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Evaluation of Antibacterial Effect of Honey on ESBL and Biofilm-Producing Enterobacterales

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ABSTRACT

Mastitis is a mammary gland inflammatory disease that causes milk yield reduction and economic losses. Mastitis is bacteriological and antibiotics are usually used for treatment. Alternative natural treatment methods such as bee products, phytotherapy, and essential oils were evaluated to reduce the use of antibiotics in the treatment of mastitis. In this study, the in vitro antibacterial effect of flower and oak honey samples dissolved in distilled water and boric acid (2%) on ESBL and biofilm-producing Enterobacterales pathogens was investigated. The aim was to establish the usability of honey/boric acid solution against mastitis as a natural antiseptic solution for bovine udder surfaces. Honey samples were studied by dissolving in distilled water and boric acid (2%) solvents. There was no significant statistical difference between honey solutions using distilled water and boric acid ($p>0.05$). Antibacterial effects were increased according to the increasing honey proportion in flower honey solutions. However, the antibacterial activity of oak honey dissolved in boric acid solution was higher than dissolved in distilled water. As a result of the statistical correlation analysis between flower and oak honey samples, antibacterial effects of flower honey samples were determined to be higher than oak honey samples ($p<0.05$) ($R=0.825$). An alternative formulation for mastitis treatment with honey and boric acid was developed for the first time in the literature.

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Balın GSBL ve Biyofilm Üreten Enterobacterales Üzerindeki Antibakteriyel Etkisinin Değerlendirilmesi

ÖZET

Mastitis, süt veriminin düşmesine ve ekonomik kayıplara neden olan bir meme bezi hastalığıdır. Mastitis bakteriyolojiktir; bu nedenle tedavilerinde genellikle antibiyotikler kullanılır. Mastitis tedavisinde antibiyotik kullanımının azaltılması için arı ürünleri, fitoterapi, uçucu yağlar gibi alternatif doğal tedavi yöntemleri değerlendirilmektedir. Bu çalışmada, distile su ve borik asit (%2) solventlerinde çözündürülen çiçek ve meşe balı örneklerinin ESBL ve biyofilm üreticisi Enterobacterales patojenleri üzerindeki in vitro antibakteriyel etkisi araştırıldı. Amaç bal/borik asit solüsyonunun büyükbaş göğüs yüzeylerinde doğal bir antiseptik solüsyon olarak mastitise karşı kullanılabilirliğini ortaya koymaktır. Borik asit ve distile su çözücülü bal çözeltileri arasında istatistiksel olarak anlamlı bir fark yoktur. Çiçek balı çözeltilerinde artan bal oranına göre antibakteriyel etkiler artmıştır. Ancak borik asit çözeltisinde çözülen meşe balının antibakteriyel etkinliği distile suda çözünenden daha yüksekti. Çiçek ve meşe balı örnekleri arasındaki istatistiksel ilişki analizi sonucunda çiçek balı örneklerinin antibakteriyel etkileri meşe balı örneklerine göre daha yüksek bulunmuştur ($p<0.05$) ($R=0,825$). Bu çalışma ile literatürde ilk defa bal ve borik asit ile mastitis tedavisine alternatif bir formülasyon geliştirilmiştir.

Mikrobiyoloji

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INTRODUCTION

Mastitis is an essential inflammation of the mammary gland that affects milking and dairy production and causes economic losses (Tepeli and Zorba, 2017; Nadia and Kheira, 2018; Krömker et al., 2019). Mastitis occurs in two types as clinical and subclinical. Clinical mastitis causes a noticeable difference in raw milk or udder quarters that can be identified by farmers. However, the subclinical version of the disease cannot be easily identified by visual symptoms (Leitner et al., 2018; Tepeli, 2020). As a result of pathologic and bacteriological changes in the mammary gland, milking channels can block continuously, and milking yield and milk quality decrease dramatically (Abdalhamed et al., 2018). Coliforms, *Streptococcus* sp., coagulase-positive *Staphylococcus* (especially *S. aureus*), and coagulase-negative *Staphylococcus* (CNS) are the main mastitis pathogens (Abdalhamed et al., 2018). Furthermore, gram-negative bacteria (mostly *E. coli*, *Klebsiell*a spp, and *Enterobacter* spp.) are an important cause of bovine mastitis around the world (Dahmen et al., 2013; Schukken et al., 2012). Subclinical mastitis infections can be prevented by early diagnosis of the disease, post-milking teat disinfection, and adequate pre-milking hygiene (Das et al., 2017). Antibiotics have been used in the treatment of mastitis for many years (Nadia and Kheira, 2018). The extensive and inappropriate use of antibiotics in dairy farms induces resistance among pathogens in mastitis. Antibiotics such as ß-lactam antibiotics (cephalosporins, penicillin), oxytetracycline, and aminoglycosides are used for the treatment of mastitis or the other bacterial infections among dairy cattle on the farm (Santioago et al., 2015; Ibrahim et al., 2016). Pathogenic bacteria, which belong to the order Enterobacterales, produce the ß-lactamase enzyme inactivating ß-lactam antibiotics (Das et al., 2017). The most clinically essential enzymes in the order Enterobacterales are known as extended-spectrum ß-lactamases (ESBLs). CTX-M, TEM and SHV are the predominant ESBL families encountered. Moreover, *bla*$_{\text{CTX-M-1}}$ is the most common genotype in animals according to available studies (EFSA, 2011; Ögedey et al., 2016; Das et al., 2017; Yıldırım and Pehlivanoğlu, 2018).

