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The Determination of Phytochemical Properties and Antimicrobial Effect of *Salvia verticillata L. subsp. amasiaca*

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***Abstract*—** *People are constantly interested in tasting, smelling and consuming flavors and aromas obtained from natural sources. At the same time, their health benefits in addition to their flavors and smells increase this interest even more. Salvia verticillata L. is one of these sources known for centuries. Salvia verticillata L. is a species belonging to the Lamiaceae family, Salvia (Lamiaceae) genus. It is widely used in the Mediterranean region not only as an ornamental plant but also in traditional medicine. In this study, the antioxidant activity, essential oil composition and antimicrobial activity of Salvia verticillata L. subsp. amasiaca were determined. While the phenolic substance concentration equivalent to gallic acid was found to be 431.70 mg, the phenolic substance concentration equivalent to ascorbic acid was found to be 351.97 mg. Copper reducing capacity was determined as 112 mmol/g and Carotene-linoleic acid emulsion as 16.35 percent, the major volatile oil ratio was found to be trans-trans-caryophyllene with 35%. It was found to be significantly effective against E. fecalis, S. aureus, P. aeruginosa among the studied microorganisms, but not against E. Coli.*

I. INTRODUCTION

The genus *Salvia L. (Lamiaceae),* comprising 900 to 1,000 species, ranks as the seventh largest genus in its family. It is part of the subfamily Nepetoideae and the Chloanthi clade. This diverse group is broadly distributed across regions including Asia, Europe, Africa, Central America, and North America. Numerous species within the genus possess pharmacological properties and are widely utilized in industries such as scent, flavoring, natural ingredients, pharmaceuticals, and cosmetics industries [1-4]. *Salvia verticillata L.* is native to southeastern Europe and is particularly prevalent in regions like Turkey, Albania, and Greece, where it disperses and undergoes evolutionary adaptationand in Turkey, the rate of endemism in genus *Salvia* is 50%. The genus *Salvia* L. is highly diverse, encompassing a range of growth forms including annuals, biennials, short-lived perennials, shrubs, subshrubs, chamaephytes, and even trees, allowing it to thrive in varied ecological and geographic conditions [5-9]. Species of *Salvia L.* have traditionally been valued as medicinal plants across various cultures. They have been used to treat a range of conditions, including infections, inflammation, fever, peptic ulcers, cough, diarrhea, vomiting, liver disorders, arthritis, and rheumatoid pain. Additionally, *Salvia* leaves have served as hair treatments, natural sweeteners, spices, and condiments. The genus has also been used to enhance the flavor of medications, improving their acceptance among patients. *Salvia* verticillata L., in particular, is

widely cultivated in gardens for its striking tubular flowers, aromatic leaves with mild essential oils, a delicate scent, a slightly bitter note, and its extended flowering period [10-12]. In this study; the antioxidant activities, antimicrobial effects, phenolic composition and essential oil composition of *Salvia verticillata L. subsp. amasiaca* grown in Hatay were determined. Specimens of *Salvia verticillata L. subsp. amasiaca* were obtained and identified by Dr. Yelda Güzel, a faculty member of the Biology Department of Mustafa Kemal University, from the Yayladağı region of Hatay. The antioxidant capacities were evaluated by using phosphomolybdenum method, cupric reducing antioxidant capacity (CUPRAC) and β-carotene-linoleic acid emulsion method. Results were compared against reference synthetic antioxidants (BHA, BHT).

II. MATERIAL AND METHODS

2.1. Preparation of Plant Extracts

After the plants provided in dry form were cut into small pieces with scissors, they were crushed and turned into powder. In order to perform antioxidant analyses, methanol extraction was performed on 30 g of powdered plant samples. For this, the samples were subjected to extraction in 70% methanol at 40ºC for 3 hours at first. This process was repeated 3 times by filtering each time. The filtrates were combined and removed under vacuum in the evaporator at 40ºC. After this process, the remaining light methanol part was taken into petri dishes in small volumes and subjected to lyophilization. Thus, the remaining methanol was completely removed at low atmospheric pressure and low temperature. The extract, which was completely dried by lyophilization, was stored at +4ºC until analyzed [1].

