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Antibiofilm Effect of Two Propolis Samples from Turkey

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Abstract

Biofilms are structured communities of bacteria, which are adhered to a surface and embedded in a self- produced matrix of extracellular polymeric substances. Since biofilms are resistant to antimicrobial agents, they are at the basis of health problems. Propolis has attracted increased interest due to its antimicrobial activity against pathogenic microorganisms.

We investigated the antibiofilm potencies of Turkish ethanol extract propolis (EEP) against some bacteria (*Listeria monocytogenes* ATCC7644, *Staphylococcus aureus* ATCC29213, *S. aureus* ATCC 33862, MRSA-20 (clinical isolate), *Pseudomonas fluorescens* ATCC55241, *Micrococcus luteus* NRRL-B1013, *Enterococcus faecalis* ATCC19433) known to form biofilms. Firstly, the antibacterial activity of EEP from Manisa-Salihli (P4) and Izmir-Foça (P10) collected in 2013 was evaluated according to Agar Well Diffusion method. Secondly, we tested with a microplate biofilms assay both the effect of antibiofilm and inhibition of biofilms of EEP. The EEP samples exhibited good antibiofilm activity against bacteria. The maximum antibiofilm activity percentage of P4 ranged from 85% for *L. monocytogenes* to 68% for *S. aureus* ATCC 29213. Also, the activity percentage of P10 ranged from 79% for *L. monocytogenes* to 48% for MRSA20. In addition, we showed that EEP samples were very effective on tested bacteria biofilms (up to 50% biofilms inhibition percentage).

Keywords: antibiofilm, biofilm reduction, propolis, Turkish

INTRODUCTION

Biofilm is a community of microorganisms that live together as buried into a hydrated matrix made up of polysaccharides, proteins and other biomolecules like DNA which was produced by themselves by sticking to a surface biotic or abiotic [1,2]. Inside the biofilm, bacteria are protected from environmental stresses, such as desiccation and disinfectants, attack by the immune system, protozoa ingestion, and antimicrobials [3]. So, biofilm formation is a successful strategy for microbial survival 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 [4-6]. Chronic infections and sepsis related to biofilms represent a major concern in nosocomial settings [7]. So, biofilms play an immensely important role in human health, as they shelter bacteria from antibiotics and host defence during infection [8]. On the other hand, developing resistance to antimicrobials and decrease in the number of newly developed antimicrobials pose significant challenges in the fight against biofilm microorganisms. Currently, there are no effective treatments that target microbial biofilms because of intrinsically resistant to conventional antibiotics [9]. This indicates the need for new antibacterial drugs active not only against planktonic bacteria but also drug resistant biofilms. Although, various bioactive compounds have shown antibiofilm activity against pathogen bacteria [10-12], 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. Propolis product of honeybees has variable and complex chemical composition due to the biodiversity of the vegetation of each region visited by bees [13-18]. Because of nontoxic natural product [19,20], biological and pharmacological properties have been researched extensively in the scientific community. Also, it has been benefited in folk medicine to maintain health. Biological and pharmacological activities of Propolis such as antibacterial [18], anti-influenza [21], anti-candida, anti-parasite [22] and antifungal [23] are known very well. Also, antitumor, anti-inflammatory, cytotoxic, antioxidant, hepatoprotective, immunomodulatory, anticancer and hematostimulative properties [24-29] of propolis have been determined.

Despite, the anti-biofilm effects of propolis are also studied in recent years [18,30,31], there is no information about the impact of the anti-biofilm Turkish propolis. With this background, the current study aimed to investigate the antibiofilm activity of propolis samples obtained from different region of Turkey against a large of pathogenic bacteria.

