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The Antimicrobial Activities of the Leaves of Some Endemic Stachys Species spreading in West Anatolia, Turkey

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Abstract

The extracts of the leaves of six endemic *Stachys* sp. (Lamiaceae) which were collected from Turkey were investigated for their antimicrobial activities. In this study, the antimicrobial activity of ethanol, aceton and hexane extracts of *Stachys annua* subsp. *cilicia*, *S. setifera* subsp. *lycia*, *S. sosnowskyi*, *S. tmolea*, *S. cretica* subsp. *anatolica*, *ve S. iberica* subsp. *iberica* var. *densipilosa* species which were collected from Kütahya and Eskişehir cities were investigated on some microorganisms. The effect of extracts on *S. aureus* ATCC 6535, *S. epidermidis* ATCC 11228, *B. cereus*, Gram (-) *E. coli* W3110, *P aeruginosa* ATCC 27853, *P. vulgaris* ATCC 13315 were investigated by using disc diffusion and microbroth dilution methods. Antibiotics were used as positive controls.

According to the results, while hexane and aceton extracts of endemic *Stachys* species exhibited no antibacterial activities, ethanol extracts of 3 endemic species (***S. tmolea, S. cretica* subsp. *anatolica, S. iberica* subsp. *iberica* var. *densipilosa)*** showed a strong antibacterial activity, especially Gram (+) *Sthaphylococcus aureus*. According to the results, it was observed that the *Stachys* extracts are more effective against Gram (+) bacteria. The results demonstrated that the ethanol extract of the leaves of ***S. tmolea, S. cretica* subsp. *anatolica, S. iberica* subsp. *iberica* var. *densipilosa*** has a strong antimicrobial activity. The secondary metabolites isolated from endemic *Stachys* sp. could be used in an effective manner to fight especially against *S. aureus*.

Key words: Lamiaceae, *Stachys*, Antimicrobial activity

INTRODUCTION

There are many plants with medical importance in the world. Chemical composition of the plants that have grown in different regions varies according to climatic and environmental factors (Radulovic et al. 2007). Although the diversity of endemic plants in Turkey is very high, the current studies on these plants are not sufficient. *Stachys* sp. which is one of these endemic genera is a member of the Lamiaceae family (Baytop, 1999).

This genus is represented by 121 species level taxa in Turkey and has a 43.4% rate of endemism (Baytop, 1999). Many of the taxa belonging to the *Stachys* genus include aromatic compounds and essential oils which have quite large medical and economic importance (Goren et al. 2011). Whole plant or leaves of *Stachys* species have been reported in folk medicine to treat a lot of diseases including genital tumors, cancerous ulcers, sclerosis of the spleen, rheumatic fever, respiratory inlammatory diseases, anxiety (Rezazadeh et al 2005, Maa et al. 2012, Laggoune et al. 2011, Kumar et al. 2012). Antimicrobial activity of *Stachys* species with many studies have been performed (Sonboli et al. 2005, Abichandani et al. 2010, Skaltsa et al. 1999). But antimicrobial features of a number of endemic *Stachys* species which are grown in different area are still unknowable.

Resistance to antibiotics used in the fight against infectious agents has become an important issue today and there is a need for more effective chemicals used in place of antibiotics. Also, new chemicals are needed for microorganisms in the infections of plants and animals. It should be found the more environmentally friendly chemicals instead of synthetic chemicals which be used in infections of plants. The plants are natural sources for these chemicals, and extracts of fresh or dried plant which has therapeutic value, or products derived from these extractions are used commonly in alternative medicine. Also this plants have anti-oxidant properties (Bakkali et al. 2008, Ebrahimabadi et al. 2010).

Therefore, extracts of six *Stachys* species which were not studied on the antimicrobial activity and were grown as endemic (plants) in Turkey were investigated for antimicrobial activity on microorganisms.

