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# Phenolic Content and Antibiofilm Activity of Propolis Against Clinical MSSA Strains

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**Abstract:** Antibiofilm properties and the phenolic composition of propolis, collected from Bartın province of Turkey in the years of 2013 and 2012, were determined. Hexane, ethyl acetate and ethanol extracts of propolis were prepared and assessed for their antibiofilm activity (inhibition of biofilm formation and reduction of established biofilm) against the clinical methicillin sensitive *Staphylococcus aureus* (MSSA) strains and *Staphylococcus aureus* ATCC 33862. Ethyl acetate and ethanol extracts of propolis presented a greater effectiveness on biofilm inhibition of the tested bacteria compared to hexane extracts. The activity patterns showed slight variations in the two years. While 0.5 mg/mL ethyl acetate, ethanol and hexane extract solutions of the product in 2013 inhibited 92.89, 82.98 and 47.42% of biofilm formation of MSSA M20 strain, the inhibition percentage of the products of 2012 were determined to be 87.14%, 75.94% and 44.89% against the same bacterium (MSSA M20), respectively. The results of the validated liquid chromatography-electrospray tandem mass spectrometry (UPLC-ESI-MS/MS) analyses showed a strong relation between the activity and the phenolic composition of the extracts. Phenolic contents of the ethyl acetate and ethanol extracts were relatively higher than hexane extracts. Caffeic acid composition of ethyl acetate, ethanol and hexane extracts of the product in 2013 was detected as 23521.0, 16881.0 and 3522.8 µg/g, respectively. On the other hand, the caffeic acid contents of the product of 2012 was found to be lower than those of 2013 (19100.0, 10416.0 and 2322.5 µg/g for ethyl acetate, ethanol and hexane extracts, respectively). Consequently, the findings have shown that propolis extracts possessed good antibiofilm activity against clinical staphylococci, and its phenolic composition has been affected by the year of collection.

**Keywords:** Antibiofilm; phenolic compounds; propolis; MSSA; principal component analysis; UPLC-ESI-MS/MS . 

## 1. Introduction

Formation of bacterial biofilms on biomaterials or host tissues leads to the development of chronic infections, as biofilms are exceptionally resistant to host immune response and chemotherapies [1]. Staphylococci are common cause of hospital-acquired infections, and biofilm formation is one of the important microbial virulence factors found in staphylococci [2-4]. As it is well known, multilayered cell clusters, embedded in a matrix of extracellular polysaccharide, which facilitates the adherence of microorganisms, creates the biofilm. Exopolysaccharide matrix limits antibiotic penetration through the biofilm and acts like a diffusion barrier [5]. The biofilm matrix consists of various chemical compositions such as proteins, extracellular DNA (eDNA),

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exopolysaccharides, teichoic acids and uronic acids, depending on bacterial species, strain type and environmental conditions [6].

In a biofilm matrix, bacteria are protected from both host immune system and large doses of traditional antimicrobial agents. Thus, biofilm related infections are difficult to eradicate [7]. Furthermore, the increase in the microbial resistance to antibiotics and the decrease in the number of newly developed antimicrobials threaten public health and cause significant problems in treatment of biofilm microorganisms. This indicates the need for novel antibacterial drugs against both planktonic bacteria and drug resistant biofilms.

Searching for alternative therapies has now become a necessity. Natural plant/animal products and/or their combinations with antibiotics or synthetic counterparts seem to be among the promising solutions [8]. Within this context, propolis has found frequent use in treatment or prevention of many infectious diseases.

Propolis from different parts of the world has different chemical compositions due to the varying conditions of the geographical regions visited by bees [9]. The primary biologically active constituents of propolis are flavonoids, (hydroxyl) cinnamic acid derivatives, alcohols, aldehydes, phenolic acids, amino acids, lignans, triterpenes, steroids and sugars. However, phenolic compounds are the most abundant ones [10]. These compounds, especially the flavonoids and phenols, render propolis biologically valuable [11-13].

In the last decades, several biological and pharmacological activities of propolis, such as antitumor, antioxidant, anti-inflammatory, antiviral, anti-parasite, antifungal, antibacterial and immunomodulatory effects, have been investigated [14]. However, there is insufficient information in the literature regarding the antibiofilm performance against Staphylococcal biofilms as well as chemical composition of the propolis obtained from North Anatolia. In the present article, inhibition effect of propolis extracts against biofilm formation and reduction of established biofilm of three different methicillin sensitive *Staphylococcus aureus* (MSSA M10, MSSA M18 and MSSA M20) and *S. aureus* ATCC 33862 have been investigated. The phenol contents of the propolis extracts were analyzed by using UPLC-ESI-MS/MS and principal component analyses. Thus, this study provides valuable and detailed information on antibiofilm activity of propolis against clinical *S. aureus* strains.

