonitor strain were determined and their QSI evaluated at sub-MIC concentrations (Table 4).

Discussion

Antibiofilm Activity

The biofilm inhibitions were determined as 82.60% for AP1 sample against *S. mutans*, 67.45% for AP2 against *L. monocytogenes*, 93.43% for AP6 against *S. typhimurium* for MIC concentration. Scazzocchio, D'Auria, Alessandrini, and Pantanella (2006) found a higher rate of *S.aureus* biofilm inhibition than our study. Koudhi, Zmantar, and Bakhrouf (2010), Dogan et al. (2014) and Capoci et al. (2015) reported higher rates of biofilm inhibition at lower concentrations compared to our results. This can be caused by the difference in the regions of collection of propolis samples.

The Antioxidant Activities

DPPH radical scavenging, β-carotene linoleic acid and ferric thiocyanate methods were used for determination of antioxidant activities. The extracts of the propolis sample from Bodrum, Gümüşlük (AP7) showed the highest antioxidant activities with IC_{50} value of 3.94 mg/mL for DPPH radical scavenging method and with 91.10% reduction rate for β-carotene linoleic acid method. The lowest antioxidant activity was determined as IC_{50} 26.33 mg/mL for DPPH radical scavenging method in AP2. The highest antioxidant activity was shown at AP1 extract with a rate of 51.77% using ferric thiocyanate method whereas AP4 extract showed the lowest prevention of lipid peroxidation (34.74%). Nieva Moreno, Isla, Sampieto, and Vattuone (2000) and Lu, Chen, and Chou (2003) reported results consistent with

Table 1. Antibiofilm activities of propolis extracts at the MIC and sub-MIC concentrations

Extracts	Conc. (mg/mL)	*C. albicans*	*S. aureus*	*S. mutans*	*L. monocytogenes*	*E. coli*	*S. typhimurium*
AP1	MIC	10.99±5.84	47.28±4.19	82.60±6.92	60.63±4.75	3.29±2.36	42.99±0.40
	MIC/2	-	29.55±3.53	73.11±0.14	48.49±5.35	-	35.50±0.60
	MIC/4	-	11.70±5.20	71.85±0.23	32.96±4.53	-	28.03±0.26
	MIC/8	-	-	62.96±0.15	18.42±1.76	-	5.59±0.88
AP2	MIC	-	35.37±2.85	46.21±7.84	67.45±1.77	8.88±0.55	60.23±4.57
	MIC/2	-	10.39±0.63	36.45±2.73	53.89±1.00	-	40.64±1.02
	MIC/4	-	1.76±3.38	21.95±1.02	45.62±0.52	-	26.63±0.21
	MIC/8	-	-	-	27.62±5.07	-	7.48±1.00
AP3	MIC	14.59±1.19	65.24±0.20	73.02±1.39	18.96±3.58	10.28±0.09	38.78±0.10
	MIC/2	5.16±4.11	53.62±0.78	56.77±1.36	-	-	28.01±2.54
	MIC/4	-	39.53±4.57	31.04±2.67	-	-	10.73±1.30
	MIC/8	-	27.19±4.42	14.03±4.57	-	-	-
AP4	MIC	10.92±1.44	32.76±1.05	61.98±1.52	64.05±2.29	9.34±0.08	58.41±0.07
	MIC/2	7.79±1.48	23.88±1.11	50.58±0.58	51.45±0.47	-	41.07±5.22
	MIC/4	6.74±2.53	7.37±0.75	32.21±3.83	38.77±6.41	-	22.88±1.18
	MIC/8	1.56±0.53	-	8.51±3.11	24.21±5.59	-	3.72±1.83
AP5	MIC	51.53±3.10	46.58±2.68	61.40±2.10	70.41±3.11	13.56±1.52	70.58±2.06
	MIC/2	26.58±1.83	31.29±0.41	40.90±3.69	62.67±5.94	-	44.88±3.22
	MIC/4	-	15.40±0.77	35.02±2.99	50.92±4.84	-	19.57±5.42
	MIC/8	-	-	10.63±0.17	34.94±0.63	-	1.88±1.88
AP6	MIC	28.14±3.37	26.41±4.46	86.47±5.38	68.43±0.79	14.50±2.47	93.43±1.93
	MIC/2	13.58±4.30	18.50±0.61	61.98±1.52	36.37±3.04	-	82.73±2.17
	MIC/4	7.29±0.07	-	32.40±1.31	12.66±4.00	-	75.22±0.69
	MIC/8	-	-	18.85±1.41	-	-	49.07±0.92
AP7	MIC	59.88±0.93	54.15±1.94	72.43±0.81	61.12±4.26	12.10±4.55	64.45±3.13
	MIC/2	41.67±0.43	44.01±0.10	56.00±3.29	46.10±1.00	-	53.24±3.24
	MIC/4	24.97±2.86	26.28±0.54	39.26±1.42	32.98±2.59	-	35.97±1.06
	MIC/8	-	12.30±0.92	21.57±4.00	15.56±3.06	-	26.15±1.62

