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Determination of Antimicrobial and Antioxidant Activities of Turkish Endemic *Salvia halophila* Hedge

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Abstract: In this study, the antioxidant activity of ethanol extract of *Salvia halophila* Hedge (Lamiaceae) was evaluated by 2 antioxidant assays, including phosphomolybdenum reduction and free radical scavenging activity. The ethanol extract showed free radical scavenging activity with IC_{50} of 67.73 µg/mL in 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. The antioxidant activity of the extract was found to be 84.87 ± 0.7 mg ascorbic acid equivalent (AAE)/g extract in phosphomolybdenum assay. In the addition to the antioxidant activity of the extract, the total phenolic content was measured. Total phenolic content of ethanol extract was found to be 8.21 ± 0.0 mg gallic acid equivalent (GAE)/g extract. Antimicrobial activity of the extract was examined against 13 bacteria and 2 yeasts by agar diffusion method. The ethanol extract of *S. halophila* had no effect against the tested yeasts. The ethanol extract showed antibacterial activity against all of the tested Gram (+) bacteria except *Listeria monocytogenes* at 1%, 2.5%, 5%, and 10% concentrations. The ethanol extract had no effect on the entire tested Gram (-) bacteria except *Aeromonas hydrophila* and *Pseudomonas aeruginosa*.

Key Words: *Salvia halophila* Hedge, antimicrobial and antioxidant activity, phenolic content, DPPH

Türkiye'de Endemik *Salvia halophila* Hedge'nin Antimikrobiyal ve Antioksidan Aktivitesinin Belirlenmesi

Özet: Bu çalışmada *Salvia halophila* Hedge (Lamiaceae)'nin etanollü özütlerinin antioksidan aktivitesi fosfomolibdenum redüksiyonu ve serbest radikal süpürücü aktivite olmak üzere 2 antioksidan analizi ile değerlendirilmiştir. 1,1-difenil-2-pikrilhidrazil (DPPH) analizinde, etanollü özüt IC_{50} = 67.73 µg/ml ile serbest radikal süpürücü aktivite göstermiştir. Fosfomolibdenum analizinde özütün antioksidan aktivitesi 84.87 ± 0.7 mg askorbik asit eşiti (AAE)/g özüt olarak bulunmuştur. Özütün antioksidan aktivitesine ilave olarak toplam fenolik içeriği ölçülmüştür. Etanollü özütün toplam fenolik içeriği 8.21 ± 0.0 mg gallik asit eşiti (GAE)/g özüt olarak bulunmuştur. Özütün antimikrobiyal aktivitesi 13 bakteri ve 2 mayaya karşı agar difüzyon metodu ile belirlenmiştir. *S. halophila*'nın etanollü özütü test edilen mayalara karşı etkili değildir. Etanollü özüt, % 1, % 2.5, % 5 ve % 10 konsantrasyonlarında *Listeria monocytogenes* hariç test edilen tüm Gram (+) bakterilere karşı antibakteriyal aktivite göstermiştir. Etanolü özüt, *Aeromonas hydrophila* ve *Pseudomonas aeruginosa* hariç test edilen Gram (-) bakterilere karşı etkili değildir.

Anahtar Sözcükler: *Salvia halophila* Hedge, antimikrobiyal ve antioksidan aktivite, fenolik içerik, DPPH

Introduction

In the recent years, essential oils and extracts of some plants have gained special interest as sources of natural antimicrobial and antioxidant agents. Since ancient times, spices and herbs have been in use for different purpose, such as food, beverages, perfumery, and drug (1). They were examined for their potential uses as alternative medicine for the treatment of many infectious diseases because of their resistance to antibiotics (2) and the preservation of foods from the side effects of lipid peroxidation during storage and processing (3). The oxidation of lipids is the major reaction responsible for the deterioration in food quality affecting the colour, flavour, texture, and nutritive value of the foods. Synthetic antioxidants are commonly used in foods to prevent or retard lipid oxidation. However, the commonly used synthetic antioxidants such as butylated hydroxy anisole (BHA) and butylated hydroxy toluene (BHT) are restricted by legislative rules because they are suspected to have some toxic effects and are possible carcinogens (4,5).