## 2. Materials and Methods

### *2.1. Microorganisms and Medium*

Three clinical isolates and a reference culture of *Staphylococcus aureus* ATCC 33862 were used as test microorganisms. The clinical isolates were identified as Methicillin Sensitive *Stahpylococcus aureus* (MSSA M10, MSSA M18 and MSSA M20), using a specific biochemical test kit (Slidex MRSA Detection). M10 and M20 were isolated from a blood and M18 from a urine samples. The bacterial strains were obtained from Bacteriology Laboratory of Pamukkale University, Biology Department. Bacterial cultures were inoculated in a growth media of Tryptic Soy Broth (TSB), which consisted of peptone from casein (17.0 g/L), peptone from soymeal (3.0 g/L), glucose (2.5 g/L), NaCl (5.0 g/L) and dipotassium hydrogen phosphate (2.5 g/L). The culture was aerobically incubated and the growth was monitored by measuring the optical density (OD) at 600 nm. The culture suspension was prepared and adjusted by comparing with the use of 0.5 McFarland turbidity standard tubes (1.5x108 cfu/mL) for all the tests.

### *2.2. Extraction Method*

Crude samples of *Apis mellifera* propolis were collected from Bartın Province, which is located in Northern Turkey, in 2013 (BP-1) and 2012 (BP-2). After propolis samples were cooled (20 °C), they were separately extracted with 96% ethanol (Merck), ethyl acetate (Merck) and hexane solutions (Merck) (1:10 w/v) at 37 °C for 5 days in a dark environment, 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, and the samples were kept at -20 °C for antibiofilm activity experiments and analysis. The yields of ethanol, ethyl acetate and hexane extracts were found to be 48.62%, 38.49% and 5.87% for BP-1 sample, and 44.69%, 41.93% and 11.29% for BP-2 sample, respectively.

### *2.3. Biofilm Inhibition*

The antibiofilm 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.

In order to determine the inhibition effectiveness against biofilm formation, a bacterial suspension at 0.5 McFarland turbidity standard was dispensed into 96-well plates in the presence of TSB, supplemented with 5% glucose (w/v), containing the propolis extracts, which were dissolved in DMSO at concentrations of 0.1-0.5 mg/mL. The plates were then incubated for 48 h at 37 °C, which was followed by washing the plates with distilled water to remove loosely attached cells. The plates were air-dried and then the wells were stained with 0.1% (w/v) crystal violet and incubated at room temperature for 15 min. Then, the plates were washed with sterile distilled water to remove any unabsorbed stain. To destain the wells, glacial acetic acid (33%) was added into each well and the absorbance at 540 nm was read on a microplate reader (Optic ivymen system 2100-C). Each experiment was performed in duplicate, and the percentage of biofilm inhibition was calculated using the following formula:

$$[(OD_{\,growth\ control} - OD_{\,sample}) / OD_{\,growth\ control}] \times 100 \qquad (1),$$

Where *OD* stands for optical density.

### *2.4. Biofilm Reduction*

The biofilms were allowed to form in 48 h prior to the addition of the propolis extracts at a final concentration of 0.5-20 mg/mL per well. Briefly, an overnight culture (0.5 McFarland turbidity), grown in TSB at 37 °C, was diluted at 1:100 rate in TSB, supplemented with 5% glucose (w/v), and 200 µl of this was transferred to 96 well plates [16]. The plates were incubated at 37 °C for 48 h to allow cell attachment. Following the 48 h incubation period, the propolis extracts in DMSO were added to 96-well plates at concentrations of 0.5-20 mg/mL. The plates were further incubated for 24 h before the crystal violet assay was performed.

### *2.5. Identification and Quantification of Phenolic Compounds*

The crude propolis samples were extracted using a similar methodology mentioned above. The residue was re-dissolved in methanol, filtered from Macherey-Nagel Chromafil Xtra PTFE 20/25 0.20 µm, and 2 µL of the solutions were injected to UPLC-ESI-MS/MS.

Analyses of the propolis samples were performed using UPLC-ESI-MS/MS, a Waters Acquity UPLC analyzer (Waters Corporation, Milford, MA USA), equipped with a Waters (Milford, MA USA) BEH C18 column (100 mm x 2.1 mm, 1.7 µm particle size) and coupled to a Waters Xevo TQ-S Triple Quadrupole tandem mass spectrometry, having electrospray ionization (Waters Corporation, Milford, MA USA). The column was eluted using a linear gradient of two mobile phases, i.e. solvent A (water:acetic acid; 99.95:0.05, v/v) and solvent B (acetonitrile:acetic acid; 99.95:0.05, v/v), which was conducted starting with 99% solvent A and subsequently decreasing it to 70% in 10 min, 5% in 2 min, increasing to 99% in 2 min, and finally maintaining 99% solvent A for 6 min with a solvent flow rate of 0.650 mL/min. The injection was performed directly into the ESI source, the temperature of which was maintained at 500 $^{o}$C, and the mass detector was measured under the optimized parameters.

