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ARAŞTIRMA MAKALESİ **RESEARCH PAPER**

Antioxidant, Antimicrobial and Anti-Quorum Sensing Activities of *Usnea filipendula* and *Viscum album*[*]

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Abstract: Many plants contain a variety of bioactive components. Therefore, it is important to know the bioactive properties of plant materials in order to be a reference for later researchers. In this study, it was investigated the antioxidant, antimicrobial and anti-quorum sensing activities of *Usnea filipendula* and *Viscum album*'s methanol extracts. To determine the antioxidant properties of the extracts; total phenolic, flavonoid and condensed tannin contents and ferric reducing antioxidant power analyses were performed. The antibacterial potential of plant extracts was tested by agar well diffusion method against *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Listeria monocytogenes*, *Candida parapsilosis* and *Candida albicans* microorganisms. Anti-quorum sensing activity was investigated on *Chromobacterium violaceum* bacteria. The highest total phenolic and ferric reducing antioxidant power was determined in *U. filipendula* extract. This extract inhibited the growth of *S. aureus, K. pneumonia* and *L. monocytogenes* microorganisms. The highest flavonoid and condensed tannin was observed in *V. album extract*. This extract was also able to prevent the growth of *K. pneumonia* and *L. monocytogenes*. None of the extracts showed anti-quorum sensing activity.

Keywords: Antioxidant, antimicrobial anti-quorum sensing, *Usnea filipendula*, *Viscum album.*

Usnea filipendula ve *Viscum album*'un Antioksidant, Antimikrobiyal ve Çoğunluğu Algılama İnhibisyonu Aktiviteleri

Öz: Birçok bitki çeşitli biyoaktif bileşenler içerir. Bu nedenle, daha sonraki araştırmacılar için referans olması amacıyla bitki materyallerinin biyoaktif özelliklerini bilmek önemlidir. Bu çalışmada, *Usnea filipendula* ve *Viscum album*'ün metanol ekstrelerinin antioksidan, antimikrobiyal ve çoğunluğu algılama inhibisyonu aktiviteleri incelenmiştir. Ekstraktların antioksidan özelliklerini belirlemek için; toplam fenolik, flavonoid ve kondanse tanen içerikleri ve demir indirgeyici antioksidan gücü analizleri yapılmıştır. Bitki ekstraktlarının antibakteriyel potansiyeli, *Staphylococcus aureus, Escherichia coli, Enterococcus faecalis, Pseudomonas aeruginosa, Salmonella typhimurium, Klebsiella pneumoniae, Proteus mirabilis, Listeria monocytogenes, Candida parapsilosis* ve *Candida albicans* mikroorganizmalarına karşı agar kuyucuk yöntemi ile ölçülmüştür. *Chromobacterium violaceum* bakterileri üzerinde ise çoğunluğu algılama inhibisyonu aktivitesi araştırılmıştır. En yüksek toplam fenolik ve demir indirgeyici antioksidan gücü, *U. filipendula* özütünde belirlenmiştir. Bu özüt, *S. aureus, K. pneumonia* ve *L. monocytogenes* mikroorganizmalarının büyümesini inhibe etmiştir. En yüksek flavonoid ve kondanse tanen *V. album* ekstraktında gözlenmiştir. Bu ekstre, *K. pneumonia* ve *L. monocytogenes*'in büyümesini önleyebilmiştir. Ekstraktların hiçbiri çoğunluğu algılama mekanizmasını inhibe edememiştir.

Anahtar sözcükler: Antioksidan, antimikrobiyal, çoğunluğu algılama inhibisyonu, *Usnea filipendula*, *Viscum albüm.*

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INTRODUCTION

The plants can be described as 'a gift of nature' because they are therapeutic. Many kinds of them have played an active role in the treatment of different diseases for centuries (Farombi, 2003). Although many drugs or medical methods have been applied to treat diseases by the development of technology and science, some governments have made it compulsory to consume natural products for many aims (Ertürk et al., 2004). Plants produce secondary metabolites in their bodies and it has been proven by many scientific studies that secondary metabolites have antioxidant, antimicrobial, anticancer, antidiabetic, etc. properties (Rao & Kingston, 1982; Mensor et al., 2001; Srinivasan, 2005; González-Lamothe et al., 2009; Kılıçkaya Selvi et al., 2019).

