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ARAŞTIRMA MAKALESI / RESEARCH ARTICLE

INVESTIGATION OF ANTIMICROBIAL AND ANTIVIRAL EFFECTS OF TÜRKIYE PROPOLIS WATER EXTRACTS: AN *IN VITRO* STUDY

Türkiye Propolis Su Ekstraktlarının Antimikrobiyal ve Antiviral Etkilerinin Araştırılması: *İn Vitro* Çalışma

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ABSTRACT

Propolis is a natural bee product used as a therapeutic agent for centuries. Propolis extracts are natural resources that attract the attention of scientists looking for new components due to the insufficiency of existing drugs. In current study, antiviral and antimicrobial activity of propolis water extracts prepared from three different raw propolis samples collected from Northeast of Türkiye (Ardahan, Rize, and Trabzon) were investigated. The total flavonoid contents (TFC) and total phenolic content (TPC) of the extracts were measured. It was determined that TPC and TFC ranged from 5.87±0.36 to 20.47±1.46 mg GAE g^{-1}, and 0.48±0.04 to 2.10±0.22 mg QUE g^{-1}, respectively. The antimicrobial activity of the extracts against 14 microorganisms (*Bacillus cereus* ATCC14579, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* ATCC 27853, *Acinetobacter haemolyticus* ATCC 19002, *Escherichia coli* ATCC 25922, *Salmonella typhimurium* ATCC 14028, *Enterobacter aerogenes* ATCC 13048, *Mycobacterium smegmatis* ATCC 607, *Klebsiella pneumoniae* ATCC 13883, *Chromobacterium violaceum* ATCC 12472, *Candida parapsilosis* ATCC 22019, and *Candida albicans* ATCC 10231) and their effect against the *Pseudomonas aeruginosa* biofilm were investigated. Additionally, anti-quorum sensing and anti-swarming activities of the extracts were tested. The antiviral activity of the extracts was examined against Herpes simplex virus type 1 (HSV-1) by MTT and qRT-PCR methods. The water extracts of propolis samples did not show antimicrobial, anti-swarming, anti-quorum sensing, and anti-viral activities. However, extracts were found to have strong anti-biofilm activities. The results show that aquatic propolis extracts can be evaluated in the treatment of biofilms.

Keywords: Antimicrobial, Biofilm, Phenolic, Propolis, RT-PCR

ÖZ

Yüzyıllardır tedavi edici ajan olarak kullanılan ve doğal bir arı ürünü olan propolis, mevcut ilaçların yetersiz kalması sonucu yeni bileşenler arayan bilim adamlarının ilgisini çeken doğal kaynaklardan biridir. Bu çalışmada Türkiye'nin kuzeydoğusundan (Ardahan, Rize, Trabzon) toplanan üç farklı propolis örneğinden su ile hazırlanan propolis ekstraktlarının antimikrobiyal ve antiviral aktivitesi araştırıldı. Ekstraktların toplam fenolik içeriği (TPC) ve toplam flavonoid içeriği (TFC) ölçüldü. TPC ve TFC'nin sırasıyla 5.87±0.36 ila 20.47±1.46 mg GAE g-^{1} ve 0.48±0.04 ila 2.10±0.22 mg QUE g-^{1} arasında değiştiği belirlendi. Ekstraktların 14 mikroorganizmaya (*Bacillus cereus* ATCC14579, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* ATCC 27853, *Acinetobacter haemolyticus* ATCC 19002, *Escherichia coli* ATCC 25922, *Salmonella typhimurium* ATCC 14028, *Enterobacter aerogenes* ATCC 13048, *Mycobacterium smegmatis* ATCC 607, *Klebsiella pneumoniae* ATCC 13883, *Chromobacterium violaceum* ATCC 12472, *Candida parapsilosis* ATCC 22019 ve *Candida albicans* ATCC 10231) karşı antimikrobiyal aktivitesi ve *Pseudomonas aeruginosa* biyofilmine etkinliği, ayrıca anti-quorum sensing ve anti-swarming aktiviteleri araştırıldı. Ekstraktların Herpes simpleks virüs tip 1 (HSV-1)'e karşı antiviral aktivitesi, MTT ve qRT-PCR yöntemleriyle test edildi. Çalışmada kullanılan propolis su ekstraktlarının antimikrobiyal, anti-swarming, anti-quorum sensing ve anti-viral aktivite göstermediği, bununla birlikte ekstraktların güçlü anti-biyofilm aktivitesine sahip olduğu tespit edildi. Sonuçlar, propolis su ekstraktlarının bakteri biyofilmi tedavisinde değerlendirilebileceğini göstermektedir.

Anahtar Kelimeler: Antimikrobiyal, Biyofilm, Fenolik, Propolis, RT-PCR

GENİŞLETİLMİŞ ÖZET

Amaç: Yüzyıllardır tedavi edici ajan olarak kullanılan ve doğal bir arı ürünü olan propolis, mevcut ilaçların yetersiz kalması sonucu yeni bileşenler arayan bilim adamlarının ilgisini çeken doğal kaynaklardan biridir. Propolisin içerisindeki biyoaktif molekül profili propolisin toplandığı botanik ve coğrafik kaynak, arı türü, toplandığı mevsim ve çevresel faktörler gibi çeşitli parametlere bağlı olarak değişmektedir. Buna bağlı olarak propolislerin terapötik etkileri değişkenlik göstereceğinden her propolis örneğinin kimyasal ve biyolojik analizinin gerçekleştirilmesi gerekmektedir. Bu çalışmada Türkiye'nin kuzeydoğusundan (Ardahan, Rize, Trabzon) toplanan üç farklı propolis örneğinden su ile hazırlanan propolis ekstraktlarının antimikrobiyal ve antiviral aktivitesi araştırıldı.