Mass spectra were acquired in negative ESI mode and executed using the Masslynx4.1 software (Waters). The spectral analysis conditions are given in Table S1 and Table S2 [17]. Analytical Parameters of UPLC-MS/MS Method Validation are given in Table S3 and the purity of the standards, calibration curve and correlation coefficient for phenolic compounds are listed in Table S4.

### *2.6. Statistical Analysis*

The analysis of phenolic compound contents in each propolis sample was conducted in triplicate and the results were expressed as mean ± standard deviation (SD). The data were analyzed by two way analysis of variance (ANOVA), followed by Tukey's HSD Test with a = 0.05, using SPSS v. 16.0 program.

## 3. Results and Discussion

### *3.1. Biofilm Inhibition Activity of Propolis Extracts*

Development of alternative therapies for curing biofilm infections is one of the attractive areas for researchers as the biofilm is more resistant to antibiotics compared to planktonic cells. *S. aureus* strains, especially methicillin sensitive *S. aureus* (MSSA) and methicillin resistant *S. aureus* (MRSA), cause a wide range of life-threatening infections by forming biofilms. Propolis is known for its antimicrobial properties and there are many related reports in the literature [9,18,19]. However, there is no adequate information related to the antibiofilm properties of propolis against MSSA or MRSA bacteria. This study is focused on antibiofilm effect of propolis against MSSA strains as well as its chemical composition.

In order to determine the biofilm inhibition activity of two propolis samples (collected in 2013 and 2012) in different concentrations (0.1, 0.2, 0.3, 0.4 and 0.5 mg/mL) against clinical pathogen bacteria, a standard quantitative biofilm assay method was used (Figure 1-2). Although a good antibiofilm activity was obtained almost for all the concentrations against the tested bacteria, the results appeared to be dose-related. While the biofilm inhibition percentages of BP-1 ranged from 23.68% to 82.00% for MSSA M10 (Figure 1A), from 37.26% to 96.45% for MSSA M18 (Figure 1B), from 35.79% to 92.89% for MSSA M20 (Figure 1C) and from 24.59% to 85.00% for *S. aureus* ATCC 33862 (Figure 1D), the biofilm inhibition rates of BP-2 ranged from 20.12% to 80.00% for MSSA M10 (Figure 2A), from 33.02% to 92.63% for MSSA M18 (Figure 2B), from 25.65% to 87.14% for MSSA M20 (Figure 2C) and from 20.35% to 78.00% for *S. aureus* ATCC 33862 (Figure 2D). These results indicated that the maximum inhibition of biofilm formation was obtained using the ethyl acetate extract. While the ethyl acetate extracts of BP-1 inhibited the biofilm formation of MSSA M18 with 96.45% rate at 0.5 mg/mL concentration, the hexane and ethanol extracts displayed 62.04% and 92.00% biofilm inhibition activities against MSSA M18, respectively, for the same concentration (Figure 1B). In addition, the biofilm biomass of MSSA M18 was inhibited at 57.80%, 85.98% and 92.63% by the hexane, ethyl acetate and ethanol extracts of BP-2, respectively, at 0.5 mg/mL concentration (Figure 2B). On the other hand, the ethanol extracts of propolis samples exhibited effective biofilm inhibition activity against the tested bacteria, as well. In a seperate study, *S. aureus* biofilm development on inert substratum was inhibited by propolis ethanol extracts at a concentration of 0.1875 mg/mL [20]. Similarly, ethanol extract of propolis, obtained from Brazil, showed 3.1 mg/mL MIC value against planktonic *Staphylococcus aureus* ATCC 25923 cells [21]. There are various studies available in the literature about antibiofilm activity of propolis against such microorganisms. For example, the ethanolic extracts of propolis (EEPs) were fractionated into hexane (H-fr), chloroform, ethyl acetate and ethanol. The ability of the four fractions and EEP to inhibit *Streptococcus mutans* and *Streptococcus sobrinus* growth and adherence to a glass surface was examined by Hayacibara et al. [22]. In another study, Bulman et al. [23] reported that propolis disrupted the QS bacterial signaling system of five *Escherichia coli* in liquid- and agar-based bioassays and in C18 reverse-phase thin-layer plate assays. Swarming motility of the opportunistic

pathogen, *Pseudomonas aeruginosa* PAO1 and its AHL-dependent LasR- and RhlR-based QS behaviors were also inhibited by propolis. On the other hand, Duarte et al. [24] showed that fatty acids (oleic, palmitic, linoleic and stearic) were the main compounds identified in EEP and EEH, and these extracts did not show major effects on the viability of mutans streptococci biofilms. Furthermore, the inhibition of *Candida albicans* biofilm formation by propolis extracts was also reported [25].

