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Antimicrobial activitiy of oak honey (*Quercus* spp.) on the biofilm microorganisms

Meşe balının (*Quercus* spp.) biyofilm mikroorganizmaları üzerindeki antimikrobiyal aktivitesi

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ABSTRACT

Objective: The aim of this study is to determine antimicrobial properties of oak honey on biofilm microorganisms and its potential usage.

Material and Methods: The oak honey was collected in 2 years from the same region (OHPH/OHSH). Susceptibility patterns of clinical strains of three bacteria (*Escherichia coli*-ATCC 35218), (*Staphyllococcus aureus*-ATCC 29213) , (*Pseudomonas aeruginosa* -ATCC 27853) and one yeast (*Candida albicans* -ATCC 10231) strains that can form biofilm communities to oak honey were assessed by the disc diffusion method.

Results: The results revealed that oak honey has high level of antimicrobial activity to the pathogens. The antifungal activity against *C. albicans* was not measured clearly though, bacteriostatic effect showed itself as a secondary zone.

Conclusion: Oak honey might have a big potential for its antimicrobial properties in near future.

Keywords: Honeydew honey, honey bee, biofilm bacteria, antimicrobial effect

ÖZ

Amaç: Çalışmanın temel amacı meşe balının patojenler üzerinde antimikrobiyal etkisinin araştırılması ve bu alanda olabilecek potansiyel kullanımına açıklık getirmektir.

Materyal ve Yöntem: Meşe balı 2 yıl süre ile aynı bölgeden toplanmıştır (OHPH/OHSH). Biyofilm oluşturma özelliği olan 3 klinik bakteri suşu (*Escherichia coli*-ATCC 35218), (*Staphyllococcus aureus*-ATCC 29213) , (*Pseudomonas aeruginosa* -ATCC 27853) ve 1 klinik maya suşunun (*Candida albicans* -ATCC 10231) meşe balına karşı duyarlılık testleri disk difüzyon yöntemiyle analiz edilmiştir.

Araştırma Bulguları: Meşe balının patojenlere karşı yüksek oranda antimikrobiyal etkisinin olduğu ortaya çıkmıştır. *C. albicans'a* karşı antifungal etki tam olarak ölçülememiş fakat bakterisidal etki sekonder zon ile ortaya çıkmıştır.

Sonuç: Meşe balının yakın gelecekte antimikrobiyal özellikleri açısından büyük bir potansiyele sahip olabileceği ortaya çıkarılmıştır.

Anahtar Kelimeler: Salgı balı, bal arısı, biofilm bakterileri, antimikrobiyal etki

INTRODUCTION

Honey has been considered the main product of honey bees. Honey has been in humans diet and also used as a medicine since its discovered. As a medicine, it has been used for treatment of skin wounds, several infections (Özkök and Sorkun, 2018; Rodriguez et al., 2015). The healing capacity of honey directly depends on its physical and chemical properties (Özkök and Sorkun, 2018). There has been lots of studies antimicrobioal activity of honey among the all biological activities (Tenover, 1986; Allen et al., 1991; Molan, 1999; Basualdo et al., 2007; Krushna et al., 2007; Malik and Sharma, 2010 ; Irish et al., 2011; Kato et al., 2012). Since the main problem in beekeeping is the marketing of bee products, the variety of products even in honey varieties should be increased (Onuç et al., 2019). Almost all of the studies have used blossom honey samples to determine antimicrobial activitiy of honey. There are few studies about honeydew honeys of those reported physicochemical and antioxidant properties.