Alternative natural treatment methods such as bee products, phytotherapy, and essential oils were evaluated to reduce the use of antibiotics in the treatment of mastitis (Bal, 2011; Nadia and Kheira, 2018). Honey and bee products were used in folk medicine since ancient times due to biological activities such as antimicrobial, antioxidant, anti-inflammatory, immune modulator, and antitumor effects (Kwakman et al., 2008; Cinar, 2020; Kolaylı et al., 2020). Moreover, honey is an important antibacterial agent in traditional medicine containing polyphenols (flavonoid, benzoic, and cinnamic acid), sugar, acid, and hydrogen peroxide and having osmotic pressure effect (Weston, 2000; Baykam, 2007; Mercan et al., 2007; Erturk et al., 2014; Al-Masaudi et al., 2021). Honey and bee products, which have an important place in Turkey, are used in food, agriculture, medicine, cosmetics, paint, and many other fields. In 2020, honey production in Turkey reached 104,077 tons, while it was 1716 tons in Çanakkale. China (24.0%) ranked first in honey production in the world in 2019 with 444 thousand tons of honey production. Turkey (6.2%) ranked second with 114 thousand tons of honey production. Since most of the honey produced in this country is consumed in the domestic market, only 6,011 tons of honey produced in 2020 were exported (Anonymous, 2021a).

Boron is a natural element which is mainly mined in Turkey (67%). Other important producers are USA and China. This element is not carcinogenic and mutagenic. Boric acid is an important boron base weak acid. Its formula is H_3BO_3. Boric acid is used as antimicrobial, antiseptic, pesticide, and food additive (E284) (İpek, 2017; Demircan and Velioğlu, 2020; Anonymous, 2021b). Moreover, boric acid was used to treat fungal infections in humans for one hundred years (Liu et al., 2021). Boric acid has broad-spectrum antibacterial and therapeutic effects, and is considered a non-antibiotic alternative treatment option for superficial bacterial, fungal, and protozoal infections of the eye, ear, and vagina because of lower expense, being widely available, easy to use, and causing little irritation (Schmidt, 2017; Liu et al., 2021; Parin et al., 2021)

In this study, the in vitro antibacterial effect of flower and oak honey samples dissolved in distilled water and boric acid (2%) on ESBL (*bla*$_{\text{CTX-M}}$ gene positive) and biofilm-producing Enterobacterales pathogens was investigated. In this research aim was to establish the usability of honey/boric acid solution against the bovine inflammatory disease mastitis as a natural antiseptic solution for bovine udder surfaces.

MATERIAL and METODS

Honey Samples

Flower and oak honey samples were provided by Çanakkale Beekeepers' Association from the Kaz Mountain region (Mount İda), Çanakkale province in the west of Turkey. Physicochemical analyses were completed by Çanakkale Beekeepers Association at Ege University Drug Development and Pharmacokinetic Research and Application Center (Table 1). The main oak cultivars grown in Kazdağı National Park and its surroundings are Turkey oak (*Quercus cerris L.*), kermes oak (*Quercus coccifera* L.), Hungarian oak (*Quercus frainetto* Ten.), Aleppo oak (*Quercus infectoria* Oliv. subsp. *Infectoria*), Georgian oak (*Quercus petraea* subsp. *iberica* (Steven ex M. Bieb.) Krassiln.), downy oak (*Quercus pubescens* Willd.) and common oak (*Quercus robur* L) as determined by Deniz and Selvi (2021). Polat and Selvi (2011) examined plants visited by bees in their study of the region that includes Kaz Mountains. It was determined that the plants most visited by honeybees are taxa belonging to the families *Asteraceae*, *Boraginacea*e, *Fabaceae*, *Lamiaceae*, and *Cistaceae*. In addition, honeybees mostly visited species including *Echium plantagineum*, *Helianthus nnuus*, *Origanum* spp., *Paliurus spina-christi*, *Vitex agnus-castus*, *Cistus* spp., *Trifolium* spp., and *Cercis siliquastrum*.

Honey samples were dissolved in distilled water and powdered boric acid (2%) (CID: 7628, 99.5%, Merck, Germany) at final concentrations of 30%, 60%, and 90% (w v^{-1}).