2.2. Antioxidant Activity

2.2.1. *Determination of Total Phenolic Substance Content*

Phosphomolybdenum Method

In addition to the Folin method, the total antioxidant capacity of the sample was also determined by the phosphomolybdenum method according to the method of Prieto et al. (1999) [13]. For this purpose, sulfuric acid, sodium phosphate, ammonium molybdate, ascorbic acid, methanol, Hitachi U.V. U1900 spectrophotometer, water bath are the chemicals and devices used. Reagent solution containing 0.6 M H_2SO_4, 28 mM Na_3PO_4 and 4 mM $(NH_4)_6Mo_7O_{24}$ (ammonium molybdate) was prepared and 3 ml of reagent solution was added to 0.3 ml of sample at a concentration of 0.4 mg/mL. After the tubes were closed, they were incubated at 95ºC for 90 minutes. The samples were cooled to room temperature and read against the control sample (0.3 ml methanol, 3 mL indicator solution) at a wavelength of 695nm. Results are given as mg ascorbic acid equivalent/gram extract.

2.2.2. CUPRAC Method

Neocuproine (2.9-dimethyl-1.10-phenanthroline), BHT, BHA, Trolox, (SigmaAldrich), $CuCl_2.2H_2O$, methanol, ethanol, ammonium acetate (Merck) were the chemicals used and Hitachi U.V. U-1900 spectrophotometer was used for measurements. The method was applied by making some changes in the method of Apak et al. (2006) [14]. 0.5 mL of plant extracts with a concentration of 0.0025 mg/ml were added to the tubes that were shaken and 1 mL of 10^{-2} M $CuCl_2$, 1 mL of $7.5x10^{-3}$ M Neocproine solution and 1 mL of 1M (pH=7) NH_4Ac buffer were added, and 0.6 mL of deionized water+trolox was added to make the total volume 4.1 mL. After the tubes were kept at room temperature for 30 minutes with their lids closed, absorbance values were measured at 450 nm. The solution prepared in the same way for all procedures but without the sample was used as a blank. Trolox solution was used as a standard at a concentration of $1.0x10^{-3}$M. After a 1:1 dilution process was performed at five different concentrations, a standard curve was created. The dry matter antioxidant capacities of each plant in terms of trolox equivalent were calculated using the calculation method given below. Since the % moisture content is different in each plant, % dry matter values were taken into account in the calculation of capacity values.

$$TEAC_{(mmol/g)} = \frac{\text{Absorbance x 4,1 x 100 x 100 x 1}}{1.67 \times 10^4 \text{ x } 0.5 \text{ x } 10 \text{ x Drog Weight (g) x Dry Matter \%}}$$

2.2.3. β- Carotene-Lineolic Acid Emulsion Method

The method was performed according to the β-carotene linoleic acid emulsion system method of Kaur and Kapoor (2002) [15]. Chloroform ($CHCl_3$), β-carotene, linoleic acid, tween 40 (SigmaAldrich), evaporator (Buchi), water bath (JSR, Korea), Hitachi U.V. U-1900 spectrophotometer are the devices and chemical materials used in the method. First, β-carotene linoleic acid emulsion solution was prepared. For this process, 0.2 mg β-carotene was dissolved in 1 ml chloroform. 0.02 ml of 60% lineolic acid solution and 200 mg Tween 40 were added. Chloroform was completely removed in the evaporator under vacuum at 40ºC. 100 ml oxygenated and dissolved in deionized water stirred vigorously. The concentration of the sample and the synthetic antioxidants BHA and BHT prepared for comparison was prepared in 70% methanol to be 1 mg/ml. 0.2 ml of each BHA and BHT solution was taken into the test tubes and 5 ml of the prepared emulsion solution was added to them. Methanol was used as the control sample. 5 ml of β-carotene linoleic acid emulsion solution was added to the tube into which 0.2 ml of methanol was taken. The absorbance of the samples in the test tubes and the control

solution was read at 470 nm (A_0). Immediately afterwards, it was left for incubation in a water bath at 50°C from this moment on, the absorbance of the solutions in the incubation was read every 15 minutes for a total of 120 minutes. Based on these absorbances, the absorbance change rate (AR) and, accordingly, the oxidation inhibition coefficients were calculated.

