Antibiofilm Activity and Chemical Contents of Propolis Samples From Manisa-Turkey

Türkiye-Manisa'dan Propolis Örneklerinin Kimyasal İçeriği ve Antibiyofilm Aktivitesi

Research Article

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ABSTRACT

In this study, the inhibition of biofilm formation and the reduction of preformed or established biofilm by ethanol extract of propolis samples (EEP) obtained from Manisa-Turkey was investigated and their chemical composition was screened. The antibiofilm effect of the propolis extracts against biofilm forming bacteria (*Listeria monocytogenes* ATCC 7644, *Methicillin Sensitive Staphylococcus aureus* (clinical isolate) MSSA M20, *S. aureus* ATCC 33862, *S. aureus* ATCC 29213, *Enterococcus faecalis* ATCC 19433, *Pseudomonas fluorescens* ATCC 55241, *Micrococcus luteus* NRRL-B1013) was tested on 96-well polystyrene plates using crystal violet assay. Also, the antibacterial activity of EEP was evaluated according to Agar Well Diffusion method. Chemical composition of extracts was detected by Gas Chromatography-Mass Spectrometry (GC/MSD) analyze. The EEP samples exhibited good antibiofilm activity against bacteria. The maximum biofilm inhibition activity percentage of MP-1 (Manisa-Köprübaşı), MP-2 (Manisa-Demirci) and MP-3 (Manisa-Kula) were found as 89.4%, 80.0% and 89.0% for *L. monocytogenes* ATCC 7644 and 66.0%, 67.0% and 74.0% for MSSA M20, respectively. According to GC/MSD analyze, triacontyl acetate was the major compound found in propolis extracts.

Key Words
Antibiofilm, Biofilm reduction, Propolis, GC/MSD.

ÖZ

Bu çalışmada, Manisa'dan elde edilen propolis örneklerinin etanol özütlerinin (EEP) biyofilm oluşumunun inhibisyonu ve oluşmuş biyofilm tabakasının giderimi üzerine etkileri ile kimyasal kompozisyonları araştırılmıştır. Propolis özütlerinin antibiyofilm etkisi biyofilm oluşturan bakterilere karşı (*Listeria monocytogenes* ATCC 7644, *Methicillin Sensitive Staphylococcus aureus* (clinical isolate) MSSA M20, *S. aureus* ATCC 33862, *S. aureus* ATCC 29213, *Enterococcus faecalis* ATCC 19433, *Pseudomonas fluorescens* ATCC 55241, *Micrococcus luteus* NRRL-B1013) 96 kuyucuklu polisitiren plakalarda kristal viole yöntemi kullanılarak test edilmiştir. Ayrıca, EEP'nin antibakteriyel aktivitesi Agar Kuyu Difüzyon yöntemine göre belirlenmiştir. Özütlerin kimyasal kompozisyonu Gaz Kromatografisi-Kütle Spektrometresi analizi (GC/MSD) ile tespit edilmiştir. EEP örnekleri bakterilere karşı iyi antibiyofilm aktivite sergilemişlerdir. MP-1 (Manisa-Köprübaşı), MP-2 (Manisa-Demirci) ve MP-3 (Manisa-Kula)'ün maksimum biyofilm inhibisyon aktivitesi oranları *L. monocytogenes* ATCC 7644 için sırasıyla %89.4; %80.0 ve %89.0 olarak, MSSA M20 için de sırasıyla %66.0; %67.0 ve %74.0 olarak bulunmuştur. GC/MSD analiz sonucuna göre triakotinil asetat propolis özütlerinde bulunan başlıca bileşiktir.

Anahtar Kelimeler
Antibiyofilm, Biyofilm indirgeme, Propolis, GC/MSD.

Article History: Received: Feb 24, 2016; Revised: Sep 6, 2016; Accepted: Oct 20, 2016; Available Dec: Jul 31, 2016.

