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Keçilerin Tükettiği Bazı Çalı Yapraklarının Antibakteriyel Aktivitesi

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ÖZ

Bu çalışmanın amacını, *Quercus coccifera*, *Phillyrea latifolia* ve *Ephedra major* çalılarından elde edilen özütlerin antibakteriyel aktivitesinin belirlemesi oluşturmuştur. Çalılardan yaprak örnekleri Nisan, Temmuz, Ekim ve Şubat aylarında hasat edilerek saf su, etanol ve aseton ile ekstrakte edilmiştir. Üç farklı çalı türünün yapraklarından elde edilen özütlerinin *S. aureus* (ATCC 25923) ve *P. aeruginosa* (ATCC 27853) bakterilerine karşı antibakteriyel aktivitesi, disk difüzyon yöntemi kullanılarak belirlenmiştir. Yapraklardan elde edilen özütler, sadece *S. aureus*'a karşı antibakteriyel aktiviteyi göstermiştir. *Phillyrea latifolia* yapraklarının saf su, etanol ve aseton ile ekstrakte edilmesi sonucu elde edilen özütler, tüm örnekleme dönemlerinde antibakteriyel aktiviteye sahip olduğu belirlenmiştir. *Q. coccifera* ve *E. Mojar* bitkilerinin sadece etanol ile ekstraksiyonu sonucu elde edilen özütler antibakteriyel aktivite göstermiştir. *E. Major*'dan Nisan ayında toplanan yaprak örneklerinin asetonla ekstrakte edilmesi sonucu elde edilen özütlerin, *S. aureus*'a karşı antibakteriyel aktivite gösterdiği belirlenmiştir. *P. latifolia* yapraklarından etanol ile ekstraksiyonu sonucu elde edilen özütler, en yüksek antibakteriyel aktiviteye sahip olmuştur. Söz konusu antibakteriyel aktivitenin gentamisin ve vankomisin ile karşılaştırılabilir düzeyde olduğu belirlenmiştir. Çalışmada genel olarak, etanolün özüt verimi açısından en etkili çözücü olduğu belirlenmiştir.

Antibacterial Activity of Some Shrub Leaves Consumed by Goats

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ABSTRACT

This study aims to determine the antibacterial activity of extracts obtained from *Quercus coccifera*, *Phillyrea latifolia*, and *Ephedra major* shrubs. Shrub leave samples were harvested in April, July, October, and February. Leaves samples were extracted with distilled water, ethanol, and acetone. The antibacterial activity of leaf extracts from three shrub species was tested against *S. aureus* (ATCC 25923) and *P. aeruginosa* (ATCC 27853) bacteria using the disc diffusion method. Shrub extracts obtained from the leaves displaced antibacterial activity against *S. aureus*. Antibacterial activity was found for water, ethanol, and acetone extracts of *Phillyrea latifolia* in all sampling periods. Only extracts obtained from *Q. coccifera* and *E. mojar* extracted with ethanol showed antibacterial activity. Leave samples harvested in April from *E. Major* extracted with acetone showed antibacterial activity against *S. aureus*. The ethanolic extracts of *P. latifolia* showed the highest antibacterial activity. This activity was comparable to gentamicin and vancomycin in this

study condition. Overall, ethanol was the most effective solvent for extract yield.

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Introduction

Natural resources have been used for a long time in traditional treatments of infections (Cowan, 1999; Abdullah et al., 2012). World Health Organization reported that 80% of people use plants for the treatment of their diseases in developing countries (Schmincke, 2003). New and natural resources crucially become important because pathogenic bacteria are resistant to antibiotics. The use of antibiotics as a "growth promoter" in animal production is avoided due to the prohibition of their uses as a growth stimulant and their long-term use as a therapeutic agent, leaving residues in animal products. Consumers' preference towards "organic" and "natural" products in animal production brings within trend towards natural products in the production process also. In this respect, the use of plants and plants' secondary compounds in animal production, as an alternative to synthetic drugs is becoming increasingly important (Rochfort et al., 2008; Hussain et al., 2012).