Gereç ve Yöntem: Propolislerin su ekstraktı hazırlanarak ekstraktların toplam fenolik içeriği (TPC) Folin-Ciocalteau yöntemi ile, ekstraktların toplam flavonoid içeriği (TFC) Fukumoto ve Mazza yöntemi kullanılarak ölçüldü. TPC ve TFC'nin sırasıyla 5.87±0.36 ila 20.47 ± 1.46 mg GAE g-^{1} ve 0.48 ± 0.04 ila 2.10±0.22 mg QUE g-^{1} arasında değiştiği belirlendi. Hazırlanan ekstraktlarıdaki su liyofilizasyon ile ortamdan uzaklaştırıldı. Kuru madde dimetilsülfoksit (DMSO) kullanılarak çözülerek antimikrobiyal ve antiviral aktivite çalışmalarında kullanıldı. Deneylerde ekstraktların DMSO oranı mikroorganizmaları, virüs partiküllerini ve hücre hatlarını etkilemeyecek konsantrasyon olan %1'in altında tutuldu. Ekstraktların 14 mikroorganizmaya (*Bacillus cereus* ATCC14579, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* ATCC 27853, *Acinetobacter haemolyticus* ATCC 19002, *Escherichia coli* ATCC 25922, *Salmonella typhimurium* ATCC 14028, *Enterobacter aerogenes* ATCC 13048, *Mycobacterium smegmatis* ATCC 607, *Klebsiella pneumoniae* ATCC 13883, *Chromobacterium violaceum* ATCC 12472, *Candida parapsilosis* ATCC 22019 ve *Candida albicans* ATCC 10231) karşı antimikrobiyal aktivitesi agar kuyucuk difüzyon yöntemi ile belirlendi. Ekstraktların *Pseudomonas aeruginosa* biyofilmine etkinliği, ayrıca anti-quorum sensing ve anti-swarming aktiviteleri araştırıldı. Ekstraktların Afrika yeşil maymun böbrek epitel hücresi (Vero) hücrelerine sitotoksik etkisi tripan mavisi boyama ve 3-(4,5-dimetiltiyazol-2-yl)-2,5-difeniltetrazolyum-bromür (MTT) yöntemleri kullanılarak araştırıldı. Antiviral aktivite testlerinde kullanılacak Herpes simpleks virüs tip 1 (HSV-1)'in titresi doku kültürü enfeksiyöz dozunun yüzde 50'si ($TCID_{50}$) testi ile belirlendi. Ekstraktların HSV-1'e karşı antiviral aktivitesi, ekstraktların Vero hücrelerine sitotoksik olmayan konsantrasyonları kullanılarak MTT ve qRT-PCR yöntemleri ile test edildi. Elde edilen verilerin istatistiksel analizi

yapılarak sonuçlar değerlendirildi.

Bulgular ve Sonuç: Çalışmada kullanılan propolis su ekstraktlarının antimikrobiyal, anti-swarming, anti-quorum sensing ve anti-viral aktivite göstermediği görüldü. Bununla birlikte Pazar ve Uzungöl ekstraktlarının güçlü anti-biyofilm aktivitesine sahip olduğu tespit edildi. Ardahan propolis ekstraktının da diğer ekstraktlar kadar güçlü olmasa da anti- biyofilm aktivitesinin olduğu görüldü. Pazar ve Uzungöl propolis ekstraktlarının gösterdiği anti-biyofilm aktiviteleri istatistiksel olarak anlamlı bulundu (p=0.001). Sonuçlar, propolis su ekstraktlarının HSV-1 enfeksiyonlarının tedavisinde yetersiz kalabileceğini, bununla birlikte propolis su ekstraktlarının bakteri biyofilmi tedavisinde değerlendirilebileceğini göstermektedir.

INTRODUCTION

Propolis is a natural mixture produced by honeybees (*Apis mellifera*). Honey bees produce propolis from saps, resins, and mucilages collected from various parts of the plants, then mix them with bee enzymes and beeswax. Honeybees use propolis to fix damage in the hive, refine internal walls, and maintain the humidity and temperature of the hive. Propolis also protects the colony against pathogenic microorganisms (Forma & Bryś, 2021). The use of propolis by humans dates back to ancient times. Propolis was used in antiquity and the Middle Ages for different purposes such as the treatment of wounds and burns, and preparation of cosmetic products. At present propolis continues to be used in alternative medicine and as a food supplement (Kocot et al. 2018).

The bioactive molecular profile of crude propolis varies according to the botanical and geographical origin, genetics of bees, season, and environmental factors. The quantity and quality of propolis collected depend on plant variety and availability, source and duration of collection, techniques and practices of beekeepers, and environmental health (Šuran et al. 2021). Raw propolis collected from hives cannot be used directly in treatment or scientific studies. To be used, the active ingredients in propolis must be extracted using various solvents. The solvents often used in the preparation of extracts are ethanol, olive oil, water, dichloromethane, and chloroform. The biological activity of the prepared extract varies according to the type and amount of active ingredients in it (Przybyłek& Karpiński, 2019; Kolayli, 2023).

Herpes simplex virus type 1 (HSV-1) is a human pathogen that replicates in peripheral tissues and then invades the nervous system and establishes latent infection (Ahmad & Wilson, 2020). Although it establishes latency, HSV-1 may later reactivate as a response to a stimuli, or spontaneously. In addition to lesions, HSV-1 can cause serious pathologies such as keratoconjunctivitis or encephalitis (Bello-Morales et al. 2021). It is estimated that 3.7 billion people under the age of 50 are infected with HSV-1 worldwide (WHO, 2015). Although there have been advances in the treatment of HSV infections with nucleoside analogs, there is a need for developed therapeutics with alternative mechanisms of action (Whitley & Baines, 2018).

The bioactive component in propolis is subject to variation depending on a number of factors. The biological, chemical and therapeutic properties of propolis exhibit regional differences. The solvents used in the preparation of the extract also ensure that different bioactive components are obtained at different rates. As a result, it can cause different pharmacological properties to be seen in extracts prepared with different solvents. For all these reasons, in the current study, it was aimed to evaluate the antimicrobial effects of water extracts of propolis samples acquired from different regions of Türkiye. It was also aimed to research the in vitro antiviral effect of propolis extracts against HSV-1.

MATERIALS and METHODS

Study Design

Propolis samples were collected from three regions on the coasts of the Eastern Black Sea in Türkiye. Biofilm activity laboratory experiments were run in duplicate (n=6, for each group). The study was completed with a total of 24 samples, including the positive sample group. Data that were inconsistent with the data obtained in the study were excluded from the evaluation.

Standard Drug, Cell Culture, and Virus

The African green monkey kidney cell line (Vero) was obtained from the University of Erciyes and was cultivated in Dulbecco's Modified Eagle's medium supplemented with antibiotics and fetal bovine serum.

HSV-1 Wal strain was first acquired from the University of Sheffield (UK). The antimicrobial activity of propolis extracts was researched against *Bacillus cereus* ATCC14579, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* ATCC 27853, *Acinetobacter haemolyticus* ATCC 19002, *Escherichia coli* ATCC 25922, *Salmonella typhimurium* ATCC 14028, *Enterobacter aerogenes* ATCC 13048, *Mycobacterium smegmatis* ATCC 607, *Klebsiella pneumoniae* ATCC 13883, *Chromobacterium violaceum* ATCC 12472, *Candida parapsilosis* ATCC 22019, and *Candida albicans* ATCC 10231.

Acyclovir (5 mg mL^{-1} dissolved in sterile dimethyl sulfoxide (DMSO) was used as positive control in antiviral activity assays. Ciprofloxacin, Gentamicin, Amphotericin B, and Ampicillin were positive controls in anti-bacterial and anti-fungal experiments.