DOI: 10.15671/HJBC.2016.131

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INTRODUCTION

Biofilms can form on most surfaces exposed to the natural environment. Inside the biofilm, bacteria are protected from environmental stresses, such as desiccation and disinfectants, attack by the immune system, protozoa ingestion, and antimicrobials [1]. So, biofilm formation is an important strategy for microbial life and for the causing of infection. Also, cells growing in biofilms are up to 1000-fold more resistant to antibiotics and biocides than planktonic cells [2-4]. In human body, biofilms can be found on many surfaces such as contact lenses, catheters, heart valves, prostheses, lung tissue, intrauterine device, and kidney stones. Especially, sepsis and chronic infections causes a major concern in nosocomial settings because of biofilm related [5]. So, biofilm plays an immensely important role in human health, as it protect bacteria from antibiotics and host defence during infection [6]. Although, various bioactive compounds have shown antibiofilm activity against pathogen bacteria [7-9] the need for the discovery of novel compounds is still very great. Since ancient times, natural products have been used as antimicrobial agents.

Among the natural products, propolis has attracted increased interest for the treatment or prevention of many infectious diseases. Because of nontoxic natural product [10,11], biological and pharmacological properties of propolis have been researched extensively in the scientific community. Also, it has been benefited in folk medicine to maintain health. Most of the biological activities of propolis have been attributed to flavonoids and phenolic compounds [12-14]. In this regard, antimicrobial activities have usually been attributed to flavonoids as well. Antibacterial [15], anti-influenza [16], anti-candida, anti-parasite [17] and antifungal [18] activities of propolis have been determined. In this study, we aimed to determine the biofilm inhibition and biofilm reduction activity of ethanol extracts of propolis samples obtained from Manisa-Turkey against a large of pathogenic bacteria and to analyse the chemical composition of propolis extracts by GC/MSD.

MATERIAL and METHODS

Bacteria

The following bacteria were used as test microorganisms: *Listeria monocytogenes* ATCC 7644, *Staphylococcus aureus* ATCC 29213, *S. aureus* ATCC 33862, *Micrococcus luteus* NRRL-B 1013, Methicilline Sensitive *Staphylococcus aureus* (MSSA) strain M20 (clinical isolate), *Enterococcus faecalis* ATCC 19433 and *Pseudomonas fluorescens* ATCC 55241. The bacterial strains were obtained from Bacteriology Laboratory of Pamukkale University Biology Department.

Extraction method

Propolis samples collected during summer 2013 were obtained from the states of Manisa-Koprubasi (MP-1), Manisa-Demirci (MP-2) and Manisa-Kula (MP-3) (Turkey). After propolis samples were cooled (20°C), extracted with 96% ethanol solution (1:10 w/v) at 37°C for 5 days in the dark, and then filtered with a Whatman No. 1 filter paper. The final filtrates were evaporated to dryness on a rotary evaporator (IKA RV 10D, Germany) under reduced pressure at 55°C and called to as ethanol extract of propolis (EEP). EEP samples were kept -20°C for antibiofilm activity experiments and analysis of GC/MSD.

Determination of biofilm formation (Congo red agar method)

The congo red method was done according to the protocol of Freeman et al. [19]. Each microorganisms was inoculated in media consist of brain heart infusion broth 37 g/L, sucrose 0.8 g/L, agar–agar 10 g/L and Congo red stain 0.8 g/L and the cultures were incubated at 37±0.1 °C for 24 h. Congo red stain was prepared as a concentrated aqueous solution, autoclaved separately and added to the media when the agar had cooled to 55°C. Biofilm positive strains produced black colored colonies while biofilm negative strains were pink colored.