Some chemical reactions in body tissues produce free radical molecules under certain conditions. Free radicals occur naturally in every step of the duration. These molecules cause metabolic problems and play a role in bringing damage to tissues. However, these unstable electron-laden chemicals are largely destroyed or removed by natural antioxidant defence systems normally found in the body. The use of antioxidant plants/foods supports the body's antioxidant defence mechanism (Gate et al., 1999; Srinivasan, 2005). Antioxidants can be considered as two major groups; synthetic and natural, generally. Despite the synthetic ones have been used in many places, there is still suspicion about their reliability (Ho & Shahidi, 2005; Taghvaei & Jafari, 2015) because of their possible toxic/side effects especially during long-term intake (Taghvaei & Jafari, 2015). On the other hand, it has been indicated that many natural additives have more antioxidants property and thermal stability than the synthetic ones.

Increased technology, unlimited consumption demand and pollution have also increased/diversified the disease. As it is known some plants species have been used to overcome the microorganisms that cause diseases. The therapeutic effects of plants are related to the synergistic effect of a large number of compounds. It has been reported that the herbal combinations provide more effective treatment against the resistance of microorganisms that are difficult to kill with a single antibiotic (Sree et al., 2010; Nazri et al., 2011).

While quorum sensing (QS) is the communication system between the bacterial cells by the signaling molecules, anti-quorum sensing is the name of the stopping this communication mechanism (Alvarez et al., 2012). Over the past few years, QS has become a very extensive field of research because of its promising results for the utilizations in industry, medicine and biotechnology (Taganna et al., 2011). According to research of some scientists that "plants are rich natural resource of quorum sensing agents" (Choo et al., 2006; Kohand Tham, 2011; Mohamed et al., 2014; Al-Haidari et al., 2016). The most likely benefit of the QS researchers is to disrupt the signal communication between microorganisms' communities and to keep their growth under control. It was seen that there is not much study in the literature about the quorum sensing-disrupting activity of plants. Therefore, this study will reveal whether the lichen and mistletoe plants investigated in this article have anti-quorum sensing activity or not.

It has been known that the lichens and mistletoe have some special bioactive properties. Bioactive natural products obtained from lichens have been utilized for medicinal and cosmetic purposes. Six lichen species involving usnic acid in various amounts were found to be effective against various (Cansaran et al., 2006; Yıldız, 2017). On the other hand, biologically active components of mistletoe have been reviewed (Ochocka and Piotrowski, 2002). Ertürk et al. (2004) investigated anti-microbial properties of mistletoe (*Viscum album* L.) against a fungus and six bacteria species. They explained that different concentration of n-hexane extract of mistletoe was effectual against micro-organisms analyzed.

The phenolic components of plant origins have attracted attention because of their useful and nutritional properties including antioxidant and antimicrobial capacity, in recent years (Bubonja-Sonje et al., 2011). The plants that are found in abundant quantities and inexpensive such as lichen or mistletoe need to be investigated, firstly. In this study it was examined the antioxidant, antimicrobial and antiquorum sensing activities of *Usnea filipendula* and *Viscum album*'s methanol extracts.

MATERIAL and METHODS

Lichen (*Usnea filipendula*) was collected from wild areas in Trabzon province, Tonya district (Fig. 1). Mistletoe (*Viscum album* L.) was collected from *Pinus sylvestris* host tree in Trabzon province, Sürmene district (Fig. 2), located in the north-eastern of Turkey (Table 1). The plants were brought to laboratory for extraction process.

Table 1. Investigated plant samples.