MATERIALS AND METHODS

Bacteria

The following five gram positive and two gram negative strains of bacteria were used as test micro-organisms respectively: *Listeria monocytogenes* ATCC 7644, *Staphylococcus aureus* ATCC 29213, *S. aureus* ATCC 33862, *Micrococcus luteus* NRRL-B 1013, Methicilline Resistance *Staphylococcus aureus* (MRSA) strain 20 (clinical isolate), *Pseudomonas fluorescens* ATCC 55241 and *Enterococcus faecalis* ATCC 19433. 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-Salihli (P4) and İzmir-Foça (P10) (Turkey). After propolis samples were cooled (20 oC), extracted with 96% ethanol solution (1:10 w/v) at 37 oC 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 ºC and called to as ethanol extract of propolis (EEP). EEP samples were kept -20 ^{o}C for antibiofilm activity experiments and analysis of GC-MS.

Determination of biofilm formation (Congo red agar method)

The congo red method was done according to the protocol of Freeman et al. [32]. 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 ^{o}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 [33]. Each microorganisms was suspended in growth media Triptic Soy Broth (TSB) consisting of pepton from casein (17.0 g/l), pepton 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 ± 0.1 ºC (30 ºC 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 Triptic 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 ºC for 1 h. Four equidistant wells (6 mm in diameter) were cut from the agar. The extracts were prepared in dimethylsulfoxide (DMSO) to a final concentration of 50 mg/ml [34]. Each compound (50 µl) was filled into the wells of agar plates directly. Plates injected with the bacteria were incubated at 37 ºC (30 ºC 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.

Antibiofilm activity assay

The antibiofilm effect of the propolis extracts against biofilm forming bacteria was tested on 96-well polystyrene plates using crystal violet assay [18]. The bacterial cultures were grown in 5 ml TSB at 37 ºC 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 ºC.

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. The semi-quantitative assessment of biofilms formation was performed by adding ethanol for gram negative bacteria and glacial acetic acid for gram positive bacteria to destain the wells. The absorbance at 540 nm was determined using a microplate reader (Optic ivymen system 2100-C). And 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

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 mg/ml per well. Biofilms formation was achieved by inoculation of a standardized (0.5 McFarland turbidity) bacterial suspension culture into a 96-well microtitre plate. The plates were incubated aerobically at 37 ºC 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 mg/ml. The plates were further incubated for 24 h before the crystal violet assay was performed.

RESULTS

Antibacterial activity of propolis samples

The extracts of two EEP were tested against indicator pathogen bacteria. Both EEP extracts showed moderate-spectrum antibacterial activity against pathogen bacteria with inhibition zone of 4.5-14.4 mm (Table 1). As seen in the table, the EEP extracts were capable of inhibiting the growth of biofilm-forming bacteria. While the propolis P4 has no effect against *Micrococcus luteus*, this strain was inhibited by propolis P10 (9.9 mm). The zones of inhibition of propolis P4 against S. aureus ATCC 29213, *P. fluorescens* ATCC 55241, *L. monocytogenes*, *S. aureus* ATCC 33862 and *E. faecalis* were 7.9 mm, 14.1 mm, 7.8 mm, 5.5 mm and 6.0 mm. Similarly, the propolis P10 also showed inhibitory activity against S. aureus ATCC 29213, *P. fluorescens* ATCC 55241, *L. monocytogenes*, *S. aureus* ATCC 33862 and *E. faecalis* were 6.0 mm, 7.6 mm, 6.1 mm, 4.9 mm and 4.5 mm.

Table 1. Antimicrobial activities of propolis extracts by using agar well diffusion method*.

Microorganisms	Propolis-4	Propolis-10
***L. monocytogenes* ATCC 7644**	7.8±1.2	6.1±0.5
MRSA 20	NT	NT
***S. aureus* ATCC 33862**	5.5±0.1	4.9±1.3
***S. aureus* ATCC 29213**	7.9±0.3	6±0.2
***E. faecalis* ATCC 19433**	6±0.2	4.5±0.1
***P. fluorescens* ATCC 55241**	14.4±0.8	7.6±1.0
***M. luteus* NRRL-B 1013**	-	9.9±0.9

*Diameter in mm of the zone inhibition, (-): No inhibition. NT: Not tested.