In general, honey is classified two types, blossom honey and honeydew honey (Can et al., 2015; Sorkun, 2008). Difference between blossom honey and honeydew honey based on its production. Blossom honey is produced from nectar. But honeydew honeys are produced in two different ways. One way is from the sweat of leaves. The another way is secretions of insects. Cause of the difference of production, honeys pysico-chemical and melissopalynological analysis have huge varieties. For these reasons, honeys differ from one another in terms of chemical composition, physical properties and melissopalynological analysis (Oses et al., 2015). Oak honey can be determined as honeydew honey and produced in one or two ways. One involves the secretions of some oak aphids, such as *Kernes guercus*, *Lachnus iliciphilus* and *Thelaxes dryophila* as reported by Simova et al., 2012. The stress factors, like nocturnal and diurnal temperature changes, sudden tempterature changes and another different factors causes oak leaves sweating. Thereby, another way to produce the oak honey is that sweating leaves (Özkök and Sorkun, 2018).

Oak honey would be the new source for antibacterial activity against pathogens. The inconvenient antibiotic usage, induce the bacterial resistance. This problem has increased in recent years and for that reason limited usage of these kinds of agents against the microorganisms (Lee et al., 2008; Brudzynski and Sjaarda, 2015). Especially for the pathogen bacteria that can form biofilm communities, biofilm allows for the cells inside to become more resistant to the body's natural antimicrobials as well as the antibiotics administered in a standard fashion. In fact, depending on the organism and type of antimicrobial and experimental system, biofilm bacteria can be up to a thousand times more resistant to antimicrobial stress (Hall-Stoodley et al., 2004). Antibacterial products, like honey, have huge interest between the researches because of he activity of pathogen microorganisims. Even though, little is known about oak honey, it has great potential for it antimicrobial property since it is dark in color and has high antioxidant capacity and the high apitherapy value (Özkök and Sorkun, 2018; Kolaylı et al., 2018). The aim of this study is to determine antimicrobial activity of oak honey on biofilm microorganisms.

MATERIAL and METHODS

Material

Oak honey production

Oak honey is produced in Thrace, northwest Anatolia, and the region of the Istıranca Mountains, with their rich oak forests. The beekeeper (Emrah Akol) who lives in this region placed the empty frames into the 8 hives at the beginning and end of the season. He reported that oak honey comes from only oak forests at the end of season between 25th July and 25th August. Each frames of each period in 25th July and 25th August 2016 (Oak Honey First Year Harvested-OHFH)- 25th July and 25th August 2017(Oak Honey Second Year Harvested-OHSH) years were harvested. 8 honey samples were collected from the Kırklareli province with the help of the General Directorate of Forestry and brought to the laboratory for analysis of antimicrobial properties. The same honey samples were analysed for chemical constituents and bioactive molecules by Özkök and Sorkun in the same project as an other researcher group. They reported that only 3 of 8 honey samples were identified as pure oak honey based on melissopalynologic and chemical analysis (Özkök and Sorkun, 2018). By using of this study as a reference, 3 honey samples were used for antimicrobial analysis. The chemical constituents and melissopalynologic properties of the all 8 honey samples are reported in this study (Özkök and Sorkun, 2018).

Bacterial strains

Susceptibility patterns of clinical strains of three bacteria (*Escherichia coli* -ATCC 35218), (*Staphyllococcus aureus* -ATCC 29213) , (*Pseudomonas aeruginosa* -ATCC 27853) and one yeast (*Candida albicans* -ATCC 10231) strain to oak honey were assessed by the disc diffusion method following the general guidelines of National Committee for Clinical Laboratory Standards (CLSI, 2013).

Method

Antimicrobial susceptibility tests

All strains of bacteria were cultured in Nutrient Broth Medium (Sigma; 42g/l) and overnight bacterial cultures (24 hours) ($1x10^8$ CFU/ml) were transferred to Nutrient Agar Medium (NA) (Sigma; 42g/l). Sabroud Dextrose Agar (SDA) was used for the yeast and followed the same methodology. After inoculation of 0.1 ml bacterial solution, the paper discs were placed the middle of the plates and 10 µl in 1:1 v/v concentration oak honey samples were inoculated to absorber paper discs in 5 mm diameter. All plates were incubated at 37^0C for 24 hours. The discs containing only sterillized distilled water (SDS) were used as negative controls. The antibiotic oxytetracycline and nystatin (antifungal) known to be effective against Gram positive and negative bacteria (wide-spectrum antibiotic) and fungi, was used as positive control. All assays were carried out in triplicate.