Sample	Scientific classification	Collected place	
		Province	District
Mistletoe	*Viscum album* L.	Trabzon	Tonya
Lichen	*Usnea filipendula*	Trabzon	Sürmene

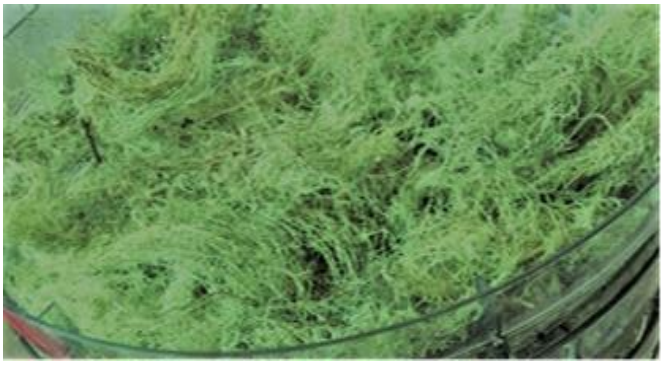

Figure 1. Lichen (*Usnea filipendula*)

Figure 2. Mistletoe (*Viscum album*)

Sample Preparation: Whole of lichen and twigs of mistletoe were used for the analyses. Samples were dried in an oven at 60°C at 24 hours before grinding. A laboratory scale Wiley mill was utilized to grind. Approximately 5 g powdered samples were dissolved in 50 mL methanol (99%). The mixture was continuously stirred using a shaker (Heidolph Promax 2020, Schwabach, Germany) at room temperature for 24 h. Particles were removed using Whatman No. 4 filter paper (pore size 20-25 µm). Then the solutions were filter sterilized using 0.45 µm hydrophilic polyvinylidene fluoride (PVDF) filters.

Antioxidant Properties

Ferric Reducing Antioxidant Power (FRAP): The antioxidant capacity was determined using ferric reducing antioxidant power (FRAP). This method is based on the reduction of tripyridyltriazine complex (Fe (TPTZ)$^{3+}$) to blue colored Fe(TPTZ)$^{2+}$ by antioxidants in acidic medium (Benzie and Strain, 1996). FRAP values were expressed in wet weight of the samples as µmol of ferrous equivalent Fe (II) per g of sample.

Determination of Phenolic Contents: The polyphenolic contents of the methanol extracts were evaluated by three different ways; total phenolic contents (TPC), total flavonoids (TF) and condensed tannin (CT) contents. For the determination of the total phenolic contents, the Folin-Ciocalteau procedure was employed and gallic acid was used as standard (Slinkard & Singleton, 1977). The results were expressed as mg Gallic Acid Equivalent (GAE) per g of methanolic extracts.

Determination of Flavonoid Contents: The concentration of the total flavonoid content in the methanol extracts was measured using a spectrometric assay. The total flavonoid concentration was expressed as mg equivalents of quercetin (QE) per g of sample (Fukumoto & Mazza, 2000).

Determination of Condensed Tannins Contents: ***T***he concentration of condensed tannins was determined according to the method previously used by Julkunen-Titto (Julkunen-Tiitto, 1985). The results were expressed as mg catechin equivalent (CE) per g of sample.

Antimicrobial Activity: The extracts were tested for antimicrobial activity by agar-well diffusion method according to the Clinical & Laboratory Standards Institute (CLSI) guidelines (Wayne, 2002) against *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* ATCC 14028, *Klebsiella pneumoniae* ATCC 13883, *Proteus mirabilis* ATCC 7002, *Listeria monocytogenes* ATCC 43251, *Candida parapsilosis* ATCC 22019 and *Candida albicans* ATCC 10231. The microorganisms were obtained from Department of Medical Microbiology, Faculty of Medicine, Karadeniz Technical University, Trabzon, Turkey. Bacteria and yeast were cultured in Luria Bertani (LB) and Sabouroud Dextrose agar (LABM, UK), respectively. Fresh cultures (18 h) of bacteria and yeast were used to make suspension in 5 mL of sterile isotonic sodium chloride and turbidity was adjusted to 0.5 McFarland. Agar plates were filled with suspension and 0.6 cm agar wells were cut out using a sterile pipette tip. 50 microliters of extracts were transferred into each agar well and cultures were incubated at 37°C for 24 hours. Ampicillin, gentamicin, cefotaxime, tetracycline and amphotericin B solutions and DMSO were used as positive and negative controls, respectively. The antimicrobial activity was determined by visual inspection and measurement of the diameter of inhibition zones around the agar-wells. The minimal inhibitory concentration (MIC) of the extracts showing a positive antimicrobial activity was determined using the liquid microdilution test method. The well with the lowest concentration that did not show any microbial growth was considered to be the MIC of the tested extract.