Table 1. Some physicochemical properties of flower and oak honey
Çizelge 1. Çiçek ve meşe balının bazı fizikokimyasal özellikleri

Analysis *(Analiz)*	Flower honey *(Çiçek balı)*	Oak honey *(Meşe balı)*
Delta C13-Proteins (*Delta C13 protein)*	-26.30 ± 0.26‰	-25.70 ± 0.26‰
Delta C13-Raw product (*Delta C13 Hambal)*	-26.50 ± 0.27‰	-25.20 ± 0.25‰
Difference protein-honey (*Protein bal farkı)*	0.20‰	-0.50‰
C4% sugar (*% C4 şeker)*	0.00%	3.13%
Activity of diastase (*Diastaz aktivitesi)*	15.1 ± 2.6 ds dn^{-1}	19.2 ± 3.3 ds dn^{-1}
Electrical conductivity (*Elektrik iletkenliği)*	0.606 ± 0.018 mS cm^{-1}	1.083 ± 0.032 mS cm^{-1}
Hydroxymethylfurfural (*Hidroksimetilfurfural)*	6.7 ± 0.7 mg/kg	Not detected
Moisture (*Nem)*	18.2 ± 0.5%	17.1 ± 0.5%
pH (*pH)*	3.90 ± 0.03	4.47 ± 0.03
Proline (*Proline)*	974.0 ± 155.8 mg kg^{-1}	705.0 ± 112.8 mg kg^{-1}
Free acidity (*Serbest asitlik)*	32 ± 5 meq kg^{-1}	27 ± 4 meq kg^{-1}
Fructose (*Fruktoz)*	43.1 ± 9.9 g $100g^{-1}$	35.4 ± 8.1 g $100g^{-1}$
Glucose (*Glukoz)*	37.8 ± 7.9 g $100g^{-1}$	29.7 ± 6.2 g $100g^{-1}$
Sucrose (*Sakkaroz)*	Not detected	Not detected
Maltose (*Maltoz)*	Not detected	Not detected
Fructose/Glucose Ration (*Fruktoz/Glikoz Oranı)*	1.13	1.19

ESBL-producing (*bla*$_{CTX-M}$ gene positive) Enterobacterales Isolates

Isolates were identified as Enterobacterales causing mastitis in raw milk with subclinical mastitis by Tepeli and Zorba (2017) in a previous study. In this previous study, the ESBL activity of isolates was determined by the agar disk diffusion method according to European Committee on Antimicrobial Susceptibility Testing Standards (EUCAST, 2013). Moreover, detection of *bla*$_{CTX-M}$ gene was carried out by polymerase chain reaction (PCR). The *bla*$_{CTX-M}$ F-5-TCTTCCAGAATAAGGAATCCC-3, R- 5-CCGTTTCCGCTATTACAAAC-3, 909 bp primers were used for genotypic identification by Tepeli et al. (2018). For this study, seven *Escherichia coli strains*, two *Morganella morganii* strains, one *Serratia liquefaciens*, and *Citrobacter braakii bla*$_{CTX-M}$ positive strains were chosen. *Klebsiella pneumoniae* ATCC 700603 was used as ESBL positive control, and *E. coli* ATCC 25922 was used as ESBL negative control. Reference cultures were provided by Çanakkale Onsekiz Mart University Food Engineering Department microbiology culture collection.

Biofilm Assay

The crystal violet 96-well microtiter plate method was used to evaluate the biofilm formation status of isolates. For this, 20 µL overnight, 10^8 CFU mL^{-1} cell density (0.5 MacFarland) isolates were dispensed into each well, containing 100 µL of TSB (tryptone soya broth, Merck, Germany). The 96-well microtiter plate was incubated at 30 °C for 24 h. The incubated microtiter plate was washed twice with distilled, sterile water, dried, and fixed in an airflow cabinet. Each well was dyed with 120 µL of 1% (v v^{-1}) crystal violet solution (Sigma-Aldrich, USA). The crystal violet solution was discarded, and wells were washed twice with distilled, sterile water, and dried at room temperature for 30 min. Then, 120 µL of ethanol (96%) was added to each well, and OD values were

read by spectrophotometer (Multiscan FC; Thermo Fisher Scientific, NY, ABD) at OD_{600}. According to the scale of Sepanovic et al. (2000), OD values were evaluated as $OD_{control} < OD \leq 2xOD_{control}$ weak, $2xOD_{control} < OD \leq 4xOD_{control}$ moderate and $4xOD_{control} < OD$ strong biofilm producers. Control was evaluated as the negative control (NC).

Agar-Wells Diffusion method

Flower and oak honey samples with three different concentrations (30, 60, 90% w v^{-1}) were prepared with distilled water and boric acid (2%). ESBL-producing (*bla*$_{CTX-M}$ gene positive) Enterobacterales and reference culture isolates stored in TSB containing 16% glycerol at –20 °C were incubated overnight at 37 °C in TSB. Overnight TSB cultures were suspended in 5 mL of sterile saline (0.85% NaCl) until 0.5 MacFarland (1–2 × 10^8 CFU mL^{-1}) standard suspension was reached (DEN-1, MacFarland densitometers, Britain). The 0.5 MacFarland standard suspensions were added to Muller Hinton agar (MHA, HiMedia, India) with sterile swabs. After inoculum absorption, 6 mm wells were prepared on MHA, and 100 µL of honey samples (at each concentration) were added to each well. Plates were incubated at 35 °C for18-24 h in a flat position. Inhibition zone diameters were measured with a digital caliper (PM, China). Positive (*K. pneumoniae* ATCC 700603) control and negative control *(E. coli* ATCC 25922), cefotaxime antibiotic (2 mg L^{-1}) and boric acid (2%) were used in the present study (Valgas et al., 2007; Boorn et al., 2010).