It has also been reported that plants have been used for many years in traditional veterinary practices (Romero et al., 2022), for instance, plant extracts have been reported to inhibit foam production in the rumen (Viegi et al., 2003) and antibiotic-like effects on the treatment of ruminal acidosis (Hutton et al., 2009). There are some reports about the use of plant extracts in the control and treatment of external parasites in cattle and sheep (Kamaraj et al., 2010). Condensed tannin, which is a phenolic secondary compound of plants, is widely distributed and has a direct antiparasitic activity of sheep gastrointestinal nematodes (Molan et al., 1999). The plants contain secondary compounds as a part of their defense mechanisms. It is reported that many plant species contain secondary compounds (tannins, phenolic compounds, flavonoids, essential amino acids, etc.) that have antimicrobial activity (Cowan, 1999). The content of secondary compounds and their chemical activity can change- by sampling period (Cheeke, 1998), plant part (Clemensen, 2018), extraction method (Altemimi et al., 2017), and polarity of the solvent used during an extraction process (Nawaz et al., 2020). Likewise, the antimicrobial activity of plants also changes by plant parts (Ginovyan et al., 2017), an extraction method (Nakatsu et al., 2000; Nostro et al., 2000), in addition solvents used during an extraction process (Sen and Batra, 2012). Several studies reported that the most commonly used solvents to investigate antimicrobial activity in plants are methanol, ethanol, and water (Lourens et al., 2004; Rojos et al., 2006; Chen et al. 2021). Şöhretoğlu et al. (2007) investigated the effects of the antimicrobial activity of different solvent extracts of *Quercus*

coccifera leaves against *Staphylacoccus aureus* and *Candida albicans* strains. The researcher found that ethyl acetate extracts of *Q. coccifera* had the highest antimicrobial activity. Methanolic extracts obtained from Ephedra major have been reported to inhibit *Aspergillus parasiticus* development and production of aflatoxin B1 (Gavkosh et al., 2009).

Plant species that are present in this study are in maquis formation and are widespread in the regions dominated by the Mediterranean climate (Aydınözü, 2008). As well as these plants are consumed by goats (Tölü et al., 2012) and contain certain levels of secondary metabolites especially condensed tannin (Tölü et al., 2012; Alatürk et al., 2014; Akbağ and Yurtman, 2022). Condensed tannin mentioned above has some special effects (antimicrobial, anti-helminthic, etc.) on animal health. Therefore shrubs that contain a certain amount of condensed tannin may potentially be used for self-medication under browsing conditions.

This study aimed to evaluate the antimicrobial activity of three shrub species which were harvested at four different sampling periods and extracted with ethanol, acetone, and water.

Material and Method

Shrub leaves samples

The shrub materials of this study were *Quercus coccifera*, *Phillyrea latifolia*, and *Ephedra major*. The leaves samples of shrub species harvested from the natural shrubby vegetation size of 30 da which were located at the Farm of Agricultural Faculty of Çanakkale Onsekiz Mart University. The shrub leaves were harvested at four sampling periods (April, July, October, and February). Leaves were harvested from 5 shrubs for each species and the same shrubs in each harvested date. For each shrub species, 5 replicate plant samples were mixed into one sample of equal weight for each sampling period. The shrub leave samples were taken to the laboratory conditions as soon as possible and subjected to drying for 10 days on a laboratory bench in a dark condition. After the drying process, the plant samples were grounded by a mill to pass a 1 mm mesh screen and ready for analysis. The milled samples were stored in a dark glass jar at room temperature and in the absence of light until the day of the analysis.

Preparation of shrub extracts and determination of yields

Extracts for each shrub sample were obtained using Soxhlet extract (Soxhlet extract system, EV6AII / 16, Gerhardt UK Ltd.). A 20 g shrub leaves sample was weighed on a filter paper and subjected to extraction by adding 200 ml of solvent. During the extraction process, the shrub samples were extracted with 3 different solvents, distilled water, ethanol, and acetone

were used. The temperature of the soxhlet extractor was fixed at the temperature where the solvents were boiled and the extraction process was continued for 8 hours after boiling was seen. After extraction, the mixture was filtered through a filter paper (Whatman No. 1) and subjected to evaporation. Evaporation was carried out with a rotary evaporator (Heidolph Laborator 4000 Efficient) at 45-50 °C and under vacuum at 150 rpm. After this process, the plant extracts were dried with a freeze dryer, and the extract yields were calculated according to the following formula (Abbas et al., 2021);

Yield (DM%) = (A1 X 100)/ A2

In the formula A1= the weight measured after freeze dryer application, and A2= the dry sample weight subjected to extraction. Plant extracts were stored at + 4 °C until the analysis.

Bacterial strains used in antibacterial susceptibility tests are gram-positive: *S. aureus* (ATCC 25923) and gram-negative: *P. aeruginosa* (ATCC 27853) obtained from Istanbul University Faculty of Pharmacy. Mueller-Hinton Agar (MHA, Merck) was used to carry out the antibacterial analysis. Select reference antibiotic discs (gentamicin and vancomycin; Oxoid) were used as positive controls, depending on the test micro-organisms.