Salvia is the largest genus consisting of about 900 species, widespread throughout the world in the Lamiaceae family. This genus is represented in Turkish flora by 88 species and 93 taxa, 45 of which are endemic (6,7). Some of these species are economically important since they are used as spices and flavouring agents. Many species of *Salvia* have been used for alimentary, pharmacological, and cosmetic purposes (8,9). They are also used as traditional herbal medicine against a variety of diseases in various parts of the world including Turkey (10). *Salvia halophila* is endemic to Turkish flora (6). As far as our literature survey could ascertain, there are only a few reports about biological activity of *Salvia halophila* (11,12), although that of many *Salvia* species have been recognized by various researchers (13,14). Furthermore, the antimicrobial activity of *S. halophila* has not previously been published. The aim of the present study was to determine and compare the antimicrobial and antioxidant activities of *S. halophila* ethanol extract.

Materials and Methods

Plant materials

S. halophila Hedge was collected from the Aksaray Eskil, near to Tuz Gölü (Salty Lake), Central Anatolia region of Turkey, in May 2006 and authenticated by Dr. Ahmet Aksoy from the Department of Biology, Faculty of Art and Science, Erciyes University, Kayseri, Turkey. The voucher specimens were preserved at the Herbarium of the Department of Biology, Faculty of Art and Science, Erciyes University, Kayseri, Turkey (Voucher No: A.Aksoy 2083).

Preparation of the plant extracts

The aerial parts of the plant material were dried in shade at room temperature and then ground to a fine powder in a mechanic grinder. Then the powdered plant materials (10 g) were extracted with 100 ml of ethanol in a Soxhlet extractor. After the filtration of the solvent, the organic phases were independently concentrated under vacuum by evaporating to dryness. The residues were dissolved in the same solvent and stored at -20 °C until studied.

Total phenolic determination

Total phenolic compound amount in extract was determined by Folin-Ciocalteu method. The procedure of Singleton and Rossi (15) has been used. The extract at the concentration of 2 mg/ml was transferred into a test tube and then mixed thoroughly with 2.4 ml distilled water and 200 µl Folin-Ciocalteu reagent. After 30 s, 600 µl sodium carbonate (20% Na_2CO_3) and 760 µl distilled water were added and mixed. The absorbance of reaction mixture was measured at 765 nm against a methanol blank after the mixture was allowed to stand for 2 h at room temperature in the dark. The result is means of the 3 readings expressed as mg of gallic acid equivalents (GAE)/g extract.

Antioxidant assays

Phosphomolybdenum reduction

The antioxidant activity of the extract was evaluated by the phosphomolybdenum method according to the procedure of Prieto et al. (16). 0.4 ml of the extract (at the concentration of 2 mg/ml) was mixed with 4 ml of reagent solution containing sulphuric acid (0.6 M), sodium phosphate (28 mM), and ammonium molybdate (4 mM). The tubes were capped and incubated in an incubator at 95 °C for 90 min. After the samples were cooled to room temperature, the absorbance of mixture was measured at 695 nm. A typical blank solution contains 4 ml reagent solution and an appropriate volume of the same solvent was used for the extract. The mean of the 3 readings was used and expressed as mg of ascorbic acid equivalents (AAE)/g extract.

DPPH radical scavenging activity

The antiradical activity of the extract was assessed on the basis of the radical-scavenging effect of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical (17). A series of extract concentration in the same extraction solvent was prepared (2, 1, 0.5, 0.25, and 0.1 mg/ml). Then, 50 µl of extract at different concentrations was mixed with 450 µl Tris-HCL and 1000 µl of 0.1 mM DPPH in methanol. The disappearance of DPPH was read spectrophotometrically at 517 nm after 30 min of incubation at room temperature in the dark. The same solvent was used as a control instead of extract. The same procedure was repeated with methanolic solutions of synthetic antioxidant butylated hydroxytoluene (BHT) as positive control. The measurements were performed in triplicate and the results were averaged. Free radical scavenging capacity was expressed as percentage inhibition of DPPH radical and was calculated by the following equation:

I (%) = 100 × (1-Absorbance of sample/Absorbance of control)

From the obtained values, the IC_{50} (defined as the concentration of extract at which 50% of maximum

scavenging activity was recorded) was calculated for each extract.