EXPERIMENTAL

Preparation of Plant Extracts

In the study, *Stachys annua* subsp. *cilicia*, *S. setifera* subsp. *lycia*, *S. sosnowskyi*, *S. tmolea*, *S. cretica* subsp. *anatolica*, and *S. iberica* subsp. *iberica* var. *densipilosa* were collected from Kütahya and Eskişehir. Plant samples have been identified according to "Flora of Turkey and the East Agean Island" (Davis 1965). The leaves of plants were dried after the collection, and they were divided into small pieces. Then, 10 g of leaves weighed, and the extraction process was performed with 100 ml of ethanol, hexane, or acetone for 8 h using a soxhlet apparatus. The extract was filtered with Whatman filter paper No.1. Then, extracts were filtered by using a 0.22 µm syringe filter, and the extraction is concentrated to 5 ml in the evaporator at 45 °C. The extracts were stored at 4 °C until the work performed.

Microorganisms

Antimicrobial activity of extracts was studied by using three Gram (+) bacteria (*S. aureus* ATCC 6535, *S. epidermidis* ATCC 11228, *B. cereus* ATCC 7064) and three Gram (-) bacteria (*E. coli* W3110, *P. aeruginosa* ATCC 27853, *P. vulgaris).*

Antimicrobial Activity Studies

In antimicrobial activity studies, the minimum inhibitory concentration values of extracts were determined by using microbroth dilution method (Zgoda & Porter. 2001). Two fold liquid dilution tests were made by nutrient broth. MIC value was determined by using microplate. Fresh cultures of the microorganisms were grown for 18 h in nutrient broth. The density of microorganisms was adjusted to 0.1 absorbance at OD_{600} as spectrophotometrically. Then the 60 µl of this culture was taken and was added to 400 ml of fresh nutrient broth media. For two-fold serial dilution, first hole of microplate contained 20 µl of each extract and 180 µl of nutrient broth containing bacteria, and 100 µl of bacterial culture was added to the other holes. Then, serial dilution was done by using 100 µl of bacterial culture from first hole to other holes. Microplate was incubated at 37 ºC for 24 h. MIC was defined as the lowest concentration which yielded no visual growth. Also antimicrobial effectiveness of extracts was tested by applying disc diffusion method.

It were taken 100 µl from microorganisms cultures which was adjusted to 0.1 absorbance at OD_{600} as spectrophotometrically, and were spreaded to nutrient agar surface in petri dish. Then, 6 mm empty sterilized antibiotic discs were placed to agar, and 15 or 20 µl of the extracts were dropped. Microorganisms were incubated at 37 ºC for 24 h. Ethanol, hexane and aceton were used as controls. All experiments were performed in triplicate. Two antibiotics have been used for controlling. When there is no similarity between the repeats, the repetition number is increased.

RESULTS

The minimum inhibitory concentration of the ethanol, acetone, hexane extract derived from *Stachys* leaves was studied by microbroth dilution method. According to the results of the studies, there was no antimicrobial activities in the studied concentrations of hexane and acetone extracts (Table 1 and 2). In contrast, ethanol extracts were determined to be quite effective in the studied concentrations on microorganisms (Table 3).

Tablo 1: The minimum inhibition concentration of hexane extracts of six *Stachys* sp

	Minimum inhibitory concentration (MIC) (µl/ml)						
	1	**2**	**3**	**4**	**5**	**6**	**Hexane**
S. aureus	**>100**	**>100**	**>100**	**>100**	**>100**	**>100**	**>100**
S. epidermidis	**>100**	**>100**	**>100**	**>100**	**100**	**>100**	**>100**
B. cereus	**>100**	**>100**	**>100**	**>100**	**>100**	**>100**	**>100**
E. coli	**>100**	**100**	**>100**	**>100**	**100**	**>100**	**>100**
P vulgaris	**>100**	**>100**	**>100**	**>100**	**>100**	**>100**	**>100**
P. aeruginosa	**>100**	**>100**	**>100**	**>100**	**>100**	**>100**	**>100**

1: *S. annua* subsp. *cilicia*, 2: *S. setifera* subsp. *lycia*, 3: *S. sosnowskyi*, 4: *S. tmolea*, 5: *S. cretica* subsp. *anatolica*, 6: *S. iberica* subsp. *iberica* var. *densipilosa*