-: No activity

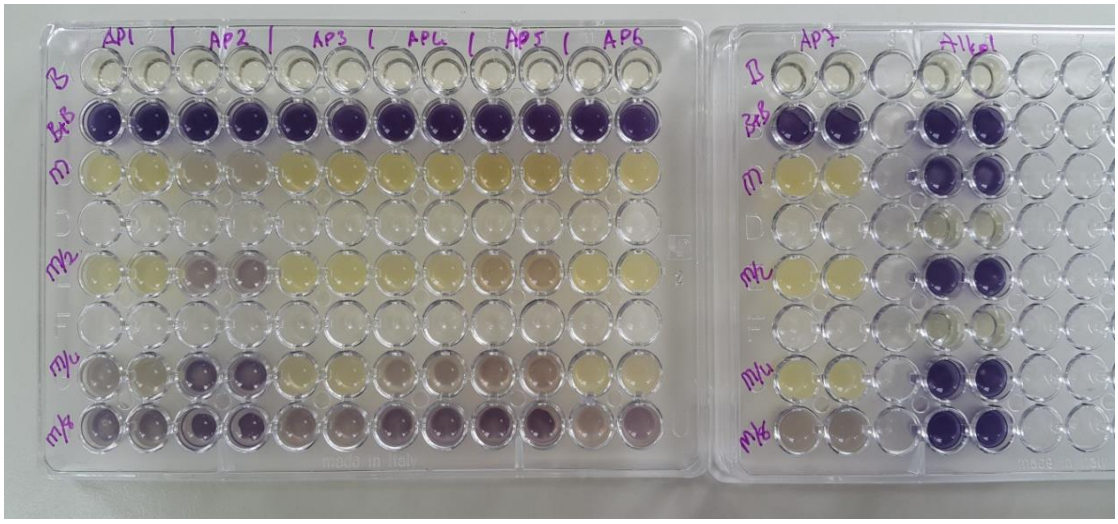

Figure 1. Violacein production inhibition results of propolis extracts against CV 12472

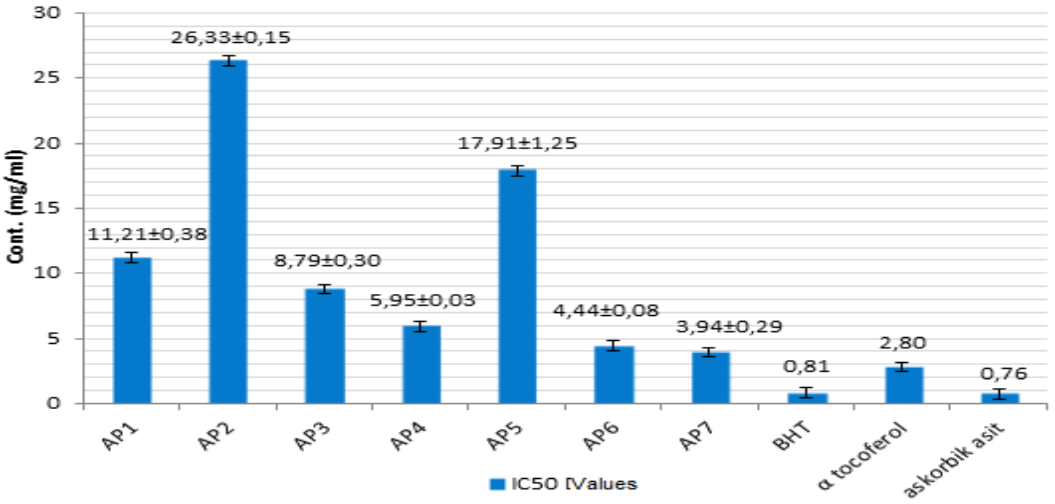

Figure 2. Results of DPPH free radical scavenging activities of propolis extracts,