Determination of antimicrobial effect

The test organisms used in this study were as follows: *Escherichia coli* ATCC 25922, *Proteus mirabilis* BC 3624, *Aeromonas hydrophila* ATCC 7965, *Bacillus subtilis* var. *niger* ATCC 10, *B. cereus* RSKK 863, *B. brevis* FMC 3, *Staphylococcus aureus* ATCC 25923, *Morganella morganii*, *Salmonella typhimurium* NRRLE 4463, *Listeria monocytogenes*1/2B, *Klebsiella pneumoniae* FMC 5, *Pseudomonas aeruginosa* ATCC 27853, *Yersinia enterocolitica* ATCC 1501, *Saccharomyces cerevisiae* BC 5461, and *Candida albicans* ATCC 1223.

Test yeasts, namely *C. albicans, S. cerevisiae*, and *Y. enterocolitica,* were grown in malt extract and nutrient broth (Merck) at 25 °C for 18 h. The other microorganisms were grown in nutrient broth at 35 °C for 18 h. All test microorganisms in nutrient broth or malt extract broth were enumerated using the serial dilution method. Their final cell concentrations were 10^6-10^7 cfu/ml. The agar diffusion method was used to detect the antimicrobial activity (18,19). Next, 250 µl of each microorganism was added into a flask containing 25 ml sterile Mueller-Hinton or malt extract agar (Merck) at 45 °C and poured into Petri dishes (9 cm diameter). Then the agars were allowed to solidify at 4 °C for 1 h. Four equidistant holes were made in the agar using sterile cork borers (Ø = 4 mm). The extracts (50 µl) were prepared at 1%, 2.5%, 5%, and 10% concentrations in the appropriate solvent and were applied to the holes using a pipettor. The extraction solvents without herb extract were used as a control. *Y. enterocolitica, C. albicans,* and *S. cerevisiae* were incubated at 25 °C for 14-24 h in the inverted position. The other microorganisms were incubated at 35 °C for 18-24 h. At the end of the period, inhibition zones formed on the medium were measured in millimetres (mm). All the tests were performed in duplicate and the results are presented as averages.

Results and Discussion

The percent yield of *S. halophila* ethanol extract was 36.35 g/100g dry herb. Folin-Ciocalteu colorimetric method is a rapid and widely used assay to investigate the total phenolic content (20). The total phenolic content of *S. halophila* ethanol extract was estimated by the Folin-Ciocalteu method as 8.21 ± 0.0 mg gallic acid equivalent (GAE)/g extract. Some, not all, phenolic compounds are very important plant constituents due to their potent antioxidant activities (21). The main antioxidant activity of *S. officinalis* was reported to be attributed mainly to its phenolic compounds, such as carnosic acid, carnasol, and rosmarinic acid (22). Akkol et al. (12) reported that the total phenolic content of hexane, ethyl acetate, methanol, %50 methanol, and aqueous extract of *S. halophila* were 29.2, 98.9, 73.2, 106.7, and 58.5 mg GAE/g extract, respectively. According to our findings, the total phenolic content of the ethanol extract was lower than that of other extracts studied by Akkol and co-workers (12). It is known that the Folin–Ciocalteu method gives different responses to different phenolic compounds, depending on the chemical structure (23).

The antioxidant activity of the ethanol extract was measured spectrophotometrically through DPPH free radical scavenging and phosphomolybdenum assay. The ethanol extract of *S. halophila* possesses free radical scavenging and antioxidant properties. The antioxidant activity of the ethanol extract was found to be 84.87 ± 0.7 mg ascorbic acid equivalent (AAE)/g extract in phosphomolybdenum assay. Aqueous alcohol is considered to be the best solvent for extracting phenolic compounds from plant materials (24).

In this study, the DPPH method was selected to evaluate the antioxidant activity of the *S. halophila* extract because it is one of the most effective methods for evaluating the concentration of radical scavenging materials that are very active in the chain breaking mechanism (25). DPPH free radical scavenging of *S. halophila* extract at 0.25 - 2 mg/mL concentrations was compared with BHT (Figure). At 0.25, 0.5, 1, and 2 mg/ml concentrations, DPPH scavenging activities of the ethanol extract were found as 2.81 ± 0.5, 11.16 ± 0.2, 24.69 ± 0.6, and 49.4 ± 0.6, respectively. The IC_{50} value, defined as the concentration of antioxidant required for 50% scavenging of DPPH radicals in this specified time period, is a parameter widely used to measure antioxidant activity; a smaller IC_{50} value corresponds to a higher antioxidant activity of the plant extract. IC_{50} value of the ethanol extract was 67.73 µg/ml. In the Figure, we observed that a dose-response relationship is found in the DPPH radical scavenging activity; the activity increased as the concentration increased. When compared to the BHT, the extract was found to be less efficient in the radical scavenging assay.