Antibiofilm activity of propolis extracts

The antibiofilm activity of propolis samples against pathogen bacteria using a standard quantitative biofilm assay method appeared to be dose-related (Tables 2 and 3). In general, Propolis samples were found to be more effective at higher concentrations. While the propolis-P4 at concentrations between 0.1 and 2.0 mg/l exhibited 3% and 85% inhibition on biofilm formation, respectively, inhibition rate of the propolis P10 ranged from 6-79% in same concentrations.

In generally, a significant decrease in biofilm formation was seen in test bacterial strains when grown in the presence of EEP extracts. A maximum of 85%, 46%, 68%, 56%, 65% and 52% reduction in biofilm biomass of *L. monocytogenes*, *S. aureus* ATCC 33862, *S. aureus* ATCC 29213, *E. faecalis* ATCC 19433, *P. fluorescence* ATCC 55241 and *M. luteus* NRRL-B 1013, respectively, was observed in propolis P4 at 1.6 mg/ml concentration. Also, Propolis P4 at 2 mg/ml efficiently dislodged the biofilm formation by 56% in MRSA-20. For the Propolis P10, a maximum of 48% inhibition in biofilm formation of MRSA-20 was shown when treated with propolis at 2 mg/ml.

Effect of propolis 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. A higher concentration of propolis was required to disrupt established biofilm than to prevent biofilm formation. Propolis-P4 was more effective than P10 (Tables 2 and 3).

GC-MS analysis

The components of propolis P4 were identified by GC-MS and the results were shown in Table 4. A total of 20 different chemical constituents were determined. The major constituents identified in propolis were Triacontyl acetate (22%), Lupeol (14%), 1-Heptacosanol (8%), 9-Butyl docasane (4%) and Flavone,5-hydroxy-7-methoxy (3%).

Table 2. Antibiofilm and biofilm inhibition effects of propolis-4 sample

Bacteria	Antibiofilm effect (%)						Biofilm inhibition effect (%)					
	Propolis concentrations (mg/ml)						Propolis concentrations (mg/ml)					
	0.1	0.2	0.4	0.8	1.6	2.0	0.1	0.2	0.4	0.8	1.6	2.0
L. monocytogenes ATCC 7644	42.0	75.0	81.0	76.0	85.0	76.0	31.2	24.5	23.1	42.6	33.4	0
MRSA 20	3.0	19.0	2.0	46.0	44.0	56.0	35.2	17.0	25.1	0	15.0	25.0
S. aureus ATCC 33862	-	-	-	9.0	46.0	7.0	33.3	0	7.3	9.4	41.6	6.2
S. aureus ATCC 29213	7.0	49.0	68.0	38.0	68.0	54.0	3.01	18.7	6.5	20.7	0	39.0
E. faecalis ATCC 19433	12.0	25.0	38.0	24.0	56.0	30.0	61.5	53.6	49.3	67.4	47.8	69.5
P. fluorescens ATCC 55241	10.0	-	16.0	30.0	65.0	31.0	61.0	0	0	0	0	0
M. luteus NRRL-B 1013	41.0	34.0	26.0	33.0	52.0	13.0	47.7	55.3	46.9	67.1	55.3	54.4