Antibacterial activity

The agar-well diffusion method was employed for the determination of antimicrobial activities of extracts [20]. Each microorganisms was

suspended in growth media Triptic Soy Broth (TSB) consisting of peptone from casein (17.0 g/L), peptone from soy meal (3.0 g/L), D(+) glucose (2.5 g/L), sodium chloride (5.0 g/L) and di-Potassium hydrogen phosphate (2.5 g/L), and the cultures were incubated at 37±0.1ºC (30°C for *M. luteus* NRRL B-1013) for 24 h. The culture suspensions were prepared and adjusted by comparing against 0.5 McFarland turbidity standard tubes (1.5x108 cfu/mL). The activated cultures were inoculated (100 μL) into each sterilized petri dishes (10x100 mm diameter) and after inoculation of bacteria, freshly prepared liquid Tryptic Soy Agar (TSA) medium was poured into each petri dishes (25 mL/petri dish) and the plates were distributed homogeneously. Then the agars were allowed to solidify at 4°C for 1 h. Four equidistant wells (6 mm in diameter) were cut from the agar. The extracts were prepared in dimethyl sulfoxide (DMSO) to a final concentration of 50 mg/mL [21]. Each compound (50 μL) was filled into the wells of agar plates directly. Plates injected with the bacteria were incubated at 37°C (30°C for *M. luteus* NRRL B-1013) for 24 h. At the end of the incubation period, inhibition zones formed on the medium were evaluated in mm.

Biofilm inhibition assay

The biofilm inhibition effect of the propolis extracts against biofilm forming bacteria was tested on 96-well polystyrene plates using crystal violet assay [15]. The bacterial cultures were grown in 5 mL TSB at 37°C under aerobic conditions for 24 h. The bacterial suspension at 0.5 McFarland turbidity standard was dispensed into each well of 96-well plates in the presence of TSB supplemented with 2% glucose (w/v) containing the propolis extracts which were dissolved in DMSO at concentrations of 0.1-2 mg/mL. The plates were then incubated for 48 h at 37°C.

Following incubation, the plates were washed with distilled water to remove loosely attached cells. The plates were air-dried and then the wells were stained with 1% (w/v) crystal violet and incubated at room temperature for 15 min after which the plates were washed with sterile distilled water to remove unabsorbed stain. To destain the wells, the semi-quantitative assessment of biofilms formation was performed by adding ethanol for gram-negative bacteria and glacial acetic acid for gram-positive bacteria. The absorbance at 540 nm was determined using a microplate reader (Optic ivymen system 2100-C). Negative controls (cells+TSB), positive control (cells+TSB+propolis) and media controls (TSB) were included. Each experiment was performed in duplicate. The percentage inhibition was obtained for each concentration of the extracts as calculated by the following formula:

[(OD *growth control* - OD *sample*) / OD *growth control*] x100

where OD stands for optical density.

Biofilm reduction assay

Biofilms were allowed to perform for 48 h before the addition of the propolis extracts at a final concentration of 0.1-2.0 mg/mL per well. Biofilms formation was achieved by inoculation of a standardized (0.5 McFarland turbidity) bacterial suspension culture into a 96-well microtiter plate. The plates were incubated aerobically at 37°C for 48 h to allow cell attachment. Following the 48 h incubation period, propolis extracts in DMSO was added to each well of 96-well plates at concentrations of 0.1-2.0 mg/mL. The plates were further incubated for 24 h before the crystal violet assay was performed.

Determination of compounds by GC/MSD

The crude propolis samples were extracted in a similar treatment as mentioned above. The residue dissolved in ethanol (99.6%) as its concentration was 100 μg/L. Then, the samples were centrifuged at 4000 rpm for 10 min and supernatant was filtered from Macherey-Nagel Chromafil Xtra PTFE 20/25 0.20 μm, and injected 2.0 μL to Agilent 7890A GC- 5975C MSD. The control parameters of GC/MSD were given in Table 1.

RESULT AND DISCUSSION

Antibacterial activity of propolis samples

Three different ethanol extracts of propolis were tested against indicator pathogen bacteria. All extracts showed moderate-spectrum antibacterial activity against pathogen bacteria with inhibition

Table 1. Control parameters of GC/MSD.