$$\text{Rate of absorbance change (AR)} = \frac{\ln(A_0 / A_t)}{t} \qquad t = 120 \text{ minute}$$

$$\text{Oxidation prevention \%} = \frac{AR_{Control} - AR_{Sample}}{AR_{Control}} \times 100$$

2.3. Essential Oil Composition

The oil in the plant was obtained with the clevenger apparatus to be used in essential oil analysis. For this purpose, approximately 50 g of the substance was taken into a one liter flask and subjected to water vapor distillation with half the volume of water in the flask for 6 hours. The essential oil obtained at the end of distillation was taken into a vial and stored at +4°C until the analysis time. The sample was analyzed with the Thermo Scientific Focus (ISQ Single Quadrupole) brand GS-MS device. Helium gas with 99.9% purity, 1 ml/min flow range and 70 eV ionization energy was used as the carrier gas. MS transfer temperature was set as 250°C and injection temperature as 220°C. The sample was injected in 1 µl volume at 250 split range. The oven temperature was increased from 50°C to 220°C with an increase of 3°C/min. The structure of each compound was determined by comparing the mass spectra of these compounds with the data obtained using the Xcalibur software program [16].

2.4. Antimicrobial Activity

The antimicrobial activity of *Salvia verticillata L. subsp. amasiaca* species was determined by disk diffusion method (CLSI). In this method, the sample was increased at a concentration of 100 mg/mL and the analysis was carried out in triplicate. DMSO (dimethylsulfoxide, Merck) was used as solvent and amikacin (Oxoid, 30 µg) was used as standard antibiotic. In the analysis, four bacterial species, two Gram positive *(Staphylococcus aureus (ATCC 29213), Enterococcus faecalis* (ATCC 29212)), two Gram negative *(Escherichia coli (ATCC 35218), Pseudomonas aeruginosa (ATCC 27853)*) and one yeast (*Candida albicans* ATCC 14053)) were recorded. For

Staphylococcus aureus, Enterococcus faecalis Bloody medium (Biomerieux), for *Escherichia coli, Pseudomonas aeruginosa* EMB (Eosin-Methylene blue) medium (Biomerieux), for Candida albicans SDA (Sabouraud Dextrose Agar) medium (Merck) album. For antimicrobial activity studies, firstly, bacterial stocks were cultured on suitable media. After culture, plants were incubated at 37°C for 24 hours. Then, bacterial potential was prepared according to McFarland 0.5 capacity standard and cultured on Müller Hinton medium. Three media were prepared for bacteria and 40 µl, 80 µl and 120 µl sample impregnated disks were placed on the medium. Inhibition zones formed around healthy discs against the characteristics of the sample were determined in mm. In addition, discs containing only DMSO were recorded as negative controls.

III. RESULTS AND DISCUSSION

3.1. Determination of Total Phenolic Substance Content

3.1.1.Phosphomolybdenum Method

The total phenolic substance amount of the sample was determined by the phosphomolybdenum method with both gallic acid and ascorbic acid standards. For this purpose, ascorbic acid calibration curve was drawn and the total phenolic substance amount was calculated in terms of ascorbic acid and gallic acid from these calibration graphs (Table 1).

Table.1: Total phenolic substance amount of Salvia verticillata L. subsp. amasiaca

Total Phenolic Substance Content	
Gallic acid equivalent	Ascorbic acid equivalent
431.70 mg GAE/g extract	351.97 mg AAE/g extract

3.2. CUPRAC Method

Trolox was used as a standard for copper ion reducing activity. Calibration graph of Trolox was drawn.