Statistical Analysis

Statistical analysis was done with the SPSS 23.0 (SPSS Inc., Chicago, IL, USA) program. The differences between means of concentrations were determined by two-way ANOVA analysis; comparisons were made with the Tukey test ($p<0.05$). Differences between concentrations of oak and flower honey samples were examined with the Paired Sample t-test.

RESULT and DISCUSSION

According to the results of the biofilm assay, in this study all *E. coli* strains (except *E.coli* II and *E. coli* III), *S. liquefaciens,* and *C. braakii* were weak biofilm producers, while *M. morganii* II and *E. coli* III produced moderate biofilms (Fig. 1). Additionally, *E. coli* II was classified as a strong biofilm producer.

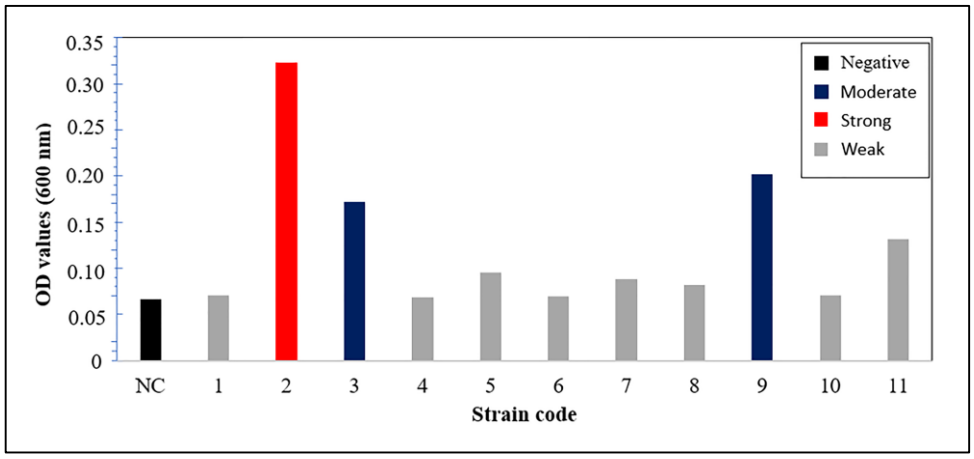

Figure 1. Biofilm status of ESBL producing Enterobacterales isolates (1-7: *E. coli*, 8-9: *M. morganii*, 10: *S. liquefaciens,* 11: *C. braakii, respectively*).
Şekil 1. GSBL üreten Enterobacterales *izolatlarının biofilm durumları*

Solutions of flower and oak honey prepared with distilled water and boric acid (2%) solvents were antibacterial against ESBL (*bla*$_{CTX-M}$ gene positive) at different levels for biofilm-producing Enterobacterales isolates (Fig. 2). The highest antibacterial activity was obtained with 90% (w v^{-1}) flower honey-distilled water solution against *C. braakii*, *M. morganii* II and all *E. coli* strains (Table 2). The highest antibacterial effect for *M. morganii* I strain was determined for the 60% flower honey-distilled water solution. However, 90% (w v^{-1}) flower honey-boric acid (2%) solution showed antibacterial activity against all strains (except *S. liquefaciens*) (Table 3).

No statistically significant difference was found between the antibacterial effect of flower honey - distilled water solutions and flower honey-boric acid (2%) solutions ($p>0.05$). Statistically significant differences were identified between both solvent concentrations ($p<0.05$). An increase in antibacterial activities for both solvents was seen when the honey amount increased.

The highest antibacterial activity was determined from 90% (w v^{-1}) oak honey-distilled water solution, except for *E. coli* II and *S. liquefaciens* (Table 4). In

addition, there was a statistically significant difference between concentrations of oak honey-distilled water samples ($p<0.05$). For oak honey-boric acid (2%), the highest antibacterial activity was determined for 30% (w v^{-1}) concentration against *M. morganii* I and *E. coli* VII. Also, the antibacterial activity of the 90% (w v^{-1}) concentration was evaluated as the highest activity against all other microorganisms (except *S. liquefaciens*) (Table 5). Differences between concentrations of oak honey–boric acid (2%) samples were determined to be statistically significant ($p<0.05$). Antibacterial activity increased with the increase in the amount of honey in oak honey–boric acid (2%) solutions. Differences between antibacterial activities of both solvents were reported to be statistically significant (distilled-water and boric-acid 2%) ($p<0.05$). Therefore, oak honey solutions prepared with boric acid (2%) had higher antibacterial activities. The antibacterial effects of oak and flower honeys were compared. The difference between the two honeys was analyzed statistically and a statistically significant difference was found ($p<0.05$) ($R=0.825$). Flower honey exhibited more antibacterial effect than oak honey.