Oven Parameters	
Equilibration Time	2 min
Max Temperature	250°C
Oven Program	70°C for 1 min then 10°C/min 175°C for 10min then 5°C/min to 210°C for 5 min then 5°C/min to 230°C for 7.5 min
Run Time	45 min
MMI Inlet Parameters	
Mode	Split
Heater	250°C
Pressure	35.299psi
Total Flow	94.8 mL/min
Septum Purge Flow	3 mL/min
Gas Saver	20 mL/min after 2 min
Split Ratio	50:1
Split Flow	90 mL/min
Thermal Aux (Tranfer Line)	
Heater	On
Temperature	250°C
Column	
Name	J&W 112-88A7 HP-88 (250°C, 60m x 250µm x 0.25µm)
Pressure	29.901 psi
Flow	1.8 mL/min
MS Acquisition Parameters	
Acquistion Mode	Scan
Solvent Delay	2.00 min
EM Voltage	1200
Low Mass	35.0
High Mass	450.0
Threshold	150
MS Source	230°C max 250°C
MS Quadrupole	150°C max 200°C

zones ranging from 3.8 to 13.4 mm (Table 2). As seen in the Table, the EEP extracts were capable of inhibiting the growth of biofilm-forming bacteria. The maximum activity was observed against *P. fluorescens* ATCC 55241. The zones of inhibition of MP-1, MP-2 and MP-3 against this pathogen bacterium were 12.8, 8.9 and 13.4 mm, respectively. While the P2 has no effect against *L. monocytogenes* ATCC 7644, P1 and P3 inhibited the growth of this strain with 7.0 and 8.2 mm inhibition zones respectively. Among the propolis samples used in this study, the most effective was found as MP-3. The zones of inhibition of MP-3 against *L. monocytogenes* ATCC 7644, *S. aureus*

Table 2. Antimicrobial activities of propolis extracts by using agar well diffusion method*.

Microorganisms	MP-1	MP-2	MP-3
L. monocytogenes ATCC 7644	7.0±1.0	-	8.2±1.2
MSSA M20	NT	NT	NT
S. aureus ATCC 33862	7.1±0.5	6.3±0.7	6.2±1.2
S. aureus ATCC 29213	3.8±1.6	-	3.8±0.0
E. faecalis ATCC 19433	-	-	-
P. fluorescens ATCC 55241	12.8±0.6	8.9±1.3	13.4±1.8
M. luteus NRRL-B 1013	5.5±0.7	7.3±0.9	8.7±2.5

Table 3. Antibiofilm and biofilm reduction effects of MP-1.

Propolis concentrations (mg/mL)	Antibiofilm effect (%)						Biofilm reduction effect (%)					
	0.1	0.2	0.4	0.8	1.6	2.0	0.1	0.2	0.4	0.8	1.6	2.0
Bacteria												
L. monocytogenes ATCC 7644	63.9	59.0	60.7	75.3	88.6	89.4	25.4	62.5	63.2	57.1	62.9	58.0
MSSA M20	24.0	16.0	24.0	66.0	26.0	46.0	-	48.6	41.0	43.5	-	-
S. aureus ATCC 33862	-	9.0	20.0	-	-	40.0	71.9	70.4	71.9	74.9	67.3	52.9
S. aureus ATCC 29213	-	1.0	-	-	20.0	42.0	29.2	40.8	26.4	32.1	19.1	-
E. faecalis ATCC 19433	24.0	-	29.0	1.0	-	-	69.3	59.3	71.1	71.6	56.3	67.5
P. fluorescens ATCC 55241	43.0	23.0	27.0	21.0	41.0	39.0	27.2	17.3	11.0	-	45.3	
M. luteus NRRL-B 1013	31.0	21.0	6.0	-	-	-	-	45.0	30.5	27.8	29.1	1.0

ATCC 33862, *S. aureus* ATCC 29213, *P. fluorescens* ATCC 55241, and *M. luteus* NRRL-B 1013 were 8.2, 6.2, 3.8, 13.4, 8.7 mm.