***Anti-Quorum Sensing Activity*:** Anti-quorum sensing activity was determined using microdilution method as described for the antimicrobial activity test above (Damte et al., 2013). The anti-QS activity of the extracts has been tested against *Chromobacterium violaceum* ATCC 12472, a violacein-producing strain. Briefly, MIC of each extract was determined as described above and sub-MIC concentrations were used for the inhibition of pigment production of *C. violaceum*. For anti-QS assay, to the fresh culture of the strains in LB broth was added for each extract and incubated for 24 h. At the end of the incubation, 1 mL of culture was centrifuged and pellet was resuspended in 1 ml of DMSO and vortexed at the high speed for pigment extraction. Supernatant was removed and absorbance values of the pigments were determined at OD 585 nm using a microplate reader (Damte et al., 2013; Norizan et al., 2013). Vanilla extract was used as positive control (Choo et al., 2006).

Statistical Analysis: The data were presented as means and standard deviations of three replicates for total phenolic content and antioxidant properties and ten replicates for metal composition analyzed by using Statistical Package for Social Sciences (SPSS version 23.0). The data were analyzed by ANOVA and tests of statistical significance were performed using Duncan's multiple range tests.

RESULTS and DISCUSSION

Antioxidant Activity: In this study, antioxidant capacity was determined using ferric reducing antioxidant power (FRAP) method. The results are given Figure 3.

As shown in Figure 3, antioxidant capacity of *U. filipendula* (54.4 µmol$FeSO_4.7H_2O$/g) was found higher than antioxidant capacity of *V. album* (51.45 µmol$FeSO_4.7H_2O$/g). Vicas et al. (2009) investigated the hydrophilic and lipophilic antioxidant activities of *V. album*. For this purpose, they collected the *V. album* leaves and stems from five host trees (*Acer campestre*, *Malus domestica*, *Fraxinus excelsior*, *Populus nigra* and *Robinia pseudoacacia*) and determined the antioxidant activity of methanol and acetone extracts of all collected samples. They reported that methanol extract of *V. album* leaves collected from *M. domestica* exhibited the highest activity.

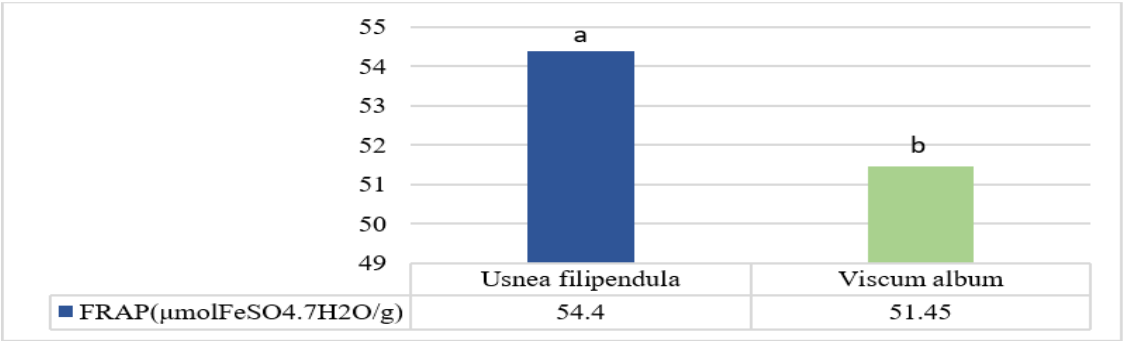

Figure 3. Antioxidant capacity of samples

Önay-Uçar et al. (2006) found that *V. album* living in different trees had different antioxidant activity. So, they reported that *V. album* extract's antioxidant capacity can vary depending on the plant's harvest time and the configuration of the main tree at the same time. In this study, *V. album* was collected from *P. sylvestris* host tree. Therefore, it can be concluded that antioxidant capacity of *V. album* collected from different host tree can be differ from our results. In a study, the reducing power activity of *V. album* crude alcoholic extract was reported at 0.10 equivalent 1mM $FeSO_4$ by (Papuc et al., 2010). It was noticed that extracts of *V. album* obtained from cashew tree demonstrated a stronger Fe chelating ability (Oluwaseun & Ganiyu, 2008).