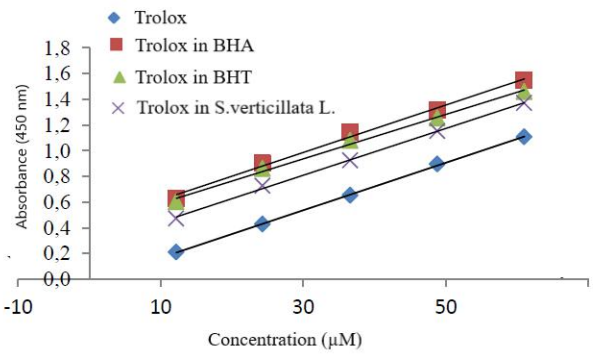

Fig.1: Comparison of antioxidant capacities of S. verticillata L. subsp. amasiaca and synthetic antioxidants.

Table.2: TEAC values of Salvia verticillata L. subsp. amasiaca and synthetic antioxidants.

Sample	**TEAC (mmol/g)**
BHA	0.760
BHT	0.717
Salvia verticillata L. subsp. amasiaca	0.112

High TEAC value indicates high antioxidant capacity. The values found are given in Table 2 and these values show that the synthetic antioxidant BHA has the highest reducing power.

3.3. β- Carotene-Lineolic Acid Emulsion Method

In the study, absorbance change rates and inhibition rates were determined at each time interval from the time-dependent absorbance values measured. Antioxidant activities against air and heat oxidation were determined according to the color fading in the mixtures. The higher the % inhibition values, the higher the oxidation inhibition activity. In other words, it is a strong antioxidant. At the end of this experiment, it was seen that the highest antioxidant capacity belonged to BHA, BHT and *S.verticillata L*. subsp. *amasiaca*, respectively.

Tablo.3: Absorbance change rates and % inhibition values of Salvia verticillata L. subsp. amasiaca and synthetic antioxidants

Sample	**Absorbance change rate**	**Inhibition %**
BHA	0.001252	93.35
BHT	0.001988	89.43
S. verticillata L. subsp. amasiaca	0.015744	16.35

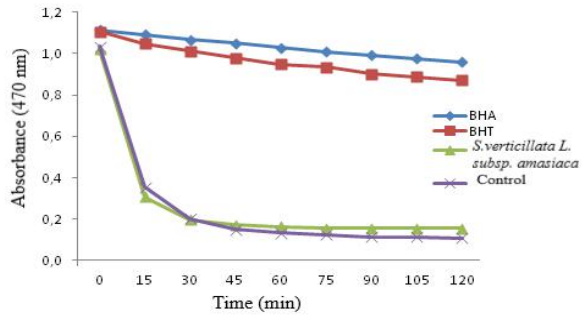

Fig.2: Change in linoleic acid peroxidation of samples with time

3.4. Analysis of Essential Oil Composition

The essential oil composition of the sample was determined by GS-MS and the chromatogram obtained for this purpose is given in Figure 3. The results of the essential oil components determined by the data obtained from this chromatogram according to retention times are given in Table 4.

Table 4: Essential oil composition of Salvia verticillata L. subsp. amasiaca species

Retention Time (min)	Retention index	Area %	Component
3.71	1033	0.54	delta-3-carene
5.24	1117	3.38	beta-pinene
5.55	1131	0.08	alpha-phellandrene
6.2	1159	0.31	sabinene
6.61	1174	0.11	alpha-myrcene
7.53	1205	0.68	dl-limonene
7.8	1216	0.18	gama-terpinene
16.41	1460	0.89	alpha-copaene
17.64	1490	0.98	alpha-cubebene
18.64	1517	2.55	beta-bourbone
19.04	1528	7.89	alpha-gurjunene
20.7	1572	1.59	alpha-ylangene
21.36	1588	4.74	germacrene-d
21.56	1593	35.07	trans-caryophyllene
23.72	1652	0.03	junipene
24.23	1665	7.07	alpha-humulene
24.33	1668	0.86	epi-bicyclosesquiphellanderene
25.02	1686	1.46	alpha-amorphene
25.7	1703	10.98	germacrene-d
25.87	1708	0.14	aromadendrene
26.61	1730	0.09	bicyclogermacrene
27.53	1756	1.75	delta-cadinene
30.18	1830	0.03	cis-calamenene
32.03	1883	0.06	cubenol
33.17	1917	0.1	butyl hydroxy
34.98	1972	5.81	caryophyllene oxide
35.86	1997	0.48	ethylchrysanthemu
36.82	2029	0.58	huuladenone
42.89	2246	0.46	beta-cadinol