Propolis Extract Preparation Protocol

Three propolis samples were collected from the Ardahan City, Uzungöl district of Trabzon City, and Pazar district of Rize City, Türkiye, and were grounded mechanically into small pieces. 5 mL of glycerol and 40 mL of distilled water were added to a 5 g powdered propolis sample in a glass bottle. The bottles were stirred in an ultrasonic bath for 2 hours with 99 amplitudes and then shaken in a magnetic stirrer at 500-600 rpm for 24 hours at 45-50˚C. After the mixture was filtered, the final volume was made up to 40 mL with distilled water. Half of the extract was reserved for chemical analysis. 20 mL of the mixture was lyophilized, and water was removed. The empty and full bottles were weighed, and the grams of sediment were calculated. The residues were dissolved with DMSO, and sheltered to 4˚C until use. In the experiments DMSO rate of the extracts was kept under 1%, which is the concentration that does not affect the microorganisms, virus particles, and Vero cells.

Determination of Total Phenolic Content (TPC) and Total Flavanoid Content (TFC) of the Extracts

The Folin-Ciocalteau procedure was applied to evaluate the TPC of the propolis extracts (Singleton & Rossi, 1965). In the procedure various concentrations of gallic acid (from 0.015 to 0.5 mg/mL) in a total volume of 20 µL and propolis samples were put in a tube. 680 µL distilled water, and 400 µL 0.2 N Folin-Ciocalteu's phenol reagent were added to the tube. After vortexing, the tubes were incubated for 3 min. Then 400 µL Na_2CO_3 (10%) was added to the mixture and the absorbance of the mixtures was measured against a blank at 760 nm.

The method of Fukumoto and Mazza was applied to evaluate the TFC of the propolis extracts (Fukumoto & Mazza, 2000). Firstly, 0.30 µL of the extract was added 50 µL of 10% $Al(NO_3)_3$ and 50 µL of 1 M ($NH_4.CH_3COO$). The mixture was then diluted to 3 mL with ethanol (99%) and incubated at room temperature. After 40 min incubation, the absorbance was then measured against a blank at 415 nm.

The standard graph was arranged with different concentrations of gallic acid, and quercetin to calculate the concentration of TPC, and TFC in the extracts, respectively. The concentration of TPC was calculated as milligrams of gallic acid equivalent (GAE) per gram (mg GAE g^{-1}), the concentration of TFC was calculated as mg quercetin equivalent (QUE) g^{-1} sample (Kolayli et al. 2022).

Anti-bacterial Activity Assay: Agar Well Diffusion Method

The agar well method published by Denev et al. was modified and used in the current study (Denev et al. 2014). *Candida* species were incubated in Mueller Hinton Agar (MHA) with 2% glucose at 35°C for two days, *M. smegmatis* in Brain-Heart Infusion Agar (BHIA) at 37°C for three days, and other bacteria in MHA at 37°C for one day. Suspensions of bacteria at a density of 0.5 McFarland and of *Candida* species at a density of 1 McFarland were prepared in Phosphate Buffered Saline (PBS). *M. smegmatis* suspension was prepared in Brain-Heart Infusion Broth (BHIB) at a concentration of 0.5 Mc Farland. The prepared bacterial suspensions were spread on MHA, the *M. smegmatis* suspension on BHIA, and the *Candida* suspensions on MHA containing 2% glucose (Woods et al. 2003; CLSI, 2009). Then, 6 mm wide wells were opened on the medium. 50 µL of the 10 mg mL^{-1} propolis extracts, positive and negative controls were placed in the wells. DMSO was used as a negative control. Ciprofloxacin, gentamicin, amphotericin b, and ampicillin were used as positive controls for *M. smegmatis*, Gram-negative bacteria, *Candida* species, and Gram-positive bacteria, respectively. Bacterial cultures were incubated for one day, *Candida* species for two days, and *M. smegmatis* for three days. Zone

diameters of <6 mm were considered ineffective, 6-14 mm were considered moderately effective, and 15 mm and above were considered as high activity (Balouri et al. 2016).

Anti-Quorum Sensing Assay

Sub-MIC concentrations of the extracts were used in the experiment. The overnight culture of the *C. violaceum* strain (50 µL) was added to 5 mL LB soft agar (0.3% w/v) prepared with LB broth (Condalab, Spain) and agar (Sigma-Aldrich, USA) and poured onto LB agar frozen in the petri dish. After the soft agar was frozen, wells were opened in the medium and 50 µL of the extracts were placed in the wells. At the end of one-night of incubation, extracts with growth around the well but no pigment formation was considered positive (Ureyen Esertas et al. 2022).

Anti-Swarming Assay

The agar was allowed to solidify by adding the final concentration of 100 µg mL^{-1} from the extracts into 5 mL of autoclaved but not solidified LB medium. A colony from the overnight culture of *P. aeruginosa* PAO1 strain was placed to the middle of the medium with a sterile toothpick and incubated for 16-18 h at 37˚C. The spread of bacteria from center to periphery was evaluated by comparing it with the *P. aeruginosa* PAO1 strain without extracts (Rashid & Kornberg, 2000).

Anti-Biofilm Assay

To identify the inhibition of the extracts on biofilm development, the *P. aeruginosa* PAO1 strain at a 0.5 McFarland density in LB medium, was diluted by 1% and used in the assay. Extract (40 µL), LB medium (125 µL), and *P. aeruginosa* PAO1 culture (35 µL) were added to each well of the 96-well plate. Wells containing only bacteria was used as control. The plates were washed three times with distilled water after 24 h of incubation at 37˚C. 0.3% crystal violet dissolved in water was added to the wells. After 15 min, the plates were washed three times with distilled water and kept in 95% ethanol for 15 minutes. The colors were measured in a spectrophotometer at 570 nm. The experiment was repeated twice and the results were averaged to create a graph (Fazli et al 2014; Ureyen Esertas et al. 2022).

Cytotoxicity Assays

Trypan blue assay: $1x10^5$ Vero cells were placed in each well of the 24-well plate and incubated until the cells adhered to the plate. Different concentrations of extracts (25-6000 µg mL-1) were placed on the plate Three wells were used for each concentration. The same amount of untreated cells was used as a negative control. Four different plates were prepared to observe the cytotoxic effect of propolis extracts on Vero cells at 24, 48, 72, and 96 hours. At the end of the designated time, the cells were trypsinized. Cells stained with trypan blue were counted using a hemocytometer. The results were evaluated concerning the number of cells in the control wells (Yildirim et al. 2016).

MTT Assay

MTT assay was carried out as described in Cora et al. 2023 (Cora et al, 2023). Briefly, Vero cells were incubated with different concentrations of propolis extracts (25-6000 µg mL^{-1}) for 24, 48, 72, and 96 hours. At the end of the time, the MTT assay was performed. The results were evaluated regarding the control wells (Mosmann, 1983). The experiment was repeated twice.

Determination of Tissue Culture Infectious Dose 50 (TCID50)

The virus was serially diluted across the 96 well plate including confluent Vero cells. The plate was incubated for 3 days at 37°C with 5% CO_2. After the incubation, the wells that were positive for cytopathic effect were counted for each dilution. The 50% infectious dose was determined by performing Spearman-Karber method (Ramakrishnan, 2016).

Antiviral Activity Assays

MTT and quantitative real-time polymerase chain reaction (qRT-PCR) methods were performed to investigate the antiviral activity of the extracts.