Oran et al. (2016) studied the antioxidant capacity of different lichen species' (*Usnea intermedia*, *Usnea filipendula* and *Usnea fulvoreagens*) methanol and ethanol extracts. They reported that methanol extracts of lichen species showed higher antioxidant capacity from the ethanol extracts.

All the previous studies and the present study are evaluated together, it can be concluded that the antioxidant capacity is affected many factors such as extract type and method, extract concentration, plant harvesting time, host tree (for mistletoe) etc.

Total Phenolic Contents: In this study, the polyphenolic contents of the methanol extracts were evaluated by three different ways; total phenolic content, total flavonoids and condensed tannin contents. The polyphenolic contents of the methanol extracts of samples are given in Figure 4.

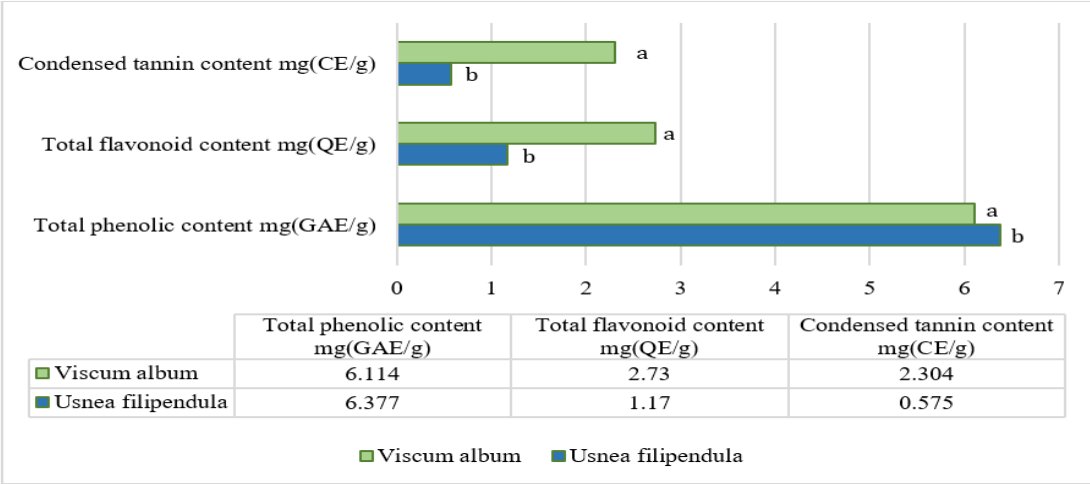

Figure 4. The polyphenolic contents of the methanol extracts of samples.

In this study, total phenolic contents of *V. album* and *U. filipendula* was determined as 6.114 and 6.377 mg (GAE)/g, respectively (Fig. 4) and the results were found to be statistically significant each other ($p<0.05$).

In a study, total phenolic contents of *V. album* methanol extract were determined as 19.43 mg GAE/g dried weight (Sengul et al., 2009). Vicas et al. (2009) reported that the total phenolic content of *V. album* methanolic and acetonic extracts' which collected from 5 host tree were between 0.40-0.65 mg GAE/g fresh weight and 0.002-0.015 mg GAE/g fresh weigh, respectively. It can be concluded that the total phenolic content of the mistletoe collected from different host trees is also different. (Papuc et al., 2010) informed that the polyphenols of *V. album* ethanolic extracts as 6.33 mg/g dry plant. Our results are comparable with just mentioned study. Total phenolic content of *U. filipendula* acetone, ethanol and methanol extracts was reported that 329.7, 197.4 and 291.5 mg GAE/100 g^{-1} of dried lichen, respectively (Oran et al., 2016).

Total Flavonoid Contents: In this study, total flavonoid content of *V. album* and *U. filipendula* was calculated as 2.73 and 1.17 mg (QE/g), respectively (Fig. 4). In a previous study, it was (Papuc et al., 2010) reported that the flavonoid content of *V. album* ethanolic extracts was 9.72 mg/g dry plant. It can be said that the solvent type affects the flavonoid content. There are some studies in the literature reported that some lichen species have important flavonoid contents (Kosanić et al., 2011).

Condensed Tannins Contents: In this study, condensed tannin content of *V. album* extract (2.304 mg (CE/g)) was found 4 times higher than the *U. filipendula* extract (0.575 mg (CE/g).