Table 3. Antibiofilm and biofilm inhibition effects of propolis-10 sample

Bacteria	Antibiofilm effect (%)						Biofilm inhibition effect (%)					
	Propolis concentrations (mg/ml)						Propolis concentrations (mg/ml)					
	0.1	0.2	0.4	0.8	1.6	2.0	0.1	0.2	0.4	0.8	1.6	2.0
L. monocytogenes ATCC 7644	32.0	13.0	32.0	55.0	79.0	77.0	37.1	4.0	0	45.4	9.8	9.0
MRSA 20	18.0	17.0	22.0	40.0	40.0	48.0	16.8	29.7	24.0	0	0	0
S. aureus ATCC 33862	-	29.0	19.0	-	3.0	-	27.5	17.9	29.3	18.8	11.0	32.8
S. aureus ATCC 29213	-	-	22.0	-	-	-	30.7	44.6	36.9	30.7	6.1	33.8
E. faecalis ATCC 19433	13.0	14.0	35.0	27.0	29.0	5.0	30.6	57.4	54.0	53.3	29.3	51.9
P. fluorescens ATCC 55241	29.0	6.0	26.0	34.0	45.0	44.0	89.2	74.5	64.2	66.6	47.6	41.9
M. luteus NRRL-B 1013	19.0	29.0	21.0	37.0	27.0	-	63.6	60.2	47.0	60.9	49.5	7.8

Table 4. Chemical compounds identified by GC-MS analyze of propolis-4 sample

Chemical compounds	% content	RT (min)
phenyl ethyl alcohol	2.43	6.94
Benzyl methyl ketone	1.33	7.15
2-propen-1-ol	0.51	12.60
Triacetin	0.30	14.12
Hexadecanoid acid methyl ester	0.33	31.50
Hexadecanal	1.21	33.71
11-octadacenoic acid methyl ester	2.46	35.62
Mycristal dehyde	0.81	36.14
cis-Bicyclo[10.8.0] eiosane	0.68	38.44
Tricosane	2.94	40.15
2-[(dodecyloxy)methyl]oxirane	1.63	42.42
Flavone,5-hydroxy-7-methoxy	3.47	44.41
Eicosane	1.99	44.71
14-Beta-H-Pregna	1.33	46.48
9-Butyl docasane	4.54	48.52
1-Heptacosanol	8.96	54.91
1-Heneicosene	1.69	55.04
Triacontyl acetate	22.15	58.09
Lupeol	14.91	58.72
A-Neogammacer-22(29)-en-3-ol	8.78	60.08

DISCUSSION

Although propolis is well known for its antimicrobial activity or other beneficial effects on humans, there are few reports that studied on propolis ability to inhibit biofilm formation [18,35,36]. Several reports demonstrating the effectiveness of honey in the treatment of various bacterial biofilms have been published [37,38]. Otherwise, many studies have been reported on antimicrobial activity of Turkish honey and propolis [17,39,40]. However, no information detailed is available on the effect of propolis on biofilm forming and biofilm inhibition. Therefore, we selected propolis for this study.

In the present study, it is proved that two propolis have anti-biofilm bacterial metabolites. On other words, the propolis extracted in ethanol was found to demonstrated noticeable antibacterial activity indicated against biofilm forming bacteria. Kouidhi et al. [18] showed anti-biofilm activity against oral streptococci after extraction ethanol.

This study has indicated that the two Turkish propolis possessed antibiofilm and biofilm inhibition action towards *Listeria monocytogenes* ATCC 7644, *Staphylococcus aureus* ATCC 29213, *S. aureus* ATCC 33862, Methicillin Resistance *Staphylococcus aureus* MRSA-20, *Pseudomonas fluorescens* ATCC 55241, *Micrococcus luteus* NRRL-B1013 and *Enterococcus faecalis* ATCC 19433. The antibiofilm activity of propolis was investigated by Stan et al. [41], they reported that inhibitory influence of propolis on *S. aureus* 13024 biofilm formation. Similar observation was made by Helaly et al. [31]. They reported that PEE inhibited *S. aureus* and *P. aeruginosa* biofilms formation too.

CONCLUSION

The obtained results indicated that propolis inhibited the biofilm forming behaviour of tested pathogens. As a known, adherence is a major step in biofilm formation. Antibiofilm effect of propolis on human pathogens and MRSA was seen in our study; therefore propolis might be used to prevent human pathogen and MRSA associated infection. But further work needs to be done to optimize the doses needed for application in bacteria causing human diseases.

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