Effect of propolis extracts on biofilm formation

The antibiofilm activity of propolis samples against pathogen bacteria using a standard quantitative biofilm assay method appeared to be dose-related (Tables 3, 4 and 5). In general, propolis samples were found to be more effective at higher concentrations and a significant decrease in biofilm formation was seen in test bacterial strains when they grow in the presence of EEP extracts. The MP-1 sample inhibited the biofilm formation of *L. monocytogenes* ATCC 7644 with 89.4% rate at 2.0 mg/mL concentration (Table 3). The biofilm biomass of MSSA M20 was inhibited at 66.0% by MP-1 at 0.8 mg/mL concentration (Table 3). Also, MP-2 showed 80.0% and 67.0% antibiofilm effect on *L. monocytogenes* ATCC 7644 and MSSA M20, respectively (Table 4). On the other hand, MP-3 was exhibited higher biofilm inhibition activity against the tested bacteria. The maximum reduction in biofilm biomass of *L. monocytogenes*, MSSA M20, *S. aureus* ATCC 33862, *S. aureus* ATCC 29213 and *E. faecalis* ATCC 19433 were respectively 88.0%, 74.0%, 66.0%, 65.0% and 56.0% by MP-3 at 1.6 mg/mL

Table 4. Antibiofilm and biofilm reduction effects of MP-2.

Propolis concentrations (mg/mL)	Antibiofilm effect (%)						Biofilm reduction effect (%)					
	0.1	0.2	0.4	0.8	1.6	2.0	0.1	0.2	0.4	0.8	1.6	2.0
Bacteria												
L. monocytogenes ATCC 7644	34.0	77.0	80.0	50.0	28.0	38.0	22.9	30.1	47.6	59.8	30.6	49.9
MSSA M20	15.0	1.0	58.0	67.0	44.0	21.0	46.0	43.0	20.0	51.0	20.0	43.0
S. aureus ATCC 33862	-	-	-	-	-	-	61.9	-	63.4	60.5	57.1	22.7
S. aureus ATCC 29213	23.0	16.0	36.0	29.0	-	16.0	47.8	39.1	49.5	40.8	46.0	44.3
E. faecalis ATCC 19433	41.0	23.0	37.0	16.0	-	-	37.2	69.0	64.1	59.2	32.4	52.6
P. fluorescens ATCC 55241	-	-	-	-	-	-	48.5	24.2	24.5	5.5	10.2	27.9
M. luteus NRRL-B 1013	35.0	44.0	12.0	-	-	-	51.0	17.9	36.4	0	28.5	32.5

Table 5. Antibiofilm and biofilm reduction effects of MP-3.

Propolis concentrations (mg/mL)	Antibiofilm effect (%)						Biofilm reduction effect (%)					
	0.1	0.2	0.4	0.8	1.6	2.0	0.1	0.2	0.4	0.8	1.6	2.0
Bacteria												
L. monocytogenes ATCC 7644	63	84	89	87	88	82	17	10	2	0	0	0
MSSA M20	8	23	40	74	74	72	21	23	23	0	0	27,5
S. aureus ATCC 33862	28	39	44	58	66	62	-	-	-	-	-	-
S. aureus ATCC 29213	56	58	-	59	65	53	-	-	-	-	-	-
E. faecalis ATCC 19433	36	26	48	30	56	32	-	-	-	-	-	-
P. fluorescens ATCC 55241	24	-	14	-	11	13		-	-	-	-	-
M. luteus NRRL-B 1013	49	48	57	43	14	7	-	-	-	-	-	-

concentration (Table 5). While the MP-3 inhibited the biofilm formation of *L. monocytogenes* ATCC 7644 with 89.0%, *M. luteus* NRRL-B 1013 biofilm was inhibited 57.0% at 0.4 mg/mL concentration. The previous reports on the influence of EEP on biofilms from different location all around the world and showing its important antibiofilm activity confirm our findings. Kouidhi et al. [15] showed ethanolic extracts of propolis exhibited antibiofilm activity against oral streptococci.