When all the polyphenolic contents and antioxidant capacity of methanol extracts of studied samples are evaluated together, the higher total phenolic content and antioxidant capacity was determined in *U. filipendula* extract while the higher total flavonoid content and condensed tannin content was determined in *V. album* extract.

Antimicrobial Activity: The antimicrobial activity of studied samples and used antibiotics are given Table 2.

Table 2. Antimicrobial activity and used antibiotics

Bacteria isolates	Agar Well Diffusion (mm zone diameter)						
	V. album	*U. filipendula*	Ampicillin	Gentamicin	Amphotericin B	Tetracycline	Cefotaxime
S. aureus	0	4	> 30	-	-	-	-
E. coli	0	0	16-17	-	-	-	-
P. aeruginosa	0	0	-	21-22	-	-	-
E. faecalis	0	0	>30	-	-	-	-
C. albicans	0	0	-	-	30		
C. parapsilosis	0	0	-	-	-	-	-
S. typhimurium	0	0	27	-	-	-	-
P. mirabilis	0	0	-	-	-	-	37
K. pneumoniae	2	1	-	-	-	-	-
L. monocytogenes	2	1	-	-	-	25	-

As can be seen in Table 2, *V. album* methanol extract inhibited *K. pneumoniae*, L. *monocytogenes* and *U. filipendula* methanol extract inhibited *S. aureus*, *K. pneumoniae* and L. monocytogenes microorganisms. Also, *V. album* extract was shown better antimicrobial zone than *U. filipendula* extract against *K. pneumoniae and L. monocytogenes* microorganisms.

Minimum inhibition concentration (MIC) values of extracts which show antimicrobial property are given in Table 3.

Table 3. Minimum inhibition concentration (MIC) values of extracts (µg/mL).

Bacteria used in the test	*V. album*	*U. filipendula*
S. aureus	-	312.5
K. pneumoniae	1250	1250
L. monocytogenes	625	625

The lower MIC value means the stronger antimicrobial effect of extract. In this study, *U. filipendula* methanol extract has the best antimicrobial activity against *S. aureus* microorganism with 312.5 µg/mL concentration. It can be said that *V. album* extract was more effective than *U. filipendula* extract, because when same MIC values of extract was tested (Table 3), *V. album* extract showed higher (twice times) antimicrobial activity (mm zone diameter) from *U. filipendula* extract (Table 2).

Sengul et al. (2009) reported that both methanol and aqueous extracts of *V. album* inhibited many organisms. Methanol extracts showed better antimicrobial activity than aqueous extracts. In a study, it was investigated the antimicrobial activity of different extracts (acetone, petroleum ether, ethyl acetate, chloroform, ethanol, methanol, water) of *V. album* collected from Rialy, Muzaffarabad Azad Jammu and Kashmir. According to the reported results all extracts inhibited many of studied microorganisms except from acetone and petroleum ether extracts (Hussain et al., 2011). In another study; it was investigated that antimicrobial activity of *V. album* against 6 bacteria and 1 fungus (*Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Proteus vulgaris* and *Candida albicans*). The results showed that the

different concentrations of n-hexane extract of mistletoe were effective against micro-organisms analyzed (Ertürk et al., 2003).

Oran et al. (2016) reported that the MIC values of all analyzed extracts ranged from 64 µg/mL to 512 µg/mL for all the bacterial strains and all the Fluoro quinolone-resistant Escherichia coli isolates (except for E101) were sensitive to the methanol extracts of the three *Usnea filipendula*. In another study, it was reported that *U. filipendula* have antimutagenic and antigenotoxic effects.

Antiquorum Sensing Activity: The communication mechanism between microorganisms has called "quorum sensing" (QS). Anti-QS activity charts of positive control (vanilla) and *U. filipendula* and *V. album* are given Figure 5-7, respectively.