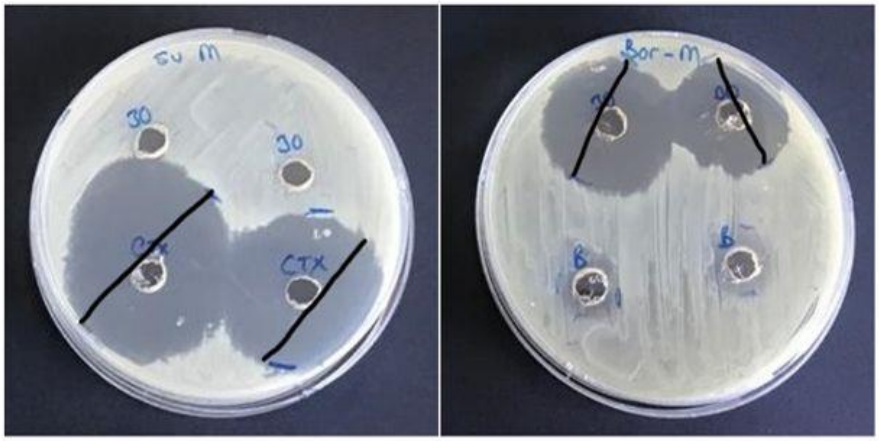

Figure 2. Inhibition zone diameter for agar-well diffusion method.
Şekil 2. Agar-kuyucuk difüzyon yöntemi inhibisyon zon çapı

Table 2. Antibacterial activity of flower honey-distilled water solutions
Çizelge 2. Çiçek balı-saf su solüsyonunun antibakteriyel etkisi

Isolate code *(İzolat kodu)*	Strain name *(Suş ismi)*	Cefotaxime 2 mg L^{-1} *(Sefotaksim)*	30% (w v^{-1})	60% (w v^{-1})	90% (w v^{-1})
1	*E. coli* I	20.13±0.53Ab	20.60±1.00Ac	15.66±0.34Acd	31.49±0.1Ac
2	*E. coli* II	18.29±0.33Ab	29.20±1.35Ac	22.99±0.17Acd	30.18±0.47Ac
3	*E. coli* III	39.59±1.58Ab	16.97±0.62Ac	27.62±0.57Acd	35.80±0.00Ac
4	*E. coli* IV	19.40±0.14Ab	15.62±2.23Ac	22.02±0.37Acd	31.33±0.32Ac
5	*E. coli* V	17.10±0.28Acb	≤ 6.00Acc	21.42±0.47Acd	21.62±0.16Acc
6	*E.coli* VI	43.27±0.37Ab	19.02±0.53Ac	20.07±0.97Acd	23.05±1.11Ac
7	*E. coli* VII	44.20±0.55Ab	22.93±0.49Ac	22.78±1.00Acd	13.51±0.47Ac
8	*M. morganii* I	42.57±0.25Ab	20.28±0.23Ac	22.56±0.12Acd	18.77±0.83Ac
9	*M. morganii* II	39.42±0.55Ab	25.72±1.14Ac	25.73±0.02Acd	35.52±1.46Ac
10	*S.liquefaciens*	45.44±0.83Adb	≤ 6.00Adc	≤ 6.00Adcd	≤ 6.00Adc
11	*C. braakii*	21.05±0.38Ab	24.85±1.38Ac	25.82±0.58Acd	35.75±0.71Ac
E.coli ATCC 25922 (ESBL negative control)		43.89±0.26Aeb	≤ 6.00Aec	≤ 6.00Aecd	21.07±1.01Aec
K. pneumoniae ATCC 700603 (ESBL positive control)		30.20±0.23Abb	≤ 6.00Abc	≤ 6.00Abcd	≤ 6.00Abc

* Capital letters (A) indicate that the difference between microorganisms is statistically significant ($p<0.05$). Lower case letters (b) show that the difference between concentrations is statistically significant ($p< 0.05$).

Antibiotics are used to treat mastitis. However, the inappropriate use of antibiotics on dairy farms can lead to resistance in mastitis pathogens. Therefore, researchers have begun to researching alternative treatment methods. Some researchers investigated alternative therapies such as ozone, essential oils, clay treatment, homeopathy, and phototherapy against microbial agents of mastitis to limit the use of antibiotics (Bal, 2011; Oral et al., 2014; Tepeli, 2020). In the present study, the in vitro antibacterial effects

of flower and oak honey samples dissolved in distilled water and boric acid (2%) were evaluated on *bla*CTX-M positive and biofilm-producing Enterobacterales isolated from bovine mastitis.

Table 3. Antibacterial activity of flower honey -boric acid solutions (2%)
Çizelge 3. Çiçek balı- borik asit (2%) solüsyonunun antibakteriyel etkisi