Table 6. Chemical compounds identified by GC/MSD analyze of propolis samples.

Chemical compounds	MP-1		MP-2	
	% content	RT (min)	% content	RT (min)
phenyl ethyl alcohol	1.46	6.94	1.05	6.94
Benzyl methyl ketone	2.11	7.15	0.81	7.15
2-propen-1-ol	0.14	12.60	0.36	12.60
Triacetin	0.23	14.12	0.13	14.12
Hexadecanoid acid methyl ester	0.74	31.50	0.74	31.50
Hexadecanal	1.34	33.71	1.03	33.71
11-octadacenoic acid methyl ester	3.81	35.62	3.03	35.62
Mycristal dehyde	1.29	36.14	1.76	36.14
cis-Bicyclo[10.8.0] eiosane	1.08	38.44	1.35	38.44
Tricosane	2.12	40.61	3.26	40.61
2-[(dodecyloxy)methyl] oxirane	2.21	42.42	3.87	42.42
Flavone,5-hydroxy-7-methoxy	2.86	44.41	4.17	44.41
Eicosane	4.69	44.71	5.95	44.71
14-Beta-H-Pregna	1.50	46.48	2.13	46.48
9-Butyl docasane	2.20	48.52	1.95	48.52
1-Heptacosanol	8.98	54.91	7.74	54.91
1-Heneicosene	1.47	55.04	1.43	55.04
Triacontyl acetate	26.92	58.09	15.63	58.09
Lupeol	10.21	58.72	6.03	58.72
A-Neogammacer22(29) en-3-ol	10.63	60.08	5.84	60.08

Similarly, the PEE inhibited the biofilm formation of *S. aureus* and *P. aeruginosa* [22].

Effect of propolis extracts on established biofilms

The effect of EEP samples was also detected on 48 h established biofilms in our study. When 48 h established biofilms were treated with different concentrations of propolis (0.1-2.0 mg/L), the biofilm established was significantly damaged at 48 h of contact with propolis. Maximum biofilm reduction was observed with 74.9% rate on *S. aureus* ATCC 29213 by MP-1 at 0.8 mg/mL concentration. However, MP-3 showed almost no biofilm reduction effect on tested bacteria. A higher concentration of propolis was required to disrupt established biofilm than to prevent biofilm formation.

GC/MSD analysis

To determine the chemical composition of propolis extracts, only MP-1 and MP-2 samples could analyse by GC/MSD. According to GC/MSD results, a total of twenty different chemical constituents were detected and quantified in MP-1 and MP-2 (Table 6). Triacontyl acetate (26.92% and 15.63%), lupeol (10.21% and 6.03%) and A-neogammacer22(29) en-3-ol (10.63% and 5.84%) were determined as the major constituents identified in MP-1 and MP-

2. On the other hand, 2-propen-1-ol, triacetin and hexadecanoid acid methyl ester were determined as the lowest amount of compounds found in the extracts.

CONCLUSION

Ethanol extracts of three samples of propolis collected from different geographical regions in Manisa (Turkey) exhibited good antibiofilm activity against the tested bacteria. All propolis samples exhibited antibiofilm activity on the bacteria with different rates. But interestingly, MP-2 did not show any antibiofilm activity on *S. aureus* ATCC 33862 and *P. fluorescens* ATCC 55241 (Table 4). On the other hand, while the MP-1 and MP-2 had notable biofilm reduction ability (maximum 74.9%), MP-3 had negligible biofilm reduction activity on the bacteria (Table 3, 4 and 5). Consequently, the propolis samples from Manisa-Turkey were very effective on tested bacterial biofilms (up to 50% biofilm inhibition percentage). Also, different chemical compounds were determined in EEP. We provide evidence that propolis, a natural product, contains constituents that inhibit biofilm formation and disrupt established biofilm.

ACKNOWLEDGMENTS

We thanks to TUBITAK-BIDEP 2209 and PAU-BAP (2013BSP025).

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