### *3.2. Effect of Propolis Extracts on Established Biofilms*

It was observed that the established biofilm was gradually damaged upon treatment with different concentrations of propolis (0.5-20 mg/L) in 24 h. Generally, the biofilm reduction rates increased with the increase in extract concentrations (Table 1). The maximum biofilm reduction of BP-1 was obtained at 60.45% against *S. aureus* ATCC 33862 with the ethyl acetate extract, and 57.97 and 57.39% against MSSA M10 with the ethyl acetate and ethanol extracts, respectively. BP-2 also showed the maximum biofilm reduction rate, 55.40%, on *S. aureus* ATCC 33862 with the ethyl acetate extract.

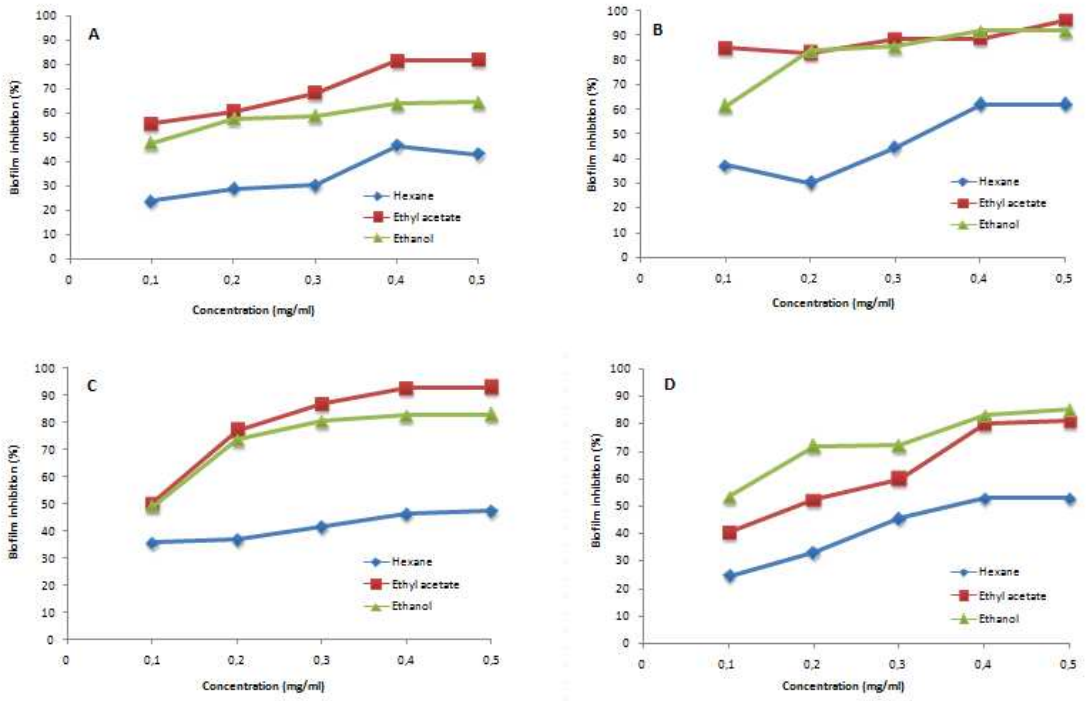


**Figure 1.** The effect of hexane, ethyl acetate and ethanol extracts of BP-1 on the biofilm formation of MSSA M10 (A), M18 (B), M20 (C) and *S. aureus* ATCC 33862 (D) bacteria, expressed as percentage inhibition evaluated by the Crystal Violet staining.

Consequently, the extracts exhibited good antibiofilm activity either by inhibition of biofilm formation (Figure 1, 2) or reduction of the preformed biofilms (Table 1) in different rates against the tested bacteria. According to the Figures (1-2) and Table 1, BP-1, collected in 2013, was slightly more active than BP-2, collected in 2012. The contents of flavonoids, flavones and flavonols show differences according to the year propolis collected [26]. Also, the ethanol and the ethyl acetate extracts of the propolis samples were more effective on *S. aureus* biofilms compared to the hexane extracts (Figure 1, 2 and Table 1).