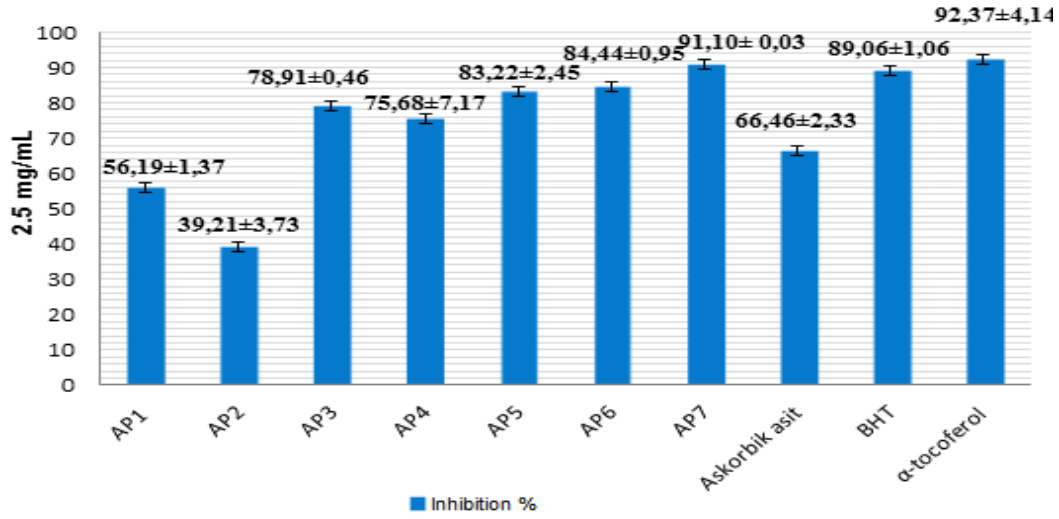

Figure 3. β-carotene-linoleic acid assay results of Propolis extracts

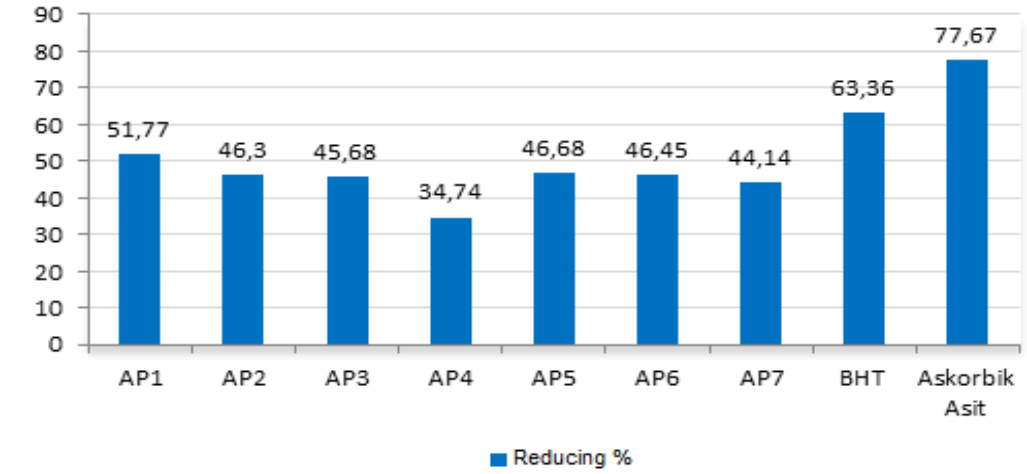

Figure 4. FTC result graph of propolis extracts (% inhibition)

Table 2. MIC concentrations against *C. violaceum* CV 12472 and *C. violaceum* CV 026 strains

Samples	CV12472	CV026
	MIC Conc.(mg/mL)	
AP1	6.25	6.25
AP2	6.25	6.25
AP3	6.25	6.25
AP4	3.12	6.25
AP5	12.5	12.5
AP6	3.12	3.12
AP7	3.12	3.12