Antibacterial susceptibility tests

In this study, the *in vitro* antibacterial activities of five shrub species were determined using the disk diffusion method (NCCLS, 1999) standardized by Akgül and Kaya (2004). Briefly, all microorganisms were grown on Mueller Hinton Agar (MHA) plates then 3 ml of MHB was inoculated with 4-5 well-isolated colonies for each strain. At the end of incubation, the density of bacteria reached 108 CFU/ml and was standardized to freshly prepared 0.5 McFarland turbidity standards. The prepared fresh bacterial cultures were sown on solid medium -prepared using MHA with the help of cotton swabs. Filter paper disks (6 mm oxoid) were soaked with 25 µl extracts. The discs were placed onto the inoculated agar surface at appropriate intervals and incubated at 37 °C for 20 hours. At the end of the incubation period, antibacterial activity was determined by measuring the diameter (mm) of inhibition zones. Gentamicin (10 µg) and vancomycin (30 µg) discs were used as positive controls. Solvents (distilled water, ethanol, and acetone) were used as a negative control. The results were expressed in terms of the diameter of inhibition zones (mm) and the presented values are the average of three separate experiments.

Results

Extract yield and their change by season

Extract yields differed within the shrub species, sampling periods, and solvents (Table 1). Totally the highest extract yield was obtained from *P. latifolia* between shrub species. The highest extract yields were obtained with ethanolic extracts of *P. Latifolia, Q. Coccifera, E. major* (Table 1). The lowest extract yields were found in *P. latifolia* harvested in October (1.78%) and *Q. coccifera* in February extracted with distiled water. The lowest extract yield was obtained for *E. major* which was extracted with acetone.

Table 1. Extraction yields of *Quercus coccifera*, *Phillyrea latifolia* and *Ephedra major* (%)

Sampling period	Shrub	Distiled water	Ethanol	Acetone
April	*P. latifolia*	4.53	14.89	2.95
July		3.97	15.58	2.89
October		1.78	13.59	2.17
February		5.65	10.45	5.42
April	*Q. coccifera*	0.47	5.42	1.35
July		0.10	3.52	2.01
October		0.53	6.41	3.26
February		0.08	5.76	3.60
April	*E. major*	7.29	6.64	4.10
July		3.24	8.15	0.74
October		1.08	4.53	2.84
February		2.12	5.62	0.81

Antibacterial Activity

In this study, different solvent extracts of *Quercus coccifera*, *Phillyrea latifolia*, and *Ephedra major* were determined against *S. aureus* and *P. aeruginosa*. Extracts obtained from the shrub leaves displace antibacterial activity against *S. aureus*. The water, ethanol, and acetone extracts of *P. latifolia* showed antibacterial activity against *S. aureus* zone of inhibition in the disc diffusion method was 15-21 mm (Table 2).

The ethanolic extract of *P. latifolia* had strong antibacterial activity against *S. aureus* in all sampling periods. The extracts obtained from *P. latifolia* in April showed the highest (21 mm) antibacterial activity and it was similar to Gentamicin (19 mm) (Table 2 and Table 3).

It has been determined that the extracts of *Q. coccifera* and *E. major* leaves extracted with ethanol showed antibacterial activity against *S. aureus* in every sampling period (Table 2). Furthermore, it was determined that *E. major* leaves harvested in April showed antibacterial activity when extracted with acetone.

Table 2. The inhibition zone diameters of *Phillyrea latifolia, Quercus coccifera and Ephedra major*, extracts against *S. Aureus* (mm)

Sampling periods	Shrub	Distiled water	Ethanol	Acetone
April	*P. latifolia*	21	21	16
Julay	*P. latifolia*	16	19	16
October	*P. latifolia*	15	20	16
February	*P. latifolia*	16	20	16
April	*Q. coccifera*	-	13	-
Julay	*Q. coccifera*	-	13	-
October	*Q. coccifera*	-	15	-
February	*Q. coccifera*	-	14	-
April	*E. major*	-	12	11
Julay	*E. major*	-	11	-
October	*E. major*	-	11	-
February	*E. major*	-	11	-

-: no inhibition zone

Solvents (distilled water, ethanol, and acetone) used as a negative control did not affect the growth of the organism in the study (Table 3). Gentamicin (10 µg) and vancomycin (30 µg) were used as positive control and the diameters of the inhibition zones were 19 mm and 17 mm respectively against *S. aureus* (Table 3).