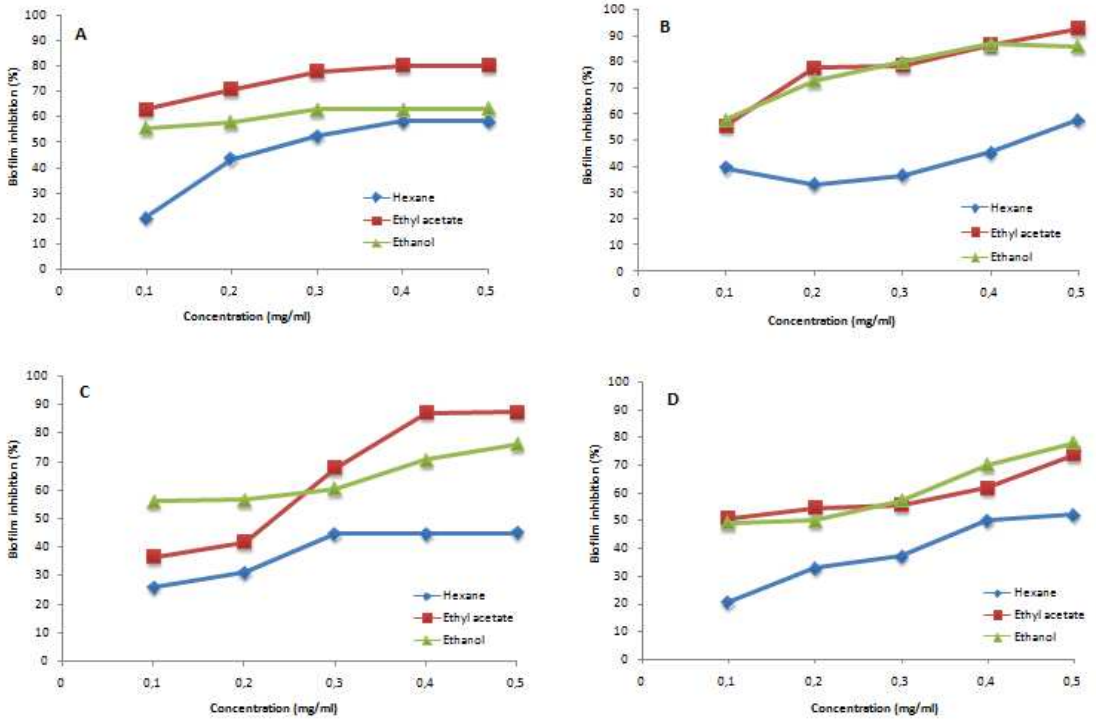


**Figure 2.** Inhibition effect of hexane, ethyl acetate and ethanol extracts of BP-2 on biofilm formation of MSSA M10 (A), M18 (B), M20 (C) and *S. aureus* ATCC 33862 (D) bacteria, expressed as percentage inhibition, evaluated by the Crystal Violet staining.

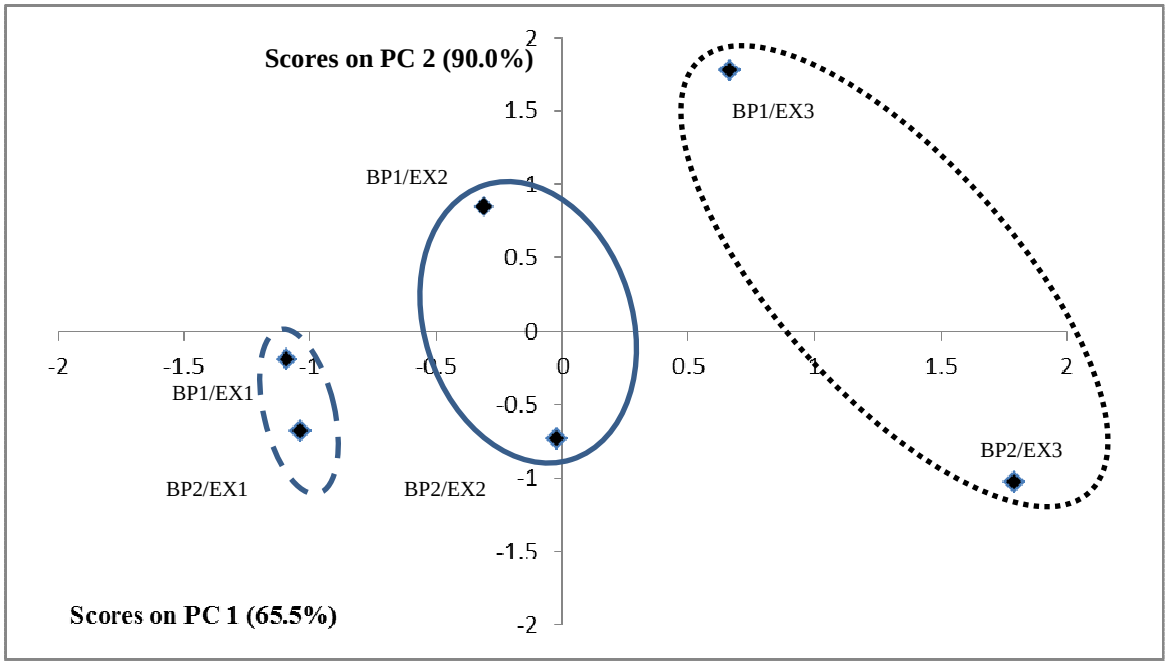


**Figure 3.** Plot of loadings of the first and second principal components, possessing different varibles: year of propolis collected: BP1, BP2; extraction solvents: EX1 (hexane), EX2 (ethanol) and EX3( ethyl acetate)