46.44	2350	1.06	spathulenol
55.78	2693	5.4	myristic acid

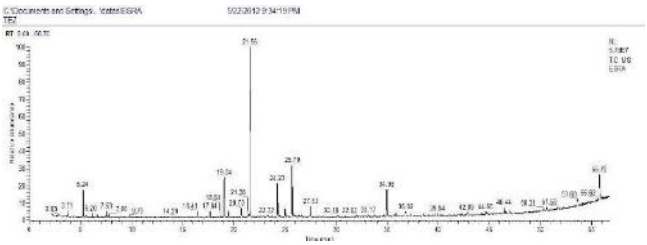

Fig.3: GS-MS chromatogram showing the essential oil composition of the sample

GS-MS analysis of *Salvia verticillata L. subsp. amasiaca* revealed that it had 31 different oil components and the main components were trans-caryophyllene (35.07%), germacrene-d (10.98%) and caryophyllene oxide (5.81%). Chromatograms of the components are given in Figure 3 and retention times of the components are given in Table 4 in detail.

3.5. Antimicrobial Activity Determination Method

Antimicrobial activity results The diameters of the inhibition zones formed by *Salvia verticillata L. subsp. amasiaca* against each microorganism were determined in mm. The lengths of the inhibition zone diameters formed by the standard antibiotic (Amikacin 30 µg) used in the tests against the microorganisms were also determined and are given in Table 5. It was determined in the study that DMSO did not form an inhibition zone.

Table 5: Effect of amikacin against the microorganisms used.

Microorganism	Zone Diameter (mm)
Staphylococcus aureus	23
Enterococcus faecalis	28
Escherichia coli (ATCC	20
Pseudomonas	21
Candida albicans	0

The inhibition zones created by the sample against bacteria in the analysis and the lengths of these zones (mm) are given in Table 6.

Table 6: Inhibition zone diameters of the sample against microorganisms (mm).

Staphylococcus aureus	disk vol(µl)	40	80	120
	Zone(mm)	24.7±1.7	30.4±1.5	32.6±1.9
Enterococcus faecalis	Disk vol(µl)	40	80	120
	Zone(mm)	21.2±0.9	20.6±1.3	23.6±0.9
Escherichia coli	disk vol(µl)	40	80	120
	Zone(mm)	-	-	-
Pseudomonas aeruginosa	disk vol(µl)	40	80	120
	Zone(mm)	23.1±1.1	26.4±0.9	31.5±2.6
Candida albicans	disk vol(µl)	40	80	120
	Zone(mm)	-	-	-

(- : no zone observed)

As seen in the Table 6, *Salvia verticillata L. subsp. amasiaca* is seen to be effective against each bacteria at different levels. In addition, as the amount of sample impregnated on the discs increased, the diameter of the formed inhibition zone also increased. It was determined that the amount of sample and the inhibition zone diameter were directly proportional. However, it was determined that *Salvia verticillata L. subsp. amasiaca* was not effective against Escherichia coli and Candida albicans.