MIC values obtained from ethanol extracts of six endemic *Stachys* species are given in Table 3. As it is shown in Table 4, it can be seen that the most effective *Stachys* species on the microorganisms was *S. cretica* subsp. *anatolica*. In addition, this species (*S. cretica* subsp. *anatolica*) was more effective on the studied Gram (+) bacteria (*S. aureus, S. epidermidis, B. cereus*) than Gram (-) bacteria (*E. coli, P. vulgaris, P. aeruginosa*), and they have been determined to be quite effective against especially *Staphylococcus* species. According to the results, MIC values of *S. cretica subsp. anatolica* ethanol extract was determined as 6,25 µl/mL on *S. aureus* and *S. epidermidis*, 12.5 µl/mL on *B. cereus*, 25 µl/mL on *E. coli*, 50 µl/mL on *P. vulgaris* and 50 µl/mL on *P. aeruginosa*. While *S. tmolea* and *S. iberica* subsp. *iberica* var. *densipilosa* were not as effective as *S. cretica* subsp. *anatolica*, they appeared to be more effective than other *Stachys* species (*S. annua* subsp. *cilicia*, *S. setifera* subsp.

lycia, *S. sosnowskyi*). These results demonstrated that *S. cretica* subsp. *anatolica*, *S. tmolea* and *S. iberica* subsp. *iberica* var. *densipilosa* were generally effective on Gram (+) bacteria (Table 3).

Tablo 2. The minimum inhibition concentration of aceton extracts of six *Stachys* sp.

	Minimum inhibitory concentration (MIC) (µl/ml)						
	1	**2**	**3**	**4**	**5**	**6**	**Aceton**
S. aureus	**>100**	**>100**	**>100**	**>100**	**>100**	**>100**	**>100**
S. epidermidis	**100**	**100**	**>100**	**>100**	**>100**	**100**	**>100**
B. cereus	**>100**	**>100**	**>100**	**>100**	**100**	**100**	**>100**
E. coli	**>100**	**>100**	**>100**	**>100**	**>100**	**>100**	**>100**
P vulgaris	**>100**	**>100**	**>100**	**>100**	**>100**	**>100**	**>100**
P. aeruginosa	**>100**	**>100**	**>100**	**>100**	**>100**	**>100**	**>100**

1: *S. annua* subsp. *cilicia*, 2: S. *setifera* subsp. *lycia*, 3: *S. sosnowskyi*, 4: *S. tmolea*, 5: *S. cretica* subsp. *anatolica*, 6: *S. iberica* subsp. *iberica* var. *densipilosa*

Tablo 3. The minimum inhibition concentration values of ethanol extracts of *Stachys* sp.

	Minimum inhibitory concentration (MIC) (µl/ml)								
	1	**2**	**3**	**4**	**5**	**6**	**Etanol**	**Cm**	**Tet**
S. aureus	**100**	**>100**	**100**	**12,5**	**6,25**	**12,5**	**100**	**15,63**	**7,81**
S. epidermidis	**50**	**100**	**50**	**12,5**	**6,25**	**25**	**100**	**15,63**	**7,81**
B. cereus	**50**	**100**	**100**	**25**	**12,5**	**12,5**	**100**	**3,90**	**15,63**
E. coli	**>100**	**>100**	**>100**	**50**	**25**	**>100**	**100**	**3,90**	**7,81**
P vulgaris	**>100**	**>100**	**>100**	**50**	**50**	**50**	**100**	**1,95**	**15,63**
P. aeruginosa	**>100**	**>100**	**>100**	**100**	**100**	**>100**	**100**	**NT**	**NT**

1: *S. annua* subsp. *cilicia*, 2: *S. setifera* subsp. *lycia*, 3: *S. sosnowskyi*, 4: *S. tmolea*, 5: *S. cretica* subsp. *anatolica*, 6: *S. iberica* subsp. *iberica* var. *densipilosa*. *Tet: Tetracycline, Cm: Chloramphenicol NT:not tested*

The data obtained in the study were conducted by disc diffusion method in order to determine the antimicrobial activity which is presented in Table 5. This data show that the ethanol extracts of three *Stachys* species have revealed strong effects on microorganisms. While extracts of aceton and hexane of six *Stachys* species did not

have any zones, ethanol extracts of three *Stachys* species had the diameter of the zone of ranging from 7-23 mm on microorganisms.