MTT assay

Different concentrations of the virus (1, 10, and 100 $TCID_{50}$) were used to infect $1x10^4$ Vero cells in 96-well plates. Infected cells were incubated with different concentrations of propolis extracts (800-12.5 µg mL^{-1}) for three days. Wells containing infected but untreated cells, acyclovir, and wells that contained just Vero cells were used as a negative control, positive control, and reproductive control, respectively. Then, MTT assay was carried out as previously described (Cora et al. 2023). The experiment was repeated twice, and the rate of cell viability was calculated by comparing them with control wells.

qRT-PCR assay

The qRT-PCR assay was achieved as previously described (Cora et al. 2023). After the Vero cells were infected with virus, different concentrations of extracts (12.5-800 µg mL^{-1}) were added to the wells. Acyclovir was used as a positive control, the wells that included infected cells were used as a negative control. After 3 days of incubation viral DNA was isolated from the wells by using a nucleic acid isolation device (Bioneer ExiPrep 16 Plus, South Korea) and isolation cartridges (ExiPrepTMPlus Viral DNA/RNA Kit, South Korea) according to the manufacturer's instructions.

HSV-1 DNA in the wells was quantified with qRT-PCR using LightCycler® 480 System (Roche Molecular Systems, USA) and the results were analyzed using the software of the device. The master mix, primer, probe, and reaction conditions previously mentioned were used in the qRT-PCR assay (Cora et al. 2023).

Statistical Analysis

All experiments were carried out in triplicates and repeated two times. Descriptive statistics were presented as mean ± standard deviation and categorical data were number (n) and percentage (%).

The suitability of the data for normal distribution was evaluated with the Kolmogorov-Smirnov Test and skewness and kurtosis coefficients. There are studies in the literature stating that variables with skewness and kurtosis coefficients between -3 and +3 meet the assumption of normality (Shao, 2002; George & Mallery, 2010; Hair et al. 2013; Tabachnick & Fidell, 2013).

In this study, besides the Kolmogorov-Smirnov test, variables with skewness and kurtosis coefficients between -1.5 and +1.5 were analyzed as having a normal distribution (Tabachnick & Fidell, 2013). The statistical significance of the anti-biofilm activity of propolis on *P. aeruginosa* PAO1 strain was determined using a one-way analysis of variance (ANOVA) test followed by Tukey's post hoc test for multiple comparisons. The *p* values were evaluated at α= 0.05 significance level, two-tailed, and 95% confidence interval.

All figures were visualized in the Microsoft Excel program and statistical analyses were performed in the IBM SPSS 23.0 (IBM SPSS Corp.; version number: 8.5.0.0021; Karadeniz Technical University, Türkiye) version (George & Mallery, 2016). Experimental (post hoc; retrospective; posterior) power analysis was performed in the study to justify the sample size. With alpha (the probability of a Type I error) = 0.05 significance level and high-level effect size (f=0.6), the power of the study (1 − β) was calculated as 0.80. The post hoc achieved power of the study was calculated in the G*Power 3.0.10 program environment.

Furthermore, The percentage of viability (%V) was determined according to the following mathematical equation (Queiroga et al. 2023):

$$\%V = ((Abs(Sample) - Abs(Blank))/(Abs(Control) - Abs(Blank))) \times 100$$

RESULTS

TPC and TFC of Extracts

The highest amount of TPC and TFC was found in Uzungöl propolis water extract with 20.47± 1.46 mg GAE g^{-1} sample, and 2.10±0.22 mg QUE g^{-1} sample, respectively. The TFC and TPC of all studied propolis extracts were summarized in Table 1.

Table 1. Total phenolic content and total flavonoid content of propolis extracts.
Tablo 1. Propolis ekstraktlarının toplam fenolik ve toplam flavonoid içerikleri

Sample	TPC	TFC
	(mg GAE g^{-1})	(mg QUE g^{-1})
Pazar Propolis	5.87± 0.36	0.48±0.04
Ardahan Propolis	6.08± 0.53	0.68±0.03
Uzungöl Propolis	20.47± 1.46	2.10±0.22

Mean ± Standard Deviation

Biological Activity Assay Results of Propolis Extracts

In the agar well diffusion method, the suppressive activity of water extracts of propolis against any of the tested microorganisms was not detected. It was also observed that propolis extracts did not have anti-swarming and anti-quorum sensing effects. However, in the anti-biofilm test performed with the *P. aeruginosa* PAO1 strain, it was determined that the Pazar and Uzungöl of the extracts had strong anti-biofilm activity and was found statistically significant (F=37.08, *p*=0.001). It was determined that Ardahan propolis had anti-biofilm activity but did not show as strong activity as other extracts. Anti-biofilm activity of extracts was shown in Figure 1. One-way ANOVA for anti-biofilm activity between groups was shown in Table 2.

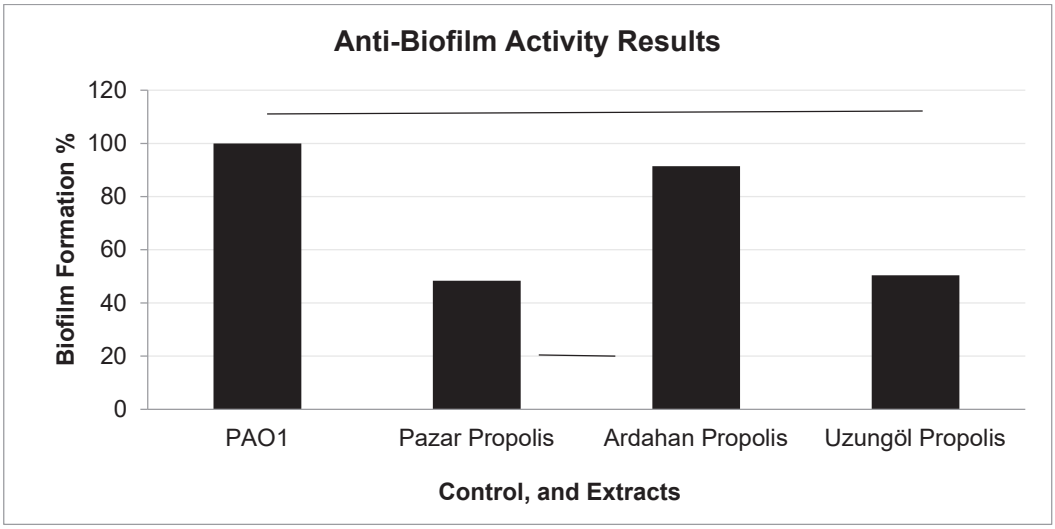

Figure 1. Anti-biofilm activity of water extracts of propolis samples. PAO1; positive control. *Asterisks indicate statistically significant differences (one-way ANOVA) between each concentration of biofilms (*p<0.05, ** p < 0.001).*

Şekil 1. Propolis su ekstraktlarının anti-biyofilm aktivitesi. PAO1; pozitif kontrol. Yıldız işaretleri, her biyofilm konsantrasyonu arasındaki istatistiksel olarak anlamlı farklılıkları (tek yönlü ANOVA) gösterir (**p*<0,05, ** *p* <0,001).