**Table 1.** Biofilm reduction percentage of propolis extracts (%) ± SD.

| Bacteria | Extracts | Concentrations of BP-1 (mg/mL) | | | | | | Concentrations of BP-2 (mg/mL) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **0.5** | **1** | **5** | **10** | **15** | **20** | **0.5** | **1** | **5** | **10** | **15** | **20** |
| **MSSA M10** | **HEX** | 15.56±0.9 | 18.79±6.3 | 24.70±3.0 | 30.18±3.2 | 38.57±1.8 | 38.04±5.0 | 15.65±5.3 | 18.97±6.6 | 23.70±5.5 | 26.18±4.6 | 30.57±6.8 | 32.04±7.0 |
| | **ETA** | 27.59±8.3 | 31.40±7.0 | 39.51±4.6 | 51.24±2.8 | 57.22±0.5 | 57.97±8.6 | 22.16±0.0 | 30.66±1.3 | 32.92±7.6 | 45.67±4.2 | 49.14±2.8 | 53.42±3.1 |
| | **ETOH** | 25.67±5.6 | 33.75±5.9 | 34.93±5.9 | 42.05±1.2 | 50.07±1.9 | 57.39±1.1 | 19.60±0.9 | 27.87±4.1 | 33.00±3.2 | 42.56±7.5 | 46.57±2.2 | 50.45±2.5 |
| **MSSA M18** | **HEX** | 20.58±4.0 | 21.48±0.2 | 22.02±0.3 | 27.34±8.8 | 29.23±5.0 | 30.50±3.1 | 11.36±3.6 | 10.95±1.9 | 13.17±2.8 | 14.70±3.5 | 25.70±2.5 | 25.88±1.8 |
| | **ETA** | 31.70±3.7 | 34.04±8.6 | 40.80±9.4 | 44.36±5.3 | 47.66±4.8 | 50.95±3.7 | 28.02±6.7 | 31.40±6.6 | 40.07±0.9 | 44.68±1.6 | 47.84±7.5 | 47.57±4.4 |
| | **ETOH** | 27.21±1.8 | 29.49±1.8 | 32.73±3.0 | 34.16±6.7 | 37.05±0.6 | 39.45±1.1 | 27.74±1.2 | 28.85±9.7 | 28.62±0.6 | 29.61±2.4 | 31.70±8.0 | 33.45±8.9 |
| **MSSA M20** | **HEX** | 16.41±1.3 | 20.71±1.5 | 21.64±3.8 | 22.20±4.8 | 22.86±2.3 | 26.89±1.4 | 20.28±7.3 | 24.34±7.3 | 25.43±1.1 | 25.30±1.4 | 25.87±1.4 | 28.05±1.8 |
| | **ETA** | 31.22±2.9 | 37.33±1.6 | 38.76±4.6 | 39.76±1.5 | 44.92±1.1 | 44.01±1.7 | 31.29±5.4 | 34.01±6.2 | 40.60±4.6 | 40.68±6.1 | 45.87±9.4 | 46.85±1.1 |
| | **ETOH** | 24.64±2.6 | 34.31±3.4 | 37.68±9.8 | 37.92±0.6 | 38.76±8.9 | 38.54±3.2 | 21.79±3.6 | 24.87±1.7 | 29.86±5.5 | 34.18±3.5 | 36.92±2.3 | 36.38±9.6 |
| **S. a*ureus* ATCC 33862** | **HEX** | 14.50±5.0 | 16.30±1.2 | 18.58±1.6 | 21.88±9.6 | 27.21±1.3 | 29.61±2.9 | 16.77±1.7 | 21.92±1.1 | 22.01±2.0 | 23.06±1.2 | 27.26±1.1 | 27.34±2.2 |
| | **ETA** | 38.41±7.1 | 49.25±1.0 | 48.55±3.5 | 52.00±6.8 | 59.49±3.4 | 60.45±6.5 | 28.58±7.3 | 34.05±3.2 | 45.43±4.4 | 50.94±5.2 | 53.81±2.0 | 55.40±2.2 |
| | **ETOH** | 29.34±0.5 | 38.82±3.2 | 41.30±1.2 | 41.87±4.0 | 42.25±1.2 | 46.51±1.6 | 18.47±1.1 | 26.44±1.2 | 35.04±9.2 | 41.46±8.4 | 45.47±0.1 | 45.34±1.7 |

### *3.3. Phenolic Compound Content of Propolis*

Phenolic compounds of the BP-1 and BP-2 samples were identified and quantified by using UPLC-ESI-MS/MS technique, based on retention time and according to their mass-to-charge ratio (Table 2) [27].

Twenty-four phenolic compounds (homogentisic, 3,4-dihydroxybenzoic (protocatechuic), gentisic, p-hydroxy benzoic, vanillic, caffeic, syringic, p-coumaric, trans-cinnamic, ferrulic and trans-2-hydroxy cinnamic acids, rutin, myricetin, 3,4-dihydroxybenzaldehyde, quercetin, naringenin, genistein, apigenin, kaempferol, chrysin and vanillin) were determined in all three fractions of the propolis samples (Table 2), and the results are given in Figure S1. On the other hand, pyrogallol, galantamine, catechin hydrate, epicatechin, catechin gallate and resveratrol were not detected, and while luteolin was not found in BP-2, no hesperetin was identified in BP-1. Also, ferulic, caffeic and gentisic acids were the most abundant phenolic compounds found in the extracts. While the ethanol extracts of propolis samples presented high quantity, the hexane extracts were lower in amount. The number of the identified phenolic compounds, obtained from Bartın propolis in the present study were found to be higher compared to that of reported by Erdogan et al. [28], who studied propolis from different regions of Turkey, and detected thirteen compounds, using a different methodology.