Isolate code *(İzolat kodu)*	Strain name *(Suş ismi)*	Boric acid 2% m V^{-1} *(Borik asit)*	Cefotaxime 2 mg L^{-1} *(Seftaksim)*	30% (w v^{-1})	60% (w v^{-1})	90% (w v^{-1})
1	*E. coli* I	13.94±0.86Aa	20.13±0.53Ab	≤ 6.00Aa	21.43±0.96Acb	28.16±0.67Ab
2	*E.coli* II	14.49±0.37AB	18.29±0.33ABb	28.91±1.36ABa	21.99±0.55ABcb	30.26±0.00ABb
3	*E. coli* III	15.72±0.13BA	39.59±1.58BAb	18.93±0.68BAa	31.79±0.43BAcb	34.95±0.35BAb
4	*E. coli* IV	≤ 6.00ABa	19.40±0.14ABb	19.87±0.19ABa	18.51±1.27ABcb	22.74±1.68ABb
5	*E. coli* V	≤ 6.00Aa	17.10±0.28Ab	≤ 6.00Aa	22.81±2.55Acb	28.50±2.33Ab
6	*E. coli* VI	≤ 6.00Aa	43.27±0.37Ab	≤ 6.00Aa	24.42±2.38Acb	28.72±1.55Ab
7	*E. coli* VII	≤ 6.00Aa	44.20±0.55Ab	≤ 6.00Aa	29.87±0.79Acb	36.14±2.06Ab
8	*M.morganii* I	≤ 6.00Aa	42.57±0.25Ab	15.43±0.48Aa	28.09±1.61Acb	29.50±0.02Ab
9	*M. morganii* II	13.05±0.64Aa	39.42±0.55Ab	≤ 6.00Aa	28.02±3.94Acb	35.75±0.25Ab
10	*S. liquefaciens*	15.44±0.54Aa	45.44±0.83Ab	≤ 6.00Aa	≤ 6.00Acb	≤ 6.00Ab
11	*C. braakii*	≤ 6.00Aa	21.05±0.38Ab	≤ 6.00Aa	26.71±1.97Acb	35.65±0.43Ab
E. coli ATCC 25922 (ESBL negative control)		≤ 6.00Aa	43.89±0.26Ab	≤ 6.00Aa	17.08±0.19Acb	23.64±1.04Ab
K. pneumoniae ATCC 700603 (ESBL positive control)		≤ 6.00ACa	30.20±0.23ACb	≤ 6.00ACa	≤ 6.00ACcb	≤ 6.00ACb

* Capital letters (A) indicate that the difference between microorganisms is statistically significant ($p< 0.05$).
Lower case letters (b) show that the difference between concentrations is statistically significant ($p< 0.05$).

Table 4. Antibacterial activity of oak honey - distilled water solutions
Çizelge 4. Meşe balı- saf su solüsyonunun antibakteriyel etkisi

Isolate code *(İzolat kodu)*	Strain name *(Suş ismi)*	Cefotaxime 2 mg L^{-1} *(Sefotaksim)*	30% (w v^{-1})	60% (w v^{-1})	90% (w v^{-1})
1	*E. coli* I	20.13±0.53Ab	≤ 6.00Aa	13.47±0.93Aa	26.86±0.60Ac
2	*E. coli* II	18.29±0.33Ab	21.60±0.06Aa	≤ 6.00Aa	≤ 6.00Ac
3	*E. coli* III	39.59±1.58Ab	≤ 6.00Aa	17.57±1.45Aa	26.07±0.96Ac
4	*E. coli* IV	19.40±0.14Ab	≤ 6.00Aa	≤ 6.00Aa	21.04±0.56Ac
5	*E. coli* V	17.10±0.28Ab	≤ 6.00Aa	≤ 6.00Aa	24.54±0.61Ac
6	*E. coli* VI	43.27±0.37Ab	≤ 6.00Aa	≤ 6.00Aa	18.99±0.36Ac
7	*E. coli* VII	44.20±0.55Ab	17.17±0.19Aa	13.43±0.95Aa	14.24±0.56Ac
8	*M. morganii* I	42.57±0.25Ab	18.59±1.20Aa	14.15±0.72Aa	15.31±0.46Ac
9	*M. morganii* II	39.42±0.55ACb	≤ 6.00ACa	19.02±0.44ACa	26.69±3.71ACc
10	*S. liquefaciens*	45.44±0.83Ab	≤ 6.00Aa	≤ 6.00Aa	≤ 6.00Ac
11	*C. braakii*	21.05±0.38Ab	≤ 6.00Aa	20.23±0.08Aa	26.61±0.73Ac
E. coli ATCC 25922 (ESBL negative control)		43.89±0.26Ab	≤ 6.00Aa	≤ 6.00Aa	10.98±0.48Ac
K. pneumoniae ATCC 700603 (ESBL positive control)		30.20±0.23ABb	≤ 6.00ABa	≤ 6.00ABa	≤ 6.00ABc

* Capital letters (A) indicate that the difference between microorganisms is statistically significant ($p< 0.05$).
Lower case letters (b) show that the difference between concentrations is statistically significant ($p< 0.05$).