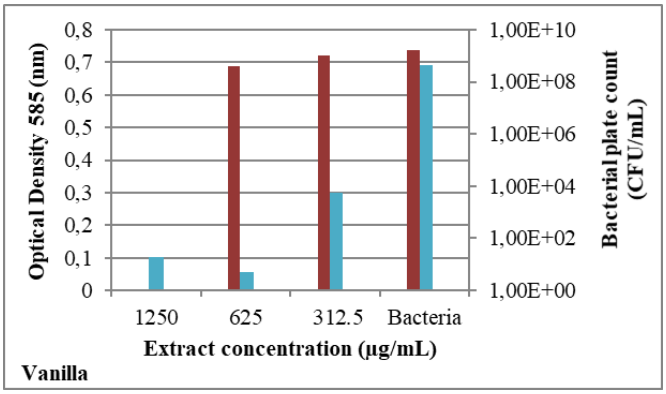

Figure 5. Anti-QS activity chart of vanilla.

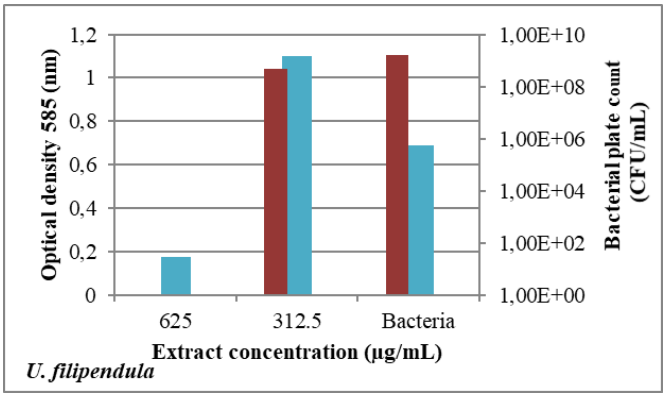

Figure 6. Anti-QS activity chart of *U. filipendula*

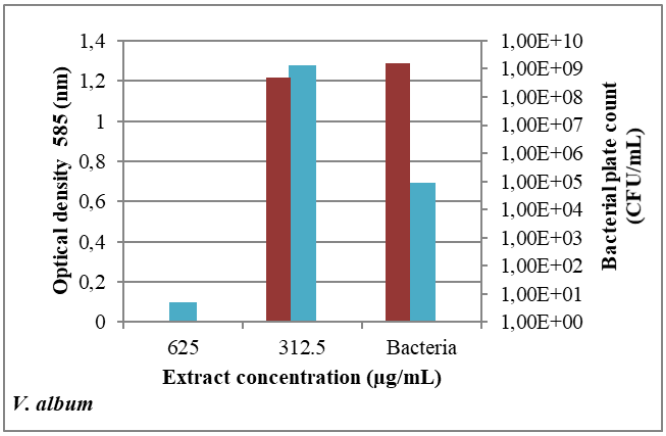

Figure 7. Anti-QS activity chart of *V. album*

Bacteria used in this study (*C. violaceum*) for anti-QS assay produces the purple pigment. If the extract we use is not killing bacteria or killing very little, the intensity of pigment production is not reduce, or if it is very low, it can be considered that used extract have anti-QS activity. A good functioning of this mentioned state is seen in the positive control extract (Fig. 5). Vanilla showed anti-QS activity from 312.5 to 625 µg/mL while inhibited the bacteria when 1250 µg/mL. In this study, both of *U. filipendula* and *V. album* inhibited the *C. violaceum* at 625 Mg/mL extract concentration so they did not show anti-QS activity.

Kenar et al. (2016) investigated that the methanolic and dicloromethanolic extracts of fruits, leaves, and stem of *V. album*. They used agar well and disc diffusion assay for anti-QS activity using *Chromobacterium violaceum* (CV12472 and CVO26) strains. They reported that the effect of *V. album* extracts on anti-biofilm and anti-QS was very effective over biofilms produced by pathogens and these extracts were good sources for new antimicrobial components.

CONCLUSION

In this study antioxidant, antimicrobial and anti-quorum sensing activities of *U. filipendula* and *V album*'s methanol extracts were investigated. As a result; the higher total phenolic content and antioxidant capacity was determined in *U. filipendula* extract. The higher total flavonoid content and condensed tannin content was determined in *V. album* extract. *U. filipendula* methanol extract has the best antimicrobial activity against *S. aureus* microorganism with 312.5 µg/mL concentration. *V. album* extract was more effective than *U. filipendula* extract against *K. pneumoniae* and *L. monocytogenes.* Both of extracts inhibited *C. violaceum*, they did not show anti-QS activity.

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