ANOVA of the data showed that the year of collection (BP-1, BP-2) and the extraction solvents (hexane, ethanol and ethyl acetate) have statistically significant effect ($p$ : 0.05) on phenolic compound contents of propolis. The effect of interaction between two main factors was also found to be significant ($p$ : 0.05) (Table 2).

Principal component analysis (PCA), i.e. a statistical process, was applied to the extracted phenolic compounds from the propolis samples, using all three extraction solvents concurrently in order to discover groupings and indicate that these results displayed significant differences, based on propolis and the extraction solvent (Table S5). PSA of the data generated two significant principal components (PCs), explaining 65.5%, 90.0% of the variance. Figure 3 (PC1 vs PC2) is a plot of principal component loadings of propolis, extraction solvents and phenolic compounds on the first and the second principal components. The maximum biofilm reduction of BP-1 and BP-2 against *S. aureus* ATCC 33862 was produced by the ethyl acetate extract (Figure 3). This level of biofilm reduction was followed by the ethanol and hexane extracts, respectively, as delineated in Figure 3.

A LSD multiple-range test was used to perform comparisons among different extraction solvents (Table S6). A statistically significant difference was obtained among 3,4-dihydroxybenzoic acid, gentisic acid, *p*-hydroxy benzoic acid, vanillic acid, caffeic acid, syringic acid, *p*-coumaric acid, ferulic acid, *trans*-cinnamic acid, naringenin, genistein, kaempferol and chrysin in terms of extraction solvent, and the higher statistical significance was observed in ethyl acetate for both propolis types (BP-1, BP-2). However, no statistically significant difference was found between hexane and ethanol extraction solvents for pyrocatechol, vanillin, rutin, *trans*-2-hydroxy cinnamic acid, myricetin and quercetin.

The antibiofilm activity results were confirmed by UPLC-ESI-MS/MS and the principal component analyses. Although twenty-four compounds were detected in the propolis samples, general phenolic contents of BP-1 was found to be higher than BP-2 (Table 2). While the ferulic acid of ethanol extract of BP-1 was 112500 µg/g, it was 98661 µg/g for BP-2 (Table 2). Similarly, Veloz et al. [26] investigated the polyphenol and flavonoid contents and antibiofilm activity of Chilean propolis samples, collected in three different years (2008, 2010 and 2011). They reported that the second extract (CEP2), which contained higher polyphenol and flavonoid contents showed the most effective biofilm inhibition activity. Specifically, the propolis samples, used in that study, contained higher amount of caffeic acid, which is reported to be an anti-quorum sensing component in propolis sample [29]. Thus, it is responsible for antibiofilm activity of propolis.

As a conclusion, as it was revealed from the analyses of the phenolic compounds of the propolis samples, the ethyl acetate and ethanol extracts had the higher amount of phenolic contents. Accordingly, it has been determined that the biofilm reduction rates of the ethyl acetate and ethanol extracts of propolis were better than that of the hexane extract of propolis. The ethyl acetate and ethanol extracts displayed high antibiofilm activity, yet hexane extracts were fairly active. This also indicated notable consistency between the phenolic content and the biofilm inhibition rate/antibiofilm activity.