Table 3. The inhibition zone diameters of solvents and gentamicin and vancomycin (mm)*

	Distiled water	Ethanol	Acetone	Gentamicine	Vancomisine
Concentration/disc	25 µl	25 µl	25 µl	10 µg	30 µg
Bacteria strains					
S. aureus	-	-	-	19	17
P. aeruginosa	-	-	-	-[a]	-[a]

-: no inhibition zone. -[a]: not determined. *: include the diameter of the disk (6 mm)

Discussion

Goats' milk and meat are important nutrient sources for humans in rural areas. The goat population was estimated at 10.634.672 head in Turkey and their milk and meat production were 561.826 tons and 67.500 tons respectively in 2018 (FAOSTAT, 2020). Subclinical mastitis is the most common disease in dairy goats (Persson and Olofsson, 2011). *S. aureus* has been reported to be an important pathogen responsible for clinical and subclinical mastitis, especially in goats (Moroni et al., 2005). It is known that *S. aureus* and *P. aeruginosa* cause several diseases in humans and goats. It has been reported that *S. aureus* is caused by skin and soft tissue, muscle, bone, lung, and heart-valve infections (endocarditis, etc.) in humans (McCaig et al., 2006). Lowy (1998) reported that *S. aureus* is a major cause of hospital infections involving bacteremia and pneumonia. *S. aureus* also causes abdominal diseases (such as lenfadenitis) with *P. aeruginosa* in sheep and goats (Al-Harbi and Mahmoud, 2012).

S. aureus is resistant to methicillin (MRSA) in some parts of Africa (WHO 2014). According to Paterson et al. (2013), *S. aureus* showed resistance to cefoxitin and oxacillin in bulk milk sampled from cattle in England and Wales. Sela et al. (2007) report that *P. aeruginosa* may cause mastitis in sheep, goats, and cattle. It is reported that *P. aeruginosa* causes cystic fibrosis and nosocomial infections in humans (Bentzmann and Pleslat, 2011).

In recent years, numerous studies have been conducted to investigate the antimicrobial activity of different plant species (Sheik et al., 2012; Abbas et al., 2021; Arif et al., 2022; Singh et al., 2023). In this study, *Quercus coccifera*, *Phillyrea latifolia Ephedra major* showed antibacterial activity against Gram-positive bacteria *S. aureus*. There is no antibacterial activity shown by Gram-negative bacteria *P. aeruginosa* due to these bacteria are more tolerant than Gram-positive bacteria (Paz et al., 1995; Chariandy et al., 1999) and having another membrane protecting them from many antibiotics and detergents (Sleigh and Timbury, 1998).

Shrublands are important forage sources for ruminants and many shrub species contain secondary compounds in different proportions (Makkar, 2003). It is reported that the most common secondary compounds contained in shrubs are tannins (Makkar and Becker, 1998). The tannin content of the shrubs can be changed seasonally and this is closely related to the intake preferences of herbivores (Rockwood, 1974). Shon et al. (2004) explain that the extract yields of plants are varied depending on the structure and polarity of the solvent used in the extract process. In addition, the chemical composition of the plant species affects extract yield. It has been reported that polar solvents are more effective than polar solvents for extracting polyphenolic compounds (Peschel et al., 2006).

In this study, the solvents used in the extract process were sequenced polar to a polar distilled water, ethanol, and acetone respectively. The highest antibacterial activity was observed in extracts that were extracted with ethanol which is the most polar solvent after distilled water in this study. This is due to the solvents with different polarities may be effective on their bioactive compounds (Parekh et al., 2005). Cowan (1999) reported that solvents with an average polarity are more effective in extracting antimicrobial compounds. Abdullah et al. (2012) using different solvents reported that in the extracts of 57 plant species, methanol is the most effective solvent for extracting antibacterial compounds. The researchers found that the extracts produced with hexane and hot water did not have any antibacterial activity. Ncube et al. (2011) reported that water extracts of *Tulbaghia violacea*, *Hypoxis hemerocallidea*, *Drimia robusta,* and *Merwilla plumbea* leaves have poor antibacterial and antifungal activity. In general, the antimicrobial activities of plant extracts are influenced mainly by environmental

and climatic factors, solvents, and extraction methods used in the extraction process (Cowan, 1999).

Conclusion

P. latifolia. Q. coccifera and *E. Major,* which are consumed by goats showed antibacterial activity against *S. aureus* in this study. Ethanol is the most effective solvent, which gives antibacterial activity in the present study. Especially ethanolic extracts of *P. latifolia* showed the highest antibacterial activity comparable to gentamicin and vancomycin. Ethanolic extracts of *P. latifolia* would potentially be used in the protection and treatment of diseases in goats and humans originating from *S. aureus* infections. Further investigations of the extracts that showed antibacterial activity in this study determined the potential usage for self-medication by goats. Especially the investigation of the anti-parasitic effects of both *in vitro* and *in vivo* conditions constitutes the subject of further studies.

Researchers' contribution rate declaration summary

The authors declare that they have contributed equally to the article.

Conflict of interest

The authors declare that they have no conflict of interest

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