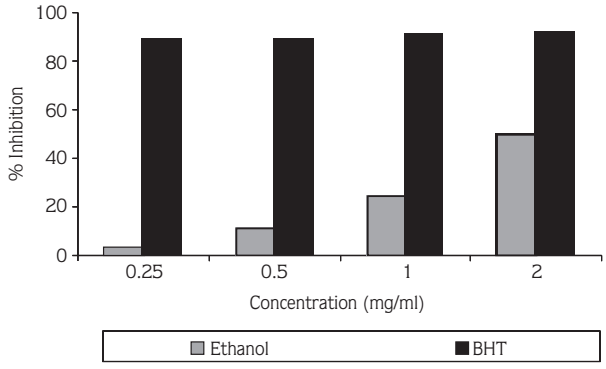

Figure. Antiradical activities of the *S. halophila* ethanol extract and BHT at different concentrations

The antioxidant and radical scavenging activities of many *Salvia* species have been thoroughly studied. In a previous paper, Bozan et al. (11) stated that the methanol extracts of *S. halophila* and other *Salvia* species exhibited antioxidant and free radical scavenging activity. Tepe et al. (14,26-28) reported that the essential oil and methanol extracts of many *Salvia* species from Turkey showed strong free radical scavenging activity. It was determined that the methanol extracts of *S. verticillata* subsp. *verticillata* and *S. verticillata* subsp. *amasiaca* had a strong free radical scavenging capacitiy with an IC_{50} value of 14.5 and 15.0 µg/ml, respectively (14). The same researcher and his co-workers reported that the essential oils, in particular, and non-polar subfractions methanolic extracts of *S. cryptantha* and *S. multicaulis* showed antioxidant activity in DPPH assay (26). Also, it was stated that the free radical scavenging activity of *S. tomentosa* aqueous methanol extract was superior to all other extracts prepared by using solvents of varying polarity (IC_{50} =18.7 µg/ml) (27). The methanol extracts of six *Salvia* species were screened for their possible antioxidant activities by Tepe et al (28) and it was determined that the most active plant was *S. euphratica* subsp. *euphratica*, an endemic species, with an IC_{50} value of 20.7 ± 1.22 µg/ml, followed by *S. sclarea* (IC_{50} = 23.4 ± 0.97 µg/ml) among the polar subfractions. DPPH scavenging activities of the extract of several *Salvia* species were evaluated previously by various researchers (13, 29). Zhao et al. (30) determined that *S. miltiorrhiza* extract possessed high scavenging activities against free radicals including superoxide anion, hydroxyl and DPPH radicals. Kamatou et al. (13) determined that the methanol extracts of *S. stenophylla*, *S. runcinata,* and *S. repens* collected from South Africa exhibited antioxidant activity with an IC_{50} value of 15.30 ± 3.21, 6.09 ± 4.63, and 8.52 ± 6.10 µg/ml, respectively.

Antimicrobial effect of the ethanol extract with the concentrations of 1%, 2.5%, 5%, and 10% were determined in vitro against 15 different microorganisms using agar diffusion method. Antimicrobial effects of the ethanol extract vary depending on the test organisms (Table). The ethanol extract of *S. halophila* had no effect against the tested yeasts. The ethanol extract showed antibacterial activity against all of the tested Gram (+) bacteria except *L. monocytogenes* at 1%, 2.5%, 5%, and 10% concentrations. The ethanol extract had no effect on the entire tested Gram (-) bacteria except *A. hydrophila* and *P. aeruginosa*. The extract had an antibacterial effect against *P. aeruginosa* only at 10% concentration.

As clearly seen in the Table, the extract of *S. halophila* was ineffective against many organisms tested especially against Gram (-) bacteria and yeasts. *B. cereus* and *B. subtilis* var. *niger* were more susceptible to all concentrations of the *S. halophila* ethanol extract than the other strains of the bacteria tested. Pure solvent used in this study had no inhibitory effects on the microorganisms tested. In this study, the antimicrobial activity of the extract was compared to standard antibiotics (Oxoid) such as Vancomycin (VA-30µg), Kanamycin (K-30µg), Amoxycillin (AML-25µg), and Gentamisin (CN-10µg), all of which were used as positive controls.