Statistical analysis

One way ANOVA and Duncan tests (SPSS 22.0 version Software programme) were performed in order to determine significant differences in the efficacy of oak honey harvested from different periods of season against clinical strains of some pathogen microorganisms.

RESULTS

The antimicrobial susceptibility tests revealed that oak honey has significant antimicrobial activity on pathogen microorganisms. There were two measured nested zones: the first circle was transparent and clear, the second was blurred which shows bacterial growth, gradually less than optimum level (Figure I.).

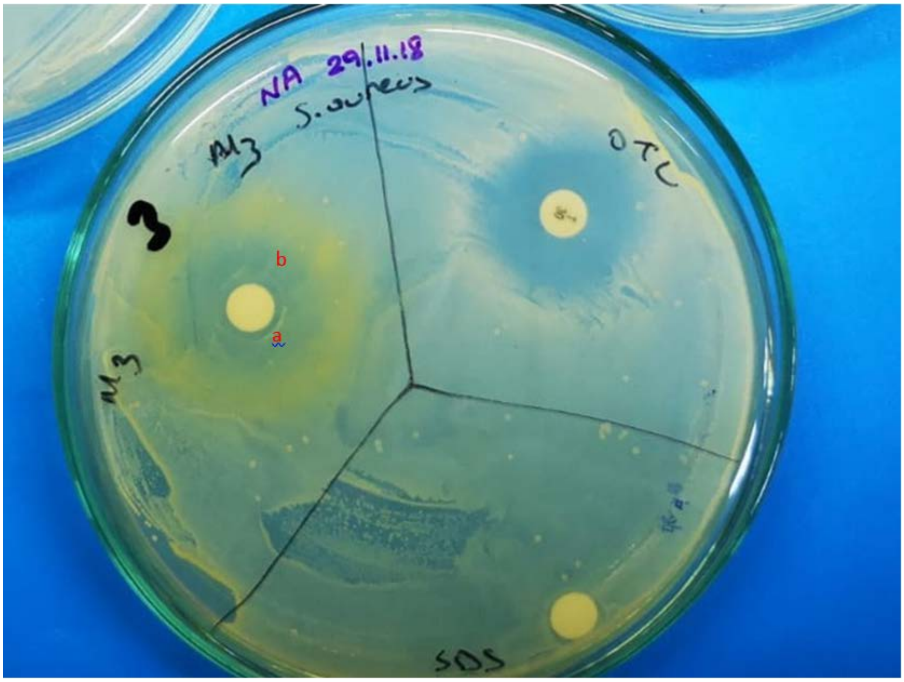

Figure 1. The bactericidal(a) and bacteriostatic(b) inhibition zones of oak honey (M3) against *S. aureus.*

***Şekil 1.** Meşe Balının (M3) S. aureus'a karşı oluşturduğu bakterisidal (a) ve bakterisitatik (b) inhibisyon zonları.*

The mechanisms of these kinds of antibacterial activities were reported as bactericidal (clear zone) and bacteriostatic (blurred zone) effects in the previous studies (Oses et al., 2015; Basualdo et al., 2007; Fidaleo et al., 2011). So, both the two kinds of zones were occured in the plates and the level of antibacterial activity of oak honey was evaluated according to inhibition zone diameters.

All results were summarized in Table 1. With regard to susceptibility of bacteria to oak honey, *S. aureus* strain was the most susceptible, then *P. aeruginosa* and *E. coli* strains have followed it respectively (ANOVA F=44.956 df=11 Sig=.000 P≤0.001).