**Table 2.** Phenolic contents (µg/g propolis extract) of propolis samples.

| | BP1 | | | BP2 | | |
|---|---|---|---|---|---|---|
| Compounds | Hexane | Ethanol | Ethyl acetate | Hexane | Ethanol | Ethyl acetate |
| Pyrogallol | < LOQ | < LOQ | < LOQ | < LOQ | < LOQ | < LOQ |
| Homogentisic acid | 903.0$^{d}$ | 3255.0$^{b}$ | 5748.3$^{a}$ | 61.29$^{f}$ | 353.41$^{e}$ | 1268.0$^{c}$ |
| 3,4-Dihydroxybenzoic acid | 253.22$^{e}$ | 652.32$^{c}$ | 984.71$^{b}$ | 51.96$^{f}$ | 453.65$^{d}$ | 1339.8$^{a}$ |
| Gentisic acid | 199.56$^{f}$ | 2122.5$^{d}$ | 5232.0$^{b}$ | 537.24$^{e}$ | 3133.4$^{c}$ | 6897.8$^{a}$ |
| Pyrocatechol | 10.73$^{f}$ | 23.49$^{e}$ | 160.97$^{c}$ | 56.84$^{d}$ | 235.66$^{b}$ | 986.72$^{a}$ |
| Galantamine | < LOQ | < LOQ | < LOQ | < LOQ | < LOQ | < LOQ |
| p-hydroxy benzoic acid | 63.45$^{f}$ | 254.60$^{d}$ | 1229.80$^{c}$ | 137.61$^{e}$ | 1755.20$^{b}$ | 3360.60$^{a}$ |
| 3,4-dihydroxybenzaldehyde | 101.86$^{b}$ | 325.76$^{b}$ | 689.46$^{b}$ | 257.73$^{b}$ | 2531.2$^{a}$ | 2342.9$^{a}$ |
| Catechin hydrate | < LOQ | < LOQ | < LOQ | < LOQ | < LOQ | < LOQ |
| Vanillic acid | 204.6$^{e}$ | 1846.1$^{c}$ | 3263.7$^{b}$ | 1159.3$^{d}$ | 3255.3$^{b}$ | 6534.2$^{a}$ |
| Caffeic acid | 3522.8$^{e}$ | 16881.0$^{c}$ | 23521.0$^{a}$ | 2322.5$^{f}$ | 10416.0$^{d}$ | 19100.0$^{b}$ |
| Syringic acid | 523.64$^{e}$ | 1546.9$^{b}$ | 1984.2$^{a}$ | 246.53$^{f}$ | 956.04$^{d}$ | 1337.3$^{c}$ |
| Vanillin | 9.31$^{f}$ | 24.97$^{e}$ | 127.45$^{c}$ | 102.98$^{d}$ | 225.16$^{b}$ | 549.40$^{a}$ |
| p-coumaric acid | 1207.5$^{f}$ | 3356.0$^{d}$ | 5977.0$^{b}$ | 2082.2$^{e}$ | 4326.1$^{c}$ | 7568.4$^{a}$ |
| Ferulic acid | 12878$^{e}$ | 112500$^{c}$ | 156600$^{a}$ | 9627.7$^{f}$ | 98661$^{d}$ | 136800$^{b}$ |
| Epicatechin | < LOQ | < LOQ | < LOQ | < LOQ | < LOQ | < LOQ |
| Catechin gallate | < LOQ | < LOQ | < LOQ | < LOQ | < LOQ | < LOQ |
| Rutin | 126.73$^{f}$ | 583.09$^{d}$ | 1046.6$^{b}$ | 236.45$^{e}$ | 646.58$^{c}$ | 3565.1$^{a}$ |
| trans-2-hydroxy cinnamic acid | 540.35$^{d}$ | 995.70$^{b}$ | 1536.1$^{a}$ | 99.78$^{f}$ | 126.39$^{e}$ | 887.14$^{c}$ |
| Myricetin | 55.87$^{f}$ | 99.63$^{e}$ | 364.44$^{c}$ | 200.71$^{d}$ | 532.69$^{b}$ | 1206.70$^{a}$ |
| Resveratrol | < LOQ | < LOQ | < LOQ | < LOQ | < LOQ | < LOQ |
| trans-cinnamic acid | 48.14$^{d}$ | 124.17$^{c}$ | 458.01$^{b}$ | 130.01$^{c}$ | 457.70$^{b}$ | 846.90$^{a}$ |
| Luteolin | 15.93$^{c}$ | 86.76$^{b}$ | 246.54$^{a}$ | 0.012$^{d}$ | 0.001$^{d}$ | 0.002$^{d}$ |
| Quercetin | 47.93$^{f}$ | 121.05$^{e}$ | 356.08$^{c}$ | 155.43$^{d}$ | 445.6$^{b}$ | 1006.1$^{a}$ |
| Naringenin | 245.43$^{e}$ | 1044.30$^{c}$ | 1986.40$^{a}$ | 114.60$^{f}$ | 345.80$^{d}$ | 1148.2$^{b}$ |
| Genistein | 364.76$^{d}$ | 997.32$^{b}$ | 1007.6$^{b}$ | 126.23$^{e}$ | 757.85$^{c}$ | 2008.2$^{a}$ |
| Apigenin | 18.85$^{d}$ | 49.06$^{c}$ | 129.42$^{b}$ | 54.45$^{c}$ | 124.59$^{b}$ | 501.41$^{a}$ |
| Kaempferol | 121.77$^{e}$ | 421.0$^{d}$ | 1126.2$^{b}$ | 100.61$^{f}$ | 647.92$^{c}$ | 1355.7$^{a}$ |
| Hesperetin | 0.0003$^{d}$ | 0.005$^{d}$ | 0.0005$^{d}$ | 12.25$^{c}$ | 39.47$^{b}$ | 125.42$^{a}$ |
| Chrysin | 9.811$^{e}$ | 28.306$^{c}$ | 89.065$^{a}$ | 9.464$^{e}$ | 19.122$^{d}$ | 60.576$^{b}$ |

Means followed by the same letter in the same column are not significantly different from each other ($p$ : 0.05)

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## Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

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