Table 2. One-way ANOVA for anti-biofilm activity between groups.

Tablo 2. Gruplar arası biyofilm aktivitesinin One-way ANOVA sonuçları.

	n	Mean±SE	Lower Bound	Upper Bound	*p*
P. aeruginosa PAO1	6	100±1.40	96.39	103.62	0.001
Pazar propolis	6	47.75±6.03	32.26	63.24	
Ardahan propolis	6	92.54±5.54	78.31	106.78	
Uzungöl propolis	6	49.81±3.64	40.46	59.16	
Total	24	72.53±26.52	61.33	83.72	

SE: Standard Error

For multiple comparisons, Tamhane's test of significance revealed a highly statistically significant difference between group PAO1 and Pazar and between group PAO1 and group Uzungöl (*P*<0.05) as depicted by Table 3.

Table 3. Multiple comparisons for anti-biofilm activity between groups.

Tablo 3. Anti-biyofilm aktivitesi için gruplar arasındaki çoklu karşılaştırmalar.

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	*p*
P. aeruginosa PAO1	Pazar propolis	52.25*	6.19	0.001
	Ardahan propolis	7.46	5.71	0.811
	Uzungöl propolis	50.19*	3.90	0.001
Pazar propolis	*P. aeruginosa* PAO1	-52.25*	6.19	0.001
	Ardahan propolis	-44.79*	8.18	0.002
	Uzungöl propolis	-2.06	7.04	1
Ardahan propolis	*P. aeruginosa* PAO1	-7.46	5.71	0.811
	Pazar propolis	44.79*	8.18	0.002
	Uzungöl propolis	42.73*	6.62	0.001
Uzungöl propolis	*P. aeruginosa* PAO1	-50.19*	3.90	0.001
	Pazar propolis	2.06	7.04	1
	Ardahan propolis	-42.73*	6.62	0.001

* The mean difference is significant at the 0.05 level.

Cytotoxicity Assay Results

Trypan blue assay results

Since there were three wells from each dilution, the number of cells in the wells was counted at the end of 24, 48, 72, and 96 h and averaged. It was observed that water extracts of propolis at concentrations of 800 µg mL^{-1} and below contained similar numbers of cells to the control. Therefore, it was determined that these concentrations did not have cytotoxic effects on Vero cells. The evaluation of the number of cells in the trypan blue test was given in Figure 2 (a, b, c).

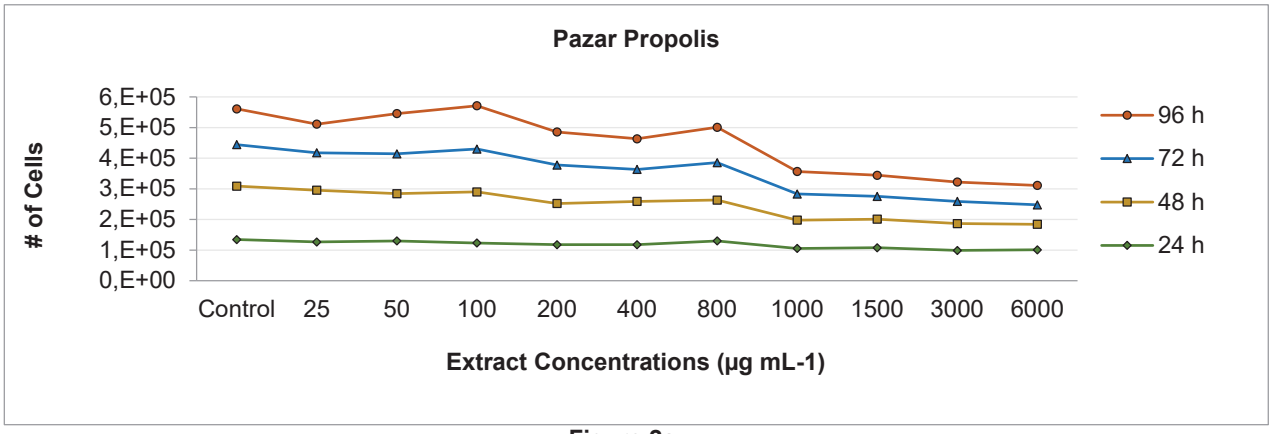

Figure 2a.

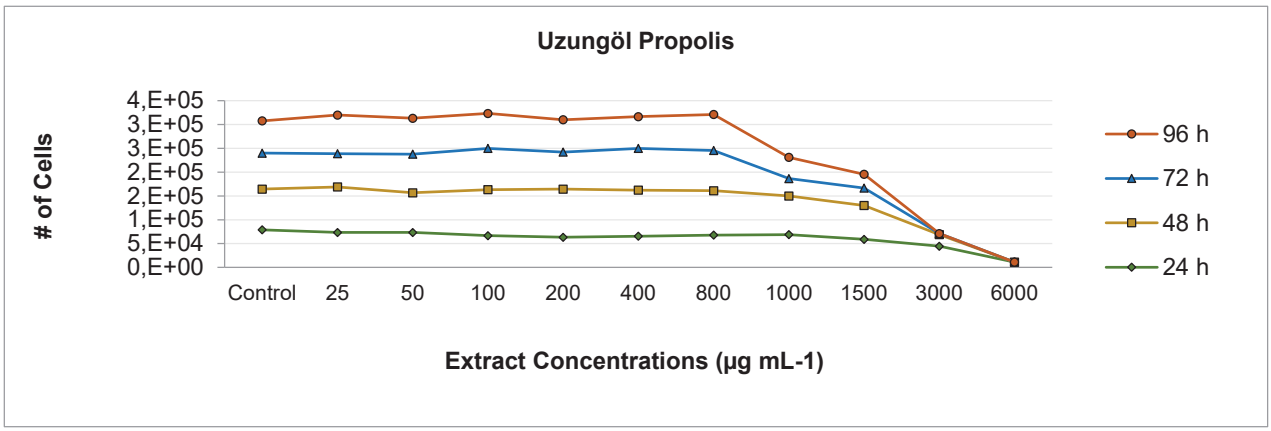

Figure 2b.