Bacterial biofilms are structured communities of cells. These structures often occur along with the production of extracellular polymers by microorganisms as a response to different environmental conditions in order to survive. These biofilms formed on surfaces are a long-term source of contamination of foodstuffs on dairy process lines and surfaces due to the available nutrients and humidity (Čabarkapa et al., 2015; İpek and Zorba, 2018). Laranjo et al. (2018) investigated the in vitro activity of propolis ethanol extracts (PEE) against biofilms produced by staphylococci isolated from the milk of small ruminants with mastitis. They and some researchers stated that biofilms associated with mammary infection should be controlled due to the fact that bacteria growing in a biofilm can become 10 to 1000 times more resistant to antimicrobials. The biofilm structure is defined as a 3-dimensional exopolysaccharide matrix in which microorganisms can interact with each other. Horizontal gene

transfers were reported in this structure between resistant bacteria in food, milk, etc., which are excellent nutrients for microorganisms. Accordingly, biofilm structures can easily form on food-related surfaces, and resistance can transfer from one microorganism to another (Magesh et al., 2013; Cengiz et al., 2014; İpek, 2017). Determination of biofilm-formation capacity is an important piece of information to understand resistance transferability status. In the current study, *E. coli* II was a strong biofilm producer, and *E. coli* II and *M. morganii* I were moderate biofilm producers. Antibacterial effects of distilled water and boric acid solutions of flower-honey samples were determined against microorganisms forming biofilms. Only 30% of flower honey-boric acid solution had an antimicrobial effect on *M. morganii* II which can form biofilms. Antibacterial effects of oak honey solutions were reported to be lower than flower honey solutions against biofilm-producing microorganisms. Milanov et al. (2015) indicated that the biofilm-producing strains might play an important role in the spread of microorganisms in the environment and milking systems. Cengiz et al. (2014) suggested that antibiotic resistance profiles in herds should be monitored. Moreover, they stated that the main cause of mastitis treatment failure is the development of antibiotic resistance. They detected that 67.8% biofilm-producing *E.coli* strains originated from cow mastitis.

Table 5. Antibacterial activity of oak honey-boric acid (2%) solutions
Çizelge 5. Meşe balı-borik asit (2%) solüsyonunun antibakteriyel etkisi

Isolate code *(İzolat kodu)*	Strain name *(Suş ismi)*	Boric acid 2% m V^{-1} *(Borik asit)*	Cefotaxime 2 mg L^{-1} *(Sefotaksim)*	30% (w v^{-1})	60% (w v^{-1})	90% (w v^{-1})
1	*E. coli* I	13.94±0.86Aa	20.13±0.53Ab	≤ 6.00Aac	16.84±0.38Acd	26.80±1.55Ad
2	*E. coli* II	14.49±0.37Aa	18.29±0.33Ab	≤ 6.00Aac	18.95±1.24Acd	28.40±1.96Ad
3	*E. coli* III	15.72±0.13Aa	39.59±1.58Ab	28.40±0.52Aac	17.18±0.62Acd	26.97±0.18Ad
4	*E. coli* IV	≤ 6.00ABa	19.40±0.14ABb	≤ 6.00ABac	≤ 6.00ABcd	23.36±0.64ABd
5	*E. coli* V	≤ 6.00ACa	17.10±0.28ACb	≤ 6.00ACac	21.12±0.44ACcd	22.80±0.22ACd
6	*E. coli* VI	≤ 6.00Aa	43.27±0.37Ab	17.75±0.17Aac	21.18±0.12Acd	24.66±1.03Ad
7	*E. coli* VII	≤ 6.00Aa	44.20±0.55Ab	19.96±0.09Aac	16.23±0.01Acd	15.97±0.29Ad
8	*M. morganii* I	≤ 6.00Aa	42.57±0.25Ab	20.66±0.36Aac	16.39±0.50Acd	15.97±1.04Ad
9	*M. morganii* II	13.05±0.64Aa	39.42±0.55Ab	12.16±0.23Aac	20.72±0.66Acd	30.23±1.03Ad
10	*S. liquefaciens*	15.44±0.54ADa	45.44±0.83ADb	≤ 6.00ADac	≤ 6.00ADcd	≤ 6.00ADd
11	*C. braakii*	≤ 6.00Aa	21.05±0.38Ab	14.36±0.96Aac	19.29±0.36Acd	26.64±0.20Ad
E. coli ATCC 25922 (ESBL negative control)		≤ 6.00Aa	43.89±0.26Ab	14.84±0.09Aac	19.79±0.37Acd	23.58±0.52Ad
K. pneumoniae ATCC 700603 (ESBL positive control)		≤ 6.00AEa	30.20±0.23AEb	30.20±0.23AEac	≤ 6.00AEcd	≤ 6.00AEd

* Capital letters (A) indicate that the difference between microorganisms is statistically significant ($p< 0.05$).
Lower case letters (b) show that the difference between concentrations is statistically significant ($p< 0.05$).

The antimicrobial activity of honey was firstly observed in 1892. There are many studies about the antibacterial activity of honey (Baltrušaitytė et al., 2007; Süerdem et al., 2018; Çakır et al., 2020; Yalazi and Zorba, 2020; Al-Masaudi et al., 2021). Basically, the antimicrobial activity of honey is associated with acidity, pH, osmotic pressure caused by sugar content, hydrogen peroxide produced enzymatically by glucose oxidase, and phenolic compounds (Ulusoy et al., 2010; Güneş et al., 2016; Nolan et al., 2019; Çil et al., 2020). The antibacterial activities of flower honey samples were higher than oak honey samples in this research. The concentration of phenolic acids and flavonoids, which differ according to plant flora constituting the source of honey, are important due to their antimicrobial effects (Nisbet and Aker, 2020). In studies conducted about the polyphenol contents of different types of honey in Turkey, catechin, luteolin, and syringic acid were detected in flower honey. However, catechin, vanillic acid, syringic acid, daidzein, and luteolin were not identified in oak honey from Thrace (Güneş et al., 2016; Kolayli et al., 2016; Nolan et al., 2019). Differences in polyphenol contents of honey can explain antibacterial activity levels.