Tablo 4. Antimicrobial activities of leaves of six *Stachys* species (mm)

	Gram (+)						Gram (-)					
	B. cereus		*S. epidermidis*		*S. aureus*		*E. coli*		*P. aeruginosa*		*P. vulgaris*	
	10 µl	20µl	10 µl	20µl	10 µl	20µl	10 µl	20µl	10 µl	20µl	10 µl	20µl
H1	-	-	-	-	-	-	-	-	-	-	-	-
H2	-	-	-	-	-	-	-	-	-	-	-	-
H3	-	-	-	-	-	-	-	-	-	-	-	-
H4	-	-	-	-	8	10	-	-	-	-	-	-
H5	7	8	-	-	7	9	-	-	-	-	-	-
H6	-	10	-	-	10	11	-	-	-	-	-	-
HC	-	-	-	-	-	-	-	-	-	-	-	-
A1	-	-	-	-	-	10	-	-	-	-	-	-
A2	-	-	-	-	7	10	-	-	-	8	-	-
A3	-	-	-	-	-	8	-	-	-	-	-	-
A4	-	-	-	-	-	9	-	-	-	-	-	-
A5	-	8	-	-	-	-	-	-	-	-	-	-
A6	-	7	-	-	-	-	-	-	-	-	-	-
AC	-	8	-	8	-	-	-	-	-	8	-	-
E1	-	8	-	-	-	-	-	-	-	-	-	-
E2	-	9	-	-	-	-	-	-	-	-	-	-
E3	-	-	-	-	-	8	-	-	-	-	-	-
E4	9	12	14	22	15	20	10	13	-	9	-	10
E5	9	13	15	23	15	24	10	14	-	11	-	-
E6	7	12	9	15	14	19	9	11	-	13	9	11
EC	7	9	7	9	7	9	7	9	-	8	-	9
Tet		23		23		22		26		NT		23
Cm		35		38		36		35		NT		39

(-): no zone; NT: not tested, 1: *S. annua* subsp.*cilicia*, 2: *S. setifera* subsp.*lycia*, 3: *S. sosnowskyi*, 4: *S. tmolea*, 5: *S. cretica* subsp. *anatolica*, 6: *S. iberica* subsp. *iberica* var. *densipilosa*, *Tet: Tetracycline, Cm: Chloramphenicol, H; Hexane, A; Aceton, E; Ethanol, HC; Hexane control, AC, Aceton control, EC; Ethanol control.*

These *Stachys* species were less effective on Gram (-) bacteria than Gram (+) bacteria. When ***S. cretica* subsp. *anatolica*** was compared with the tested standard antibiotics, it seemed to be more effective on *S. aureus*. These results demonstrated that ***S. cretica* subsp. *anatolica*** endemic species contained more effective matter than antibiotics to combat with *S. aureus*. According to these results, it was observed that ***S. tmolea***, ***S. iberica subsp. iberica var. densipilosa*** and mainly ***S. cretica subsp. anatolica*** were quite serious candidates for the treatments of *Staphylococcus aureus* infections. Furthermore, the effects of *S. cretica* subsp. *anatolica* was determined to be more effective when it was compared to such antibiotics.

DISCUSSION

In studies with some of endemic *Stachys* species were determined to have a highly effective antimicrobial properties (Duarte et al. 2005, Saeedi et al. 2008). However, there is not enough information about antimicrobial acitivities of a member of this species. In particular, they may contain different chemicals and chemicals at different rates because of the climate features and geographical area that the endemic species grow, and this is quite natural. Climatic differences may change the amount and types of secondary metabolites of *Stachys* sp (Khanavi et al. 2009, Vundac et al. 2007). In the some study, the kind and the amount of content of *Stachys lavandulifolia* essential oils are analyzed, and when they are compared with the members of the same plant that grow in different regions, it was determined that they are quite different in accordance with their chemical compounds (Mahzooni-kachapi et al. 2012, Feizbaksh et al. 2003). In addition to, Vundac et al. demosntrated that the essential oil composition of two subspecies of *S. recta* (*S. recta* subsp. *recta* and *S. recta* subsp. *subcrenata*), growing under almost identical conditions have been found to be quite different (Vundac et al. 2006). Therefore, we studied the antimicrobial activity of the six *Stachys* endemic species in Turkey (Kütahya and Eskişehir) on some microorganisms.