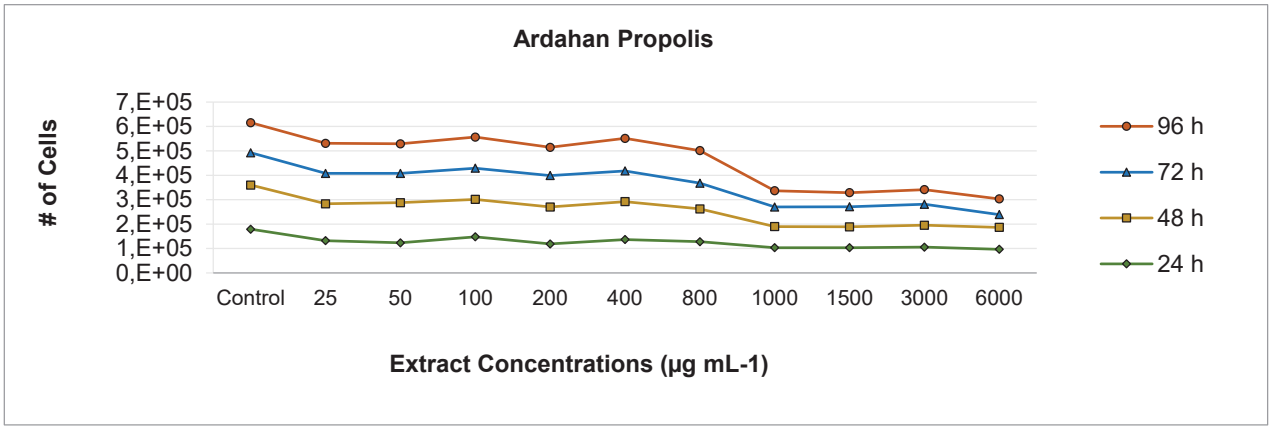

Figure 2c.

Figure 2a, b, c. The cytotoxic effect of Pazar (2a), Uzungöl (2b), and Ardahan (2c) propolis water extracts on Vero cells by trypan blue staining method.

Şekil 2a, b, c. Pazar (2a), Uzungöl (2b) ve Ardahan (2c) propolisi su ekstraktının tripan mavisi boyama yöntemi ile Vero hücreleri üzerine sitotoksik etkisi.

MTT assay results

The viability of Vero cells incubated with dilutions of propolis was evaluated at the end of 24, 48, 72, and 96 h. Cell viability in the wells containing the propolis dilutions was calculated as % by evaluating compared to the control. Since there were three wells from each dilution and control, the average of the viability rate in the wells was calculated. It was determined that no cytotoxic effect was observed in the water extracts of Pazar and Ardahan propolis at concentrations of 800 µg mL^{-1} and below. However, it was observed that Uzungöl propolis had no cytotoxic effect at 1500 and below concentrations. The results were summarized in Figure 3 (a, b, c).

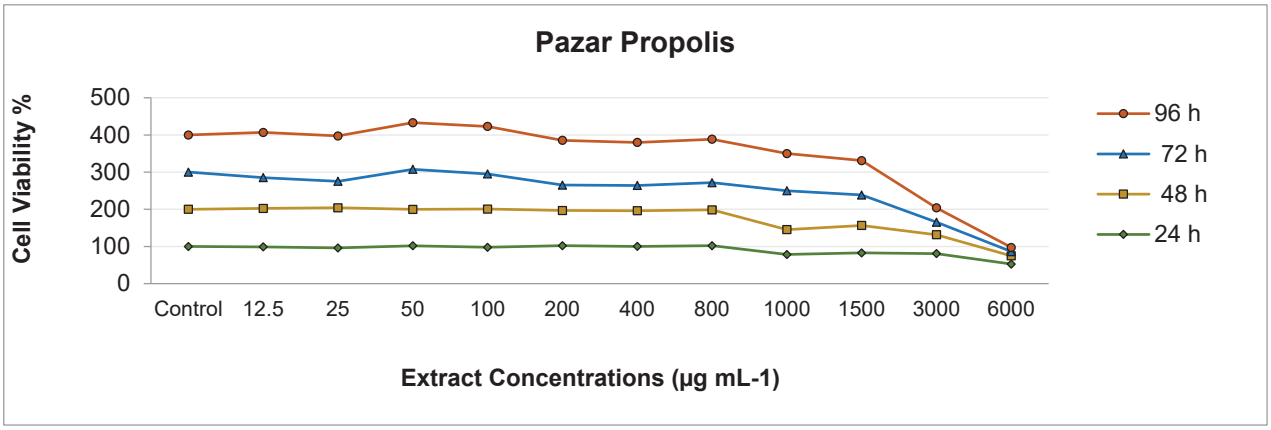

Figure 3a.

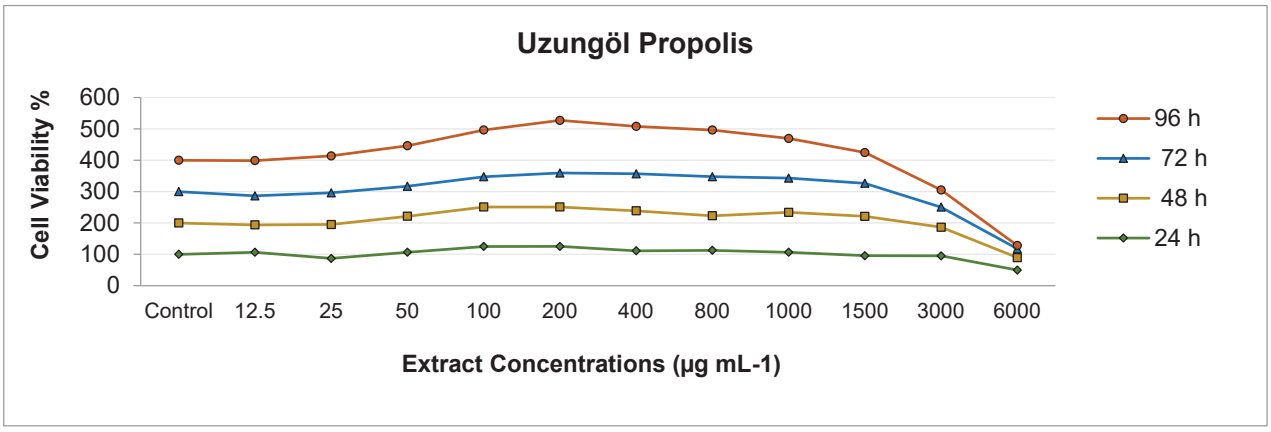

Figure 3b.

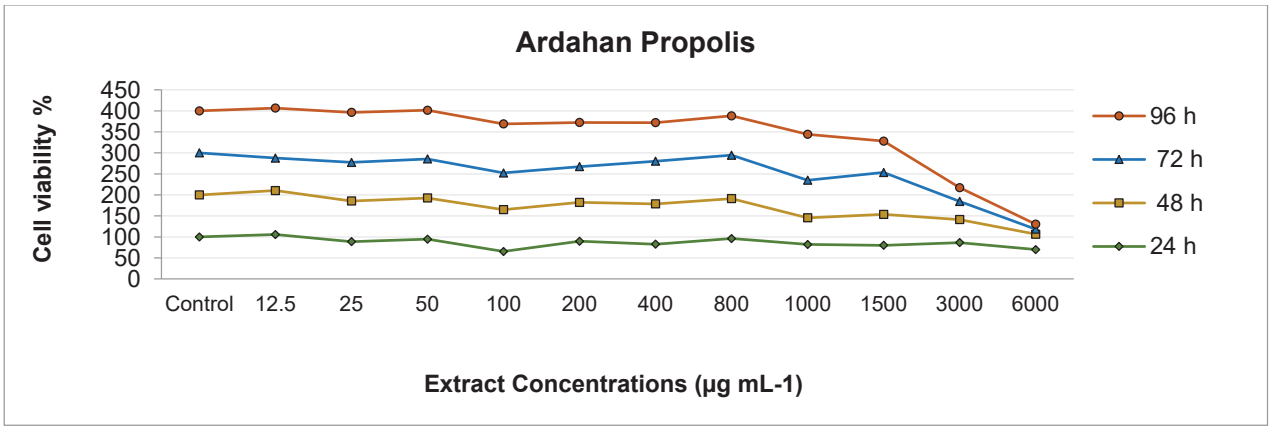

Figure 3c.