Table 1. The diameter of inhibition zones (mm)± standard deviation of oak honey samples with negative and positive controls

Çizelge 1. *Negatif ve pozitif kontrol deney gruplarında meşe balı örneklerinin inhibisyon zon çaplarının (mm)± standart sapması*

Test Discs / Microorganisms	**OHFH/H1**	**OHFH/ H2**	**OHFH/ H3**	**OHSH/ H1**	**OHSH/ H2**	**OHSH/ H3**	**OTC/ Nystatin** (Positive control)	**SDS** (Negative control)
Escherichia coli **ATCC 35218**	nz	6±0.16 9**	5.5±0.1	6±0.26	6±0.17	6±0.19	16	nz
	nz	5.5±0.1	6±0.19 11**	6±0.17	6±0.19	7±0.16	15	nz
	nz	6±0.13 14**	5.5±0.1	6±0.19	6±0.21	6±0.15	17	nz
Staphyllococcus aureus **ATCC 29213**	nz 10**	nz 9**	nz 11**	9±0.21 19**	10±0.26 19**	9±0.11 20**	20	nz
	nz 13**	nz 11**	nz 9**	10±0.15 18**	7.5±0.16 14**	9±0.14 20**	18	nz
	nz 11**	nz 11**	nz 15**	10±0.19 20**	5.5±0.09 10**	8±0.12 20**	19	nz
Pseudomonas aeruginosa **ATCC 27853**	7±0.19 15**	3±0.07 17**	3±0.06 15**	8±0.24	6±0.16 16**	10±0.17 17**	8	nz
	8±0.13 13**	3±0.04 13**	nz 16**	12±0.22	7±0.14	7±0.12	11	nz
	7±0.15 10**	5.5±0.1 16**	nz 16**	9±0.19	7±0.22 19**	7±0.16 15**	10	nz
Candida albicans **ATCC 10231**	nz 7**	nz 9**	nz	nz 7**	nz 6**	Nz 10**	Nz	nz
	nz 5**	nz	nz	nz 8**	nz 7**	Nz 8**	Nz	nz
	nz	nz 7**	nz 5**	nz 10**	nz 9**	Nz 9**	Nz	nz

**The measurements includes the disc diameter (5 mm). All assays were carried out in triplicate)*

***bz: Bacteriostatic zone diameter, ***nz: no zone,*

*****OHFH/H1-2-3: Oak Honey First Year Harvested /Hive 1-2-3,*

******OHSH/H1-2-3-: Oak Honey Second Year Harvested/ Hive 1-2-3*

All strains were classified according to their susceptibilities and the groups which have different inhibition zone values (mm) were created by means of Duncan test (Table 2.).

Table 2. The comparative data of the antimicrobial activities of OHFH and OHSH samples by Duncan test. Microorganisms are listed from the highest to the lowest antimicrobial activities of oak honey samples

Çizelge 2. *OHFH ve OHSH örneklerinin antimikrobiyal aktivitelerinin Duncan testi ile karşılaştırmalı verileri. Mikroorganizmalar meşe balının gösterdiği en yüksek aktiviteden en düşük aktiviteye göre sıralanmıştır*

			Inhibition zone diameters (mm) of OHFH and OHSH samples				
Subgroups	**OHFH/OHSH**	**Subset for alpha= .05**	**a***	**b***	**c***	**d***	**e***
Microorganisms		**N**					
***Staphyllococcus aureus*-ATCC 29213**	OHSH	9	10.001				
***Pseudomonas aeruginosa* -ATCC 27853**	OHSH	9		7.783			
***Escherichia coli*-ATCC 35218**	OHSH	9			6.003		
***Escherichia coli*-ATCC 35218**	OHFH	9			5.500		
***Pseudomonas aeruginosa* -ATCC 27853**	OHFH	9				4.969	
***Staphyllococcus aureus*-ATCC 29213**	OHFH	9					0.000
***Candida albicans* -ATCC 10231**	OHSH	9					0.000
***Candida albicans* -ATCC 10231**	OHSH	9					0.000

**The Duncan significance test groups are indicated in parenthesis. Means for groups in homogenous subsets are displayed.*

DISCUSSION

The interesting result of classifying according to Duncan test was that while *S. aureus* is the most susceptible group of OHSH, it is more resistant group to OHFH. This result might show the negative effect of mixture of the nectar flow contribution to oak honey.