Several studies have been conducted for antimicrobial activities of essential oils and extracts of many *Salvia* species (26,27,31,32). As far as our literature survey could ascertain, there is no study about antimicrobial activity of *S. halophila* extract. No or slight activity was observed when the polar and non-polar subfractions of the methanol extracts of *S. cryptantha* and *S. multicaulis* were tested, whereas essential oils exhibited antimicrobial activity (26). It is reported that the essential oil of *S. tomentosa* was particularly found to possess strong antimicrobial activity while other non-polar extracts and subfractions of *S. tomentosa* showed moderate activities and polar extracts remained almost inactive (27). In a previous study, it is reported that the essential oil of *S. tomentosa* had remarkable activity against all the tested bacteria except for *P. aeruginosa* (31). Delamare et al. (32) reported the essential oils of *S. officinalis* and *S. triloba* exhibited remarkable bacteriostatic and bactericidal activities against *B. cereus*, *B. megaterium*, *B. subtilis*, *A. hydrophila*, *A. sobria,* and *K. oxytoca*. It was stated that *S. jaminiana*

Table. Antimicrobial activity of *S. halophila* ethanol extract at different concentrations (inhibition zones, mm).

Microorganisms	(%) Concentrations of the ethanol extract				Antibiotics (µg)			
					VA	K	AML	CN
	10	5	2.5	1	30	30	25	10
Gram (-)								
A. hydrophila	6.0 ± 0.0[a]	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	18.0 ± 0.0	13.0 ± 0.0	33.0 ± 0.0	8.5 ± 0.0
E. coli	-	-	-	-	7.0 ± 0.0	7.0 ± 0.0	12.0 ± 0.0	9.0 ± 0.0
M. morganii	-	-	-	-	7.0 ± 0.0	6.5 ± 0.0	-	-
K. pneumoniae	-	-	-	-	17.0 ± 0.0	11.0 ± 0.0	16.0 ± 0.0	6.5 ± 0.0
Y. enterocolitica	-	-	-	-	8.5 ± 0.0	14.0 ± 0.0	13.0 ± 0.0	9.0 ± 0.0
P. mirabilis	-	-	-	-	-	13.0 ± 0.0	31.0 ± 0.0	8.0 ± 0.0
P. aeruginosa	5.0 ± 0.0	-	-	-	6.5 ± 0.0	12.0 ± 0.0	30.0 ± 0.0	12.0 ± 0.0
S. typhimurium	-	-	-	-	-	10.0 ± 0.0	29.0 ± 0.0	8.0 ± 0.0
Gram (+)								
B. cereus	6.5 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	5.0 ± 0.0	19.0 ± 0.0	15.0 ± 0.0	34.0 ± 0.0	11.0 ± 0.0
B. brevis	6.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	27.0 ± 0.0	20.0 ± 0.0	12.0 ± 0.0	16.0 ± 0.0
B. subtilis var. *niger*	6.5 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	5.0 ± 0.0	13.0 ± 0.0	16.0 ± 0.0	7.0.0 ± 0.0	10.0 ± 0.0
S. aureus	6.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	14.0 ± 0.0	12.0 ± 0.0	6.5 ± 0.0	10.0 ± 0.0
L. monocytogenes	-	-		-	24.0 ± 0.0	15.0 ± 0.0	33.0 ± 0.0	13.0 ± 0.0
Yeasts								
C. albicans	-	-	-	-	nt	nt	nt	nt
S. cerevisiae	-	-		-	-	-	-	-

-: not detected;
nt: not tested;
VA: Vancomycin, K: Kanamycin, AML: Amoxycillin, CN: Gentamisin;
[a]the zone of inhibition including diameter of hole (4 mm) and disc (6 mm), mean value ± SD

inhibited the growth of *B. subtilis*, *S. aureus*, and S*treptococcus alfa*-hemolitic (33). Kamatou et al. (34) reported that *S. chamelaeagnea* extract had good antibacterial activity against 2 Gram positive bacteria (*B. cereus* and *S. aureus*) and 2 Gram negative bacteria (*E. coli* and *K. pneumoniae*).

Our results suggest that the *S. halophila* ethanol extract may be used as a natural antioxidant and as antimicrobial agents. Further studies are warranted for the isolation and identification of individual phenolic compounds and also in vivo studies are needed for understanding their mechanism of action as an antioxidant and antimicrobial agent better.

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