As a result of the analyses, the antioxidant, antimicrobial capacity, phenolic substance composition and essential oil components of *Salvia verticillata L. subsp. amasiaca* species, a member of the sage family, were determined. As a result of the study, it was determined that the antioxidant and antimicrobial effects of the sample were considerably strong and that it had a rich component content in terms of phenolic content and essential oil. According to the total phenolic compound method which is intended to determine

the total phenolic substance amount in the sample, the total phenolic substance amount contained in *Salvia verticillata L. subsp. amasiaca* species was found to be 351.94 mg AAE/g extract and 431.7 mg GAE/g extract according to the phosphomolybdenum method. Along with all other species of the *Salvia* family, it is considered a promising source of polyphenols with remarkable diversity in both chemical structure and biological activity. In previous studies on *Salvia* species, spectrophotometric determination of total phenols, phenolic acids and/or flavonoids has been frequently performed and a direct relationship has been established with antioxidant activity. The results of total phenolic substance content in terms of gallic acid and ascorbic acid also explain this [17-20]. High TEAC value obtained in the CUPRAC method indicates high reduction. Copper ion reducing capacity is higher than the sample in synthetic antioxidants. These values were found as 0.760; 0.717; 0.112 for BHA, BHT and *Salvia verticillata L. subsp. amasiaca*, respectively. Therefore, copper ion reducing capacity is BHA>BHT>*Salvia verticillata L. subsp. amasiaca*. The results obtained in β-carotene Linoleic acid emulsion system were consistent with the results of the analyses and the % inhibition value was found as BHA>BHT>*Salvia verticillata L. subsp. amasiaca*. Considering these data, it has been determined that *Salvia verticillata L. subsp. amasiaca* species can prevent the formation of damaged cells caused by free radicals and therefore can be used as an alternative to synthetic antioxidants that have carcinogenic effects in the protection of human health. The *Salvia* genus is a species rich in essential oil content. Therefore, the use of this species is widespread in cosmetics, perfumery and food industry. In this study, it was determined that *Salvia verticillata L. subsp. amasiaca* species had 31 different essential oil components as a result of GS-MS analysis and therefore it was concluded that it is one of the species that can be widely used in the mentioned areas. According to the data obtained as a result of the essential oil analysis of the sample, its main components were determined to be trans-caryophyllene (% 35.07), germacrene-d (% 10.98) and caryophyllene oxide (% 5.81). Antimicrobial activity analysis of *Salvia verticillata L. subsp. amasiaca* was studied by disk diffusion method and its effect against four bacteria and one yeast species was tested. Multiple previous studies have shown antibacterial activity of Salvia spp. Predominantly against Gram-positive strains, generally attributed to the rich content of polyphenolic active compounds, which supposedly mechanism of action consists in damaging the structure of the cellular membrane and its functions, consequently. A possible explanation for the poor susceptibility of the Gram-negative bacteria to various extracts and antibiotics would be the barrier formed against lipophilic compounds, due to the lipopolysaccharides and lipoproteins present in their cellular walls [21-25]. In this study, it was understood that *Salvia verticillata L. subsp. amasiaca* was ineffective against the gram-negative *bacteria E Coli*. When the results were taken into consideration, it was seen that it showed significant effect against *E. fecalis, S. aureus, P. aeruginosa* from the studied microorganisms, but it was not effective against C. albicans and *E. Coli*. When the analysis results were evaluated, it could be concluded that *Salvia verticillata L. subsp. amasiaca* could be evaluated as an alternative in the development of drugs that can be used in the treatment of diseases caused by *E. fecalis, S. aureus and P. aeruginosa.* Members of the plant genus Salvia have a long and rich history of use as both culinary and medicinal herbs [26-27]. *Salvia* species, perhaps one of the most well-known members of the medicinal aromatic plant family, can be used both in the kitchen and as a medicinal plant. It is clear that similar studies will be useful in revealing the medicinal aromatic properties of *Salvia* species.

Statistical Analysis

All the above-mentioned experiments were carried out in triplicates and the results are expressed as mean ± standard deviation. The differences between the obtained results were determined by the ordinary one-way ANOVA and post hoc Tukey's multiple comparisons test. Pearson's correlation coefficient was calculated to find the degree of association between the two variables. All statistical analyses were accomplished by the GraphPad Prism software (version 8.4.3) as well as Microsoft Excel. All values with $p<0.05$ were considered statistically significant.

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