Table 3. Violacein inhibition rates of propolis extracts against *C. violaceum* CV 12472

	Violacein inhibition (%)						
Conc.	AP1	AP2	AP3	AP4	AP5	AP6	AP7
MIC	100	64.8±0.5	100	100	100	100	100
MIC/2	100	39.8±0.2	100	100	51.8±1.8	100	100
MIC/4	47.1±1.2	29.2±0.5	100	50.4±3.7	48.9±0.6	100	100
MIC/8	41.2±0.8	23.1±0.3	49.7±1.0	34.6±1.1	37.3±0.5	45.6±2.8	51.0±0.4

Table 4. Antiquorum sensing activity results of propolis extracts

Propolis extracts	Concentrations (mg/mL)	*C. violaceum* CV026	
		Antimicrobial zone (mm)	QS inhibition zone (mm)
AP1	MIC	-	-
	MIC/2	-	-
	MIC/4	-	-
AP2	MIC	-	-
	MIC/2	-	-
	MIC/4	-	-
AP3	MIC	-	-
	MIC/2	-	-
	MIC/4	-	-
AP4	MIC	-	9
	MIC/2	-	-
	MIC/4	-	-
AP5	MIC	-	-
	MIC/2	-	-
	MIC/4	-	-
AP6	MIC	8	10
	MIC/2	7	-
	MIC/4	-	-
AP7	MIC	-	12
	MIC/2	-	10
	MIC/4	-	8
C_{10}HSL		-	31
Ethanol		-	-

-: No effect

our DPPH results in their study. Chen, Weng, Wu, and Lin (2004), Russo et al. (2004), Choi et al. (2006), Alencar et al. (2007), Moreira, Dias, Pereira, and Estevinho (2008), Miquel, Nunes, Dandlen, Cavaco, and Antunes (2010), Piccinelli et al. (2013) and Silva Frozza et al. (2013) reported lower IC_{50} values compared to our results. Sheng, Zhou, Wang, Xu, and Hu (2007) reported results consistent with our study in the antioxidant activity experiments they conducted with the FTC method.

Quorum Quenching Activities

In the study, MIC values of biomonitor strains against propolis extracts were determined prior to the determination of antiquorum sensing activity. The highest antimicrobial effect was found in AP4, AP6 and AP7 extracts against C. violaceum CV12472 strain. The highest antimicrobial effect was seen in AP7 extract against C. violaceum CV026 strain. All of the propolis extracts used in the study showed inhibition of violacein production at different concentrations. Also, the highest inhibition of violacein production was determined in 100% at concentrations of MIC, MIC/2 and MIC/4, and 51.0% at a concentration of MIC/8 for AP7 extract.

It was determined that AP4, AP6 and AP7 extracts among the propolis extracts used in the study have antiquorum sensing activity. However, there were no activity detected for AP1, AP2, AP3, AP5 for antiquorum quenching activity. The results reported by Savka et al. (2015) confirm our anti-quorum sensing results.

Conclusion

The antimicrobial effect of propolis samples collected from Milas and Bodrum (Gümüşlük) was revealed in this study. It was found that the propolis sample collected from the Milas region highly inhibited the *S.mutans* and *S.typhimurium* biofilm formation, especially at MIC concentrations. DPPH and β-carotene linoleic acid method results also revealed that Milas and Bodrum (Gümüşlük) propolis samples have high antioxidant activity as well as antimicrobial activity. Considering the antiquorum sensing results, it was determined that the Bodrum (Gümüşlük) propolis sample was effective in terms of quorum sensing inhibition.

Amongst the propolis ethanol extracts used in the study, Milas and Bodrum (Gümüşlük) samples showed the feature of being alternative in the treatment of many infections due to their antimicrobial and antibiofilm activities, and to eliminate the harmful effects of free radicals that cause many health problems, according to the antioxidant activity results. In addition, these two propolis extracts were found to be able to prevent the quorum sensing communication system used by most pathogenic bacteria to cause disease and especially to control biofilm production. Accordingly, it has been revealed that these two extracts have a potential in alternative treatment studies to be applied in the field of medicine.

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