Three different solvents for endemic *Stachys* sp. were used, because different secondary metabolites may be obtained from different solvents. Consequently, no antimicrobial activity was observed in the extract obtained from the hexane and acetone solvent, but extract obtained by the ethanol has been found to be highly effective on microorganisms. Three *Stachys* species (*S. cretica* subsp. *anatolica*, *S. tmolea* and *S. iberica* subsp. *iberica* var. *densipilosa*) from six endemic species in our study have been identified to be highly effective antimicrobial activity, especially on *S. aureus*.

In the literature, there are a lot of data on antimicrobial activity of some of the extract of endemic *Stachys* species obtained in different solvents, such as *S. sivasica, S. anumurensis, S. cydnia, S. aleurites, S. pinardii. S. lavondulifolia, S. byzantia, S. inflanta, S. laxa, S. pseudopinardii, S. woronowii, S. plumosa, S. alopecuros, S. scardia, S. cretica* subsp *cretica, S. germanica, S. recta, S. euboica, S. menthifola, S. cretica* subsp *anatolica, S. pumilia, S. schtschegleevii, Stachys annua* ssp. *annua* var. *annua, S. thirkei, S. officinalis, S. persica* (Abichandani et al., 2010, Mahzooni-kachapi et al., 2012, Dulger & Gonuz, 2004, Dulger et al., 2005, Skaltsa et al., 2003, Bulduruç et al., 2007, Sarac & Ugur, 2007, Kürşat & Erecevit 2009, Oztürk et al.

2009, Petrovic et al., 2005, Yildirim et al., 2012, Dığrak et al. 2001, Dulger & Aki 2009, Ünsal et al., 2010, Dulger & Dulger 2015, Jasbi et a.,l 2014, Taheri et al., 2014, Azami et al., 2016). Benli et al., (2007) demonstrated that the methanol extract of *Stachys cretica* subsp. *anatolica* was effective on only *B. subtilis* between the studied microorganisms (Benli et al., 2007).

In our study, *S. cretica* subsp. *anatolica* was determined to be quite effective on the *S. aureus* and *S. epidermidis*. In the literature, many researchers studied the antimicrobial properties of *Stachys* species by using different solvents. In these studies, there exists many different results. While some of the *Stachys* species are considered as quite effective against bacterial species, in some others it is not effective. Some of the types of *Stachys* were determined as effective against fungi (Mohammadi et al., 2014). The ethanol or methanol is used as a solvent for *Stachys* species, and the antimicrobial activity appears to be more effective. Ethanol was observed as the best solvent for extracting antimicrobial substances. These differences arises from the plants that grow in different geographical regions, from climatic differences and intraspecific or interspecific variation. This genus contains different natural products, including monoterpenes, sesquiterpenes, diterpenes, triterpene, flavonoids, biflavonoids, glycosides, phenolic acids (Goren et al., 2011, Vundac et al., 2006, Tundis et al., 2014). These substances are the reasons of antimicrobial activity.

CONCLUSION

This research showed that ethanol extracts of especially *S. cretica* subsp. *anatolica, S. tmolea* and *S. iberica* subsp. *iberica* var. *densipilosa* have the significant activity on Gram positive bacteria between studied microorganisms. Active substances in this endemic species identified as more effective than the ones that must be detected in the antibiotics. Especially, this chemical subtances can be used effectively in the fight against methicillin-resistant S*taphylococcus aureus* infections. For the first time in the literature, this study has demonstrated the antimicrobial activity of *S. tmolea* ethanol extract on *S. aureus*.

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