Figure 3 a, b, c. The cytotoxic effect of Pazar (3a), Uzungöl (3b), and Ardahan (3c) propolis water extract on Vero cells by MTT method.

Şekil 3 a, b, c. Pazar (3a), Uzungöl (3b) ve Ardahan (3c) propolis su ekstraktının MTT yöntemi ile Vero hücrelerine sitotoksik etkisi.

Antiviral Activity Assay Results

MTT assay results

When HSV-1 is produced in Vero cells, it creates a lytic infection and causes the cells to lyse. However, if acyclovir is added to the breeding medium, the reproduction of the virus is prevented, the existing cells in the medium are not destroyed, and continue to grow. It was noticed that the viability rate of cells in wells containing only viruses decreased as the number of viruses increased. The viability rate of the cells was calculated as 4.8%, 8.2%, and 7% in the wells containing the virus at 1 $TCID_{50}$, 10 $TCID_{50}$, and 100 $TCID_{50}$, respectively. The viability rate in the wells containing acyclovir at 25 µg mL^{-1} concentration was 92.8%, 72.8%, and 36.8% in the wells containing 1 $TCID_{50}$, 10 $TCID_{50}$, and 100 $TCID_{50}$, respectively. Because the viability rate of the cells in the water extracts was similar to the viability rate in the cells containing only the virus, it was understood that the water extracts did not have an antiviral effect on HSV-1. MTT assay results of antiviral activities of water extracts of propolis was shown in Figure 4.

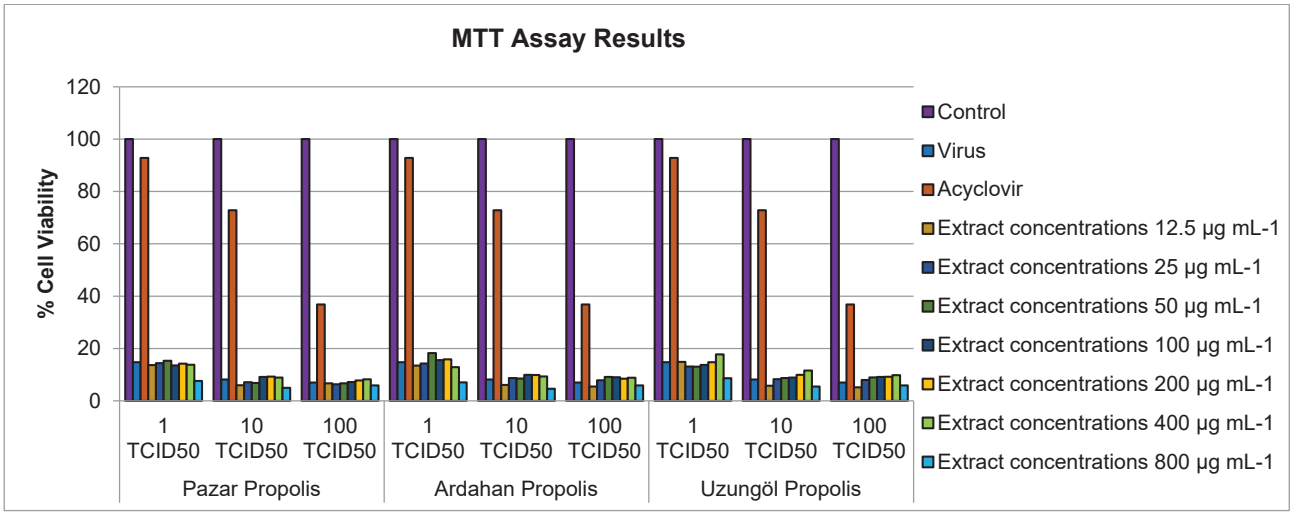

Figure 4. The antiviral activity of propolis samples against HSV-1 with MTT assay. Control; Wells that contain only Vero cells used as reproductivity control, Virus; wells that Vero cells infected with virus used as negative control, Acyclovir; positive control.

Şekil 4. Propolislerin HSV-1 üzerine antiviral etkisinin MTT yöntemi ile arştırılması. Konrol; sadece Vero hücresi içeren kuyucuklar üreme kontrolü olarak kullanıldı, Virüs; virüs ile enfekte edilmiş Vero hücreleri negatif kontrol olarak kullanıldı, Asiklovir; pozitif kontrol.

qRT-PCR assay results

The qRT-PCR method was used to evaluate the effect of propolis extracts on the reproduction of the virus. In this method, the virus was added to the wells of the plate containing confluent Vero cells. Only virus-infected cells were used as negative control and acyclovir was used as positive control. The amount of viral DNA in the samples was determined using standards that included a known amount of virus. It was observed that there was no difference between the water extracts of propolis and the negative control, therefore the water extracts did not affect the reproduction of the virus. qRT-PCR assay results of antiviral activities of water extracts of propolis were shown in Figure 5.