Özkirim et al. (2021) reported the antibacterial activity of oak honey against biofilm-producing, antibiotic-resistant clinical *E. coli* ATCC 35218 strains. Similarly, in this study, oak honey samples prepared with distilled water and boric acid solvent had antibacterial activity against the weak, medium, and strong biofilm producing *E. coli* strains. Süerdem et al. (2018) reported that oak honey was the more effective honey type because activity was measured against some bacterial strains (*E. faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 29213, *S. aureus* ATCC 29213, *S. aureus* ATCC 6538P, *Listeria monocytogenes*, and *Salmonell*a Typhimurium ATCC 51812). Wasihun and Kasa (2016) reported the antibacterial activity of red and white honey samples

against multi-drug resistant clinical *E. coli* and *K. pneumonia* strains. Kwakman et al. (2008) determined bactericidal activity of 10-40% (v v^{-1}) Revamil honey prepared with phosphate buffer against ESBL-producing *E. coli* and *K. oxytoca.* Bacayo et al. (2018) studied the antibacterial activity of 25% (w v^{-1}) concentrate prepared with Mueller Hinton broth and Tualang honey against *K. pneumonia,* and they identified antibacterial activity. Mercan (2007) reported that honey samples from the Sivas region had antimicrobial effects on *M. morganii* strains. Similar to the results of these studies, antibacterial activities of honey samples were determined against *E. coli*, *K. pneumonia,* and *M. morganii* in this research.

In the literature, no study evaluated the antibacterial activity of boric acid against the strains tested in this study. Boric acid contains boron, a natural element, which has been found in many foods like pistachio nuts, plums, tea and coffee. Boron amounts were determined in the order 13.8-18 µg g^{-1}, 21.5-27 µg g^{-1}, 21.5-27 µg g^{-1}, and 14.33 µg g^{-1} (İpek, 2017). Boric acid at 4 g kg^{-1} can be used as preservative for fish caviars. It was emphasized that there was no cause for concern in terms of genotoxicity (EFSA, 2013; Demircan and Velioğlu 2020; Liu et al., 2021). In practice, 2% boric acid solutions are usually used for human inflammatory treatments and 2% boric acid solutions are not cytotoxic as an inflammatory surface antiseptic agent (Anonymous, 2006; Anonymous, 2019; Liu et al., 2021). In this, 20 mg mL^{-1} boric acid was prepared as 2% solution against the animal inflammatory disease mastitis as a natural antiseptic solution for udder surface of the animal. Schmidt (2017) stated that intact skin does not absorb boric acid and boric acid in elemental form is safe for oral ingestion up to 18 mg day^{-1} for an adult. The Panel on Food Additives and Nutrient Sources organized by the European Food Safety Authority (EFSA) concluded that boric acid does not raise concerns in terms of genotoxicity (EFSA, 2013). Furthermore, İlhan et al. (2019) reported the antibacterial activity of boric acid against antibiotic-resistant *L. monocyto*genes and *S. aureus*. This study by İlhan et al. (2019) provided significant information about the antibacterial activity of boric acid against different types of resistant bacteria. Liu et al. (2021) measured the therapeutic effect of 3% boric acid on skin microflora. They concluded that boric acid inhibited candida, reduced microbial diversity, and improved the microecological flora of mouse skin. Parin et al. (2021) reported the antimicrobial activity of polyamide 6/honey fibers loaded with boric acid against *Escherichia coli* and *Staphylococcus aureus*. Almost all common bacteria or fungi were inhibited with 10–20 mg mL^{-1} boric acid (Hui et al., 2016). The specific mechanisms of boric acid versus cells are not clear. But studies indicate that boric acid increases the permeability of the pathogen cell wall, destroys cell membranes, and inhibits cell membrane formation (Liu et al., 2021). Nevertheless, the effect of boric acid on microflora was rarely reported.

CONCLUSIONS

Today, the use of natural products for the treatment of antibiotic resistant bacteria and the diseases caused by these pathogens has attracted the attention of researchers. This study showed that flower and oak honey samples dissolved in distilled water and boric acid (2%) solvents had significant antibacterial activity potential against ESBL and biofilm producing Enterobacterales. In conclusion, it is thought that honey/boric acid solutions will be an alternative method instead of antibiotics for the prevention or treatment of mastitis caused by environmental pathogens. This is a preliminary study before cytotoxicity studies. Further studies are needed about the applicability of honey/boric acid solutions.

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Author(S) Contribution

S. Özdikmenli Tepeli participated conception and design of the work, data collection, data analysis and interpretation, performing the analysis, drafting the article, critical revision, final approval of the version to be published. B. Kaya was involved in data collection, data analysis and interpretation, performing the analysis, drafting the article. D. İpek was involved in data analyses, critical revision and final approval of the version to be published.

Conflict of Interest

The authors declare no conflict of interest.

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