In general, the antibacterial effects of different honey varieties on *S. aureus* have been previously demonstrated by several studies (Oses et al., 2015; Voidarou et al., 2011; Perez-Martin et al., 2008; Sherlock et al., 2010). In other words, *S. aureus* is susceptible to honey's antibacterial effect, and the result obtained in this study coincides with other studies conducted so far. *E. coli* and *P. aeruginosa* are relatively more resistant to oak honey and can be interpreted as the clinical strains isolated from patients. Even though these strains isolated from patients are defined as either resistant pathogens due to the antibiotics used before or resistance to the immune system, the antibacterial effect of oak honey on these strains was found to be statistically significant.

The anti-fungal effect on *C. albicans* was found to be negligible. Therefore, it would be true to say that oak honey has an intense antifungal effect. On the other hand, the bacteriostatic activity on *C. albicans* was measured in all assays. So, oak honey might be suitable for the long-term used against Candida infections in order to observe anti-fungal effect.

Similar significant results have been obtained from the ANOVA ($F=53712$ $df=14$ Sig=.000 $P\leq 0.001$) analysis between OHFH samples and OHSH samples. The OHSH has higher antimicrobial activity than OHFH significantly according to inhibition zones of each honey sample. This result confirmed the beekeepers observation about the source of oak honey (mixed with nectar of other flowers) at the beginning of season. So, it means that the pure oak honey comes from only oak forests at the end of season (25th July and 25th August) has the higher antimicrobial activity statistically. By this study it could be suggested to produce pure oak honey between the dates mentioned above to get more benefits from antimicrobial properties.

The antibacterial nature of honey is dependent on various factors. These factors working either singularly or synergistically. The most prominent are; hydrogen peroxide (produced by the glucose oxidase added to honey by bees), phenolic compounds, pH of honey; osmotic pressure exerted by the honey etc. (Sherlock et al., 2010). Compared with pine and some of blossom honeys, oak honey contains higher flavonoid content (Fidaleo et al., 2011; Can et al., 2015; Kolaylı et al., 2018). And its antioxidant activity and phenolic compounds has higher similarity (Özkök and Sorkun, 2018). The results of this study revealed the "antimicrobial capacity of oak honey" as a parallel with its physcicochemical and antioxidant properties reported in the studies listed above.

Low moisture content of oak honey may also be a cause of high antibacterial effect (Simova et al., 2012; Mavric et al., 2008; Sherlock et al., 2010). In fact, studies conducted to date have shown that honeydew honeys are compared with multifloral blossom honeys have more antibacterial effects and are directly proportional to their moisture content (Özkök and Sorkun, 2018; Simova et al., 2012). Since microorganims need water to be alive and also growth, moisture level of honey is critical parameter to inhibit microorganism growth. The moisture content of oak honey was measured as 15% which is enough not to allow bacterial growth in honey (Molan, 1999).

Besides the physicochemical and antioxidant properties of oak honey which have been studied so far, this study would be complementary literature by also determining the antimicrobial properties of oak honey. Accordingly, oak honey has a high antimicrobial activity, with mostly bacteriostatic mechanisms so it may be suitable for long-term use in daily human diet. In addition, the antimicrobial effect of oak honey on clinical strains of human-pathogens showed that oak honey can also be used in apiteraphy.

CONCLUSION

In this study, the antimicrobial properties, as well as the physicochemical and antioxidant properties of oak honey has been studied so far, have also been contributed to the literature. The identification of antimicrobial activity, mechanism (bactericidal / bacteriostatic) and duration of activity will be the reference source for the determination of the usage areas of oak honey. Accordingly, oak honey has a high antimicrobial activity, with mostly bacteriostatic mechanisms so it may be suitable for long-term use in daily human diet. In addition, the antimicrobial effect of oak honey on clinical strains of human-pathogens showed that oak honey can also be used in apiteraphy.

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