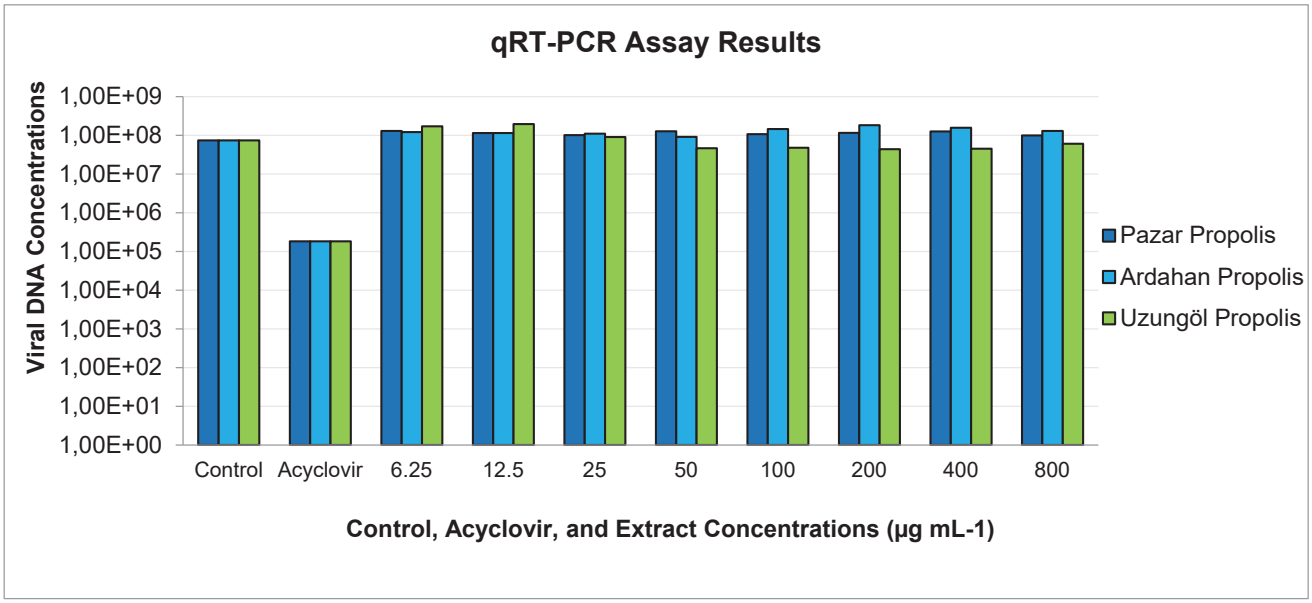

Figure 5. The antiviral activity of propolis samples against HSV-1 with qRT-PCR assay.

Şekil 5. Propolis örneklerinin qRT-PCR testi ile HSV-1 üzerine antiviral etkisi.

DISCUSSION

TFC and TPC values of propolis vary depending on many parameters, therefore these values should be revealed in every study. The TPC and TFC values of propolis samples In Egyptian brown propolis water extract, the total phenolic content was found as 210.33 mg GAE g-1 (Ibrahim & Alqurashi, 2022). Salleh et al. found the TPC as 7.60-13.21 mg GAE mL-1 in three different Malaysian stingless bee propolis water extracts (Salleh et al. 2021). In a study on several Indonesian stingless bee propolis, TPC was found to range between 10 and 28.65 mg GAE mL-1 (Fikri et al 2019). Kubiliene et al. demonstrated that the TPC of Lithuania propolis water extract was 1.2 mg GAE mL-1 (Kubiliene et al. 2018). Abogharip et al. state that the TPC of Egyptian propolis water extract was 5.23 mg GAE g-1 (Abogharip et al 2023). In a study conducted by Omer et al., the TPC value of water propolis extract from the West Blacksea region of Türkiye was found to range between 9.90 and 13.99 mg GAE g-1 (Omer et al. 2023). In the current study, the TPC values of water extract of propolis samples were investigated and it was found as 5.87, 6.08, and 20.47 mg GAE g-1 in Pazar, Ardahan,and Uzungöl propolis, respectively. The TPC values were found to be compatible with most of the studies in the literature.

TFC in three different Malaysian stingless bee propolis water extracts was investigated using rutin as the standard reference. TFC in the samples was found between 34.17-34.53 mg rutin equivalent (RE) mL-1 (Salleh et al. 2021). The TFC in several Indonesian stingless bee propolis was found to range between 1.42 and 1.80 mg QUE g-1 (Fikri et al. 2019). The TFC of Egyptian propolis water extract was found as 5.55 mg QUE g-1 (Abogharip et al. 2023). Omer et al. stated that the TPC value of water propolis extract from the West Blacksea region of Türkiye was found to range between 2.25 and 3.09 mg QUE g-1 (Omer et al. 2023). In the current study, the TFC values of water extract of propolis samples were detected as 0.48, 0.68, and 2.10 mg QUE g-1 in Pazar, Ardahan, and Uzungöl propolis, respectively.

The biological and pharmacological properties of propolis have been revealed in many studies (Milojkovic, 2018). Omer et al. state that in antibacterial studies water extract from the West Black Sea region of Türkiye had activity against *P. aeruginosa*, *E. faecalis*, *S. enterica*, *L. monocytogenes*, and *B. cereus* (Omer et al.2023). In a study, it was demonstrated that Romanian propolis water extract has weak antimicrobial activity against *C. albicans*, *B. subtilis*, and *E. coli* (Nichioti et al, 2023). While Elgin et al. suggest that Turkish propolis water extract inhibits the growth of *E. coli*

BW25113 by affecting nucleic acid metabolism, Campos et al. state that the antimicrobial activity of Brazilian propolis water extract was not observed against *E.coli* and *S. aureus* (Elgin et al.2023; Campos et al. 2020). In the current study antimicrobial activity of water extract of propolis samples was investigated against 14 microorganisms. However, no activity was found against any of the microorganisms studied.

The biofilm is an aggregation of bacteria covered with a self-generated matrix. This is a strategy for bacteria to survive during unsuitable living conditions. The biofilm allows bacteria to escape from the immune system and makes bacteria 1000 times more resistant to antibiotics. *P. aeruginosa* is an opportunistic Gram-negative bacterium that forms biofilm. It is known that the biofilms of *P. aeruginosa* are responsible for 90% of wound infections and complicate the healing of wounds. Therefore, it is important to develop new therapeutic strategies and alternative methods against *P. aeruginosa* biofilms (Thi et al 2020). Among the three propolis water extracts Pazar and Uzungöl propolis extracts significantly inhibited *P. aeruginosa* biofilm formation, while Ardahan propolis inhibited biofilm formation less than others. One of the limitations of our study is the small sample size, which is insufficient to determine the average biofilm activity across propolis levels. Therefore, these results cannot be used to generalize the results to the entire population. Further research with a larger sample size may contribute to our knowledge as an indicator of changes in the anti-biofilm activity of propolis.

In the current study, antiviral activity against HSV-1 was not observed in any of the extracts prepared with water. Similarly, antimicrobial activities of the extracts were not found. However, two of the extracts have been shown to have strong antibiofilm activity. It is thought that the reason for this may be the fact that the components found in propolis are seen in the extract in different solvents at different rates. Kara et al. stated that some phenolic acids (such as gallic acid and protocatechuic) present in propolis can completely pass into aqueous solutions but may not be present in ethanolic extracts. They also showed that these two phenolic acids were mostly obtained through aqueous extracts and were not detected in 70% ethanol by the HPLC–PDA assay (Kara et al. 2022).

Conclusion: In this study, it has been shown that the water extracts of studied propolis samples cannot play a significant role in the development of antibacterial and antiviral agents or in increasing their effectiveness, however, they can be evaluated in the treatment of *P. aeruginosa* biofilms, which prolong the healing process by forming a biofilm in wounds. As a future study, a more detailed investigation can be carried out with different extraction methods and increased number of samples to futher reveal the therapeutic effects of propolis.

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Ethics: This study was approved by the local Ethics Committee of Karadeniz Technical University School of Medicine (Protocol/plan code of the research: 2017-166).

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