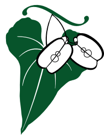
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Original Scientific Paper

Phytochemical analysis and biological activities of *Salvia candidissima* subsp. *candidissima* mericarps

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ABSTRACT:
The sterol, amino acid, mineral, total phenolic, and total flavonoid contents, and the antioxidant, antimicrobial, and antibiofilm activities of *Salvia candidissima* subsp. *candidissima* mericarps were investigated. The mericarps were collected in the province of Bayburt, Turkey. Gas chromatography was used for sterol analysis, Prominence ultra-fast liquid chromatography for amino acid analysis, and ICP-MS for mineral analysis. Folin-Ciocalteu, $Al(NO_3)_3$, and DPPH radical scavenging activity assays were performed on ethanol extracts of the mericarps to evaluate the total phenolic and flavonoid contents, and antioxidant potential, respectively. The ethanol and hexane extracts of the mericarps were tested for their antimicrobial activity against seven bacterial and three fungal strains using the microdilution method and for antibiofilm activity against *Pseudomonas aeruginosa* biofilm using the crystal violet staining method. The sterol, amino acid, and mineral contents and antibiofilm activity of the mericarps were studied for the first time. The most abundant components of the mericarps are determined as β-sitosterol (69.8%), glutamic acid (4895 mg/100 g), and potassium (11474 µg/g). The results of total phenolic and flavonoid contents and DPPH free radical scavenging activity were 3.47 µg GAE/mg extract, 0.35 µg QE/mg extract, and 79.79%, respectively. Both of the tested extracts exhibited antimicrobial activity against all the tested microorganisms, however, the efficiency of the extracts was not as strong as the reference drugs ampicillin and fluconazole. While both extracts were effective in preventing biofilm formation, the ethanol extract was found to be more effective than the hexane extract in preformed biofilm inhibition. In conclusion, *S. candidissima* subsp. *candidissima* mericarps have good nutritional potential with high amounts of amino acids, sterols (especially β-sitosterol), minerals, and phenolics and flavonoids. Additionally, our findings provide important preliminary data for the literature in terms of the antibiofilm activity of *Salvia candidissima* subsp. *candidissima* mericarps.

Keywords:
Salvia candidissima subsp. *candidissima*, mericarp, chemical content, DPPH, antimicrobial activity, antibiofilm activity

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INTRODUCTION

Salvia (sage) is the most species-rich genus in Lamiaceae, comprising approximately 1000 species, more than half of which are found in North and South America (Drew *et al.* 2017). *Salvia* species have been traditionally used in the treatment of colds, aches and pains, infections, wounds, bronchitis, flu, tuberculosis, haemorrhage, insomnia, some cardiovascular and menstrual disorders, and stomach, liver, and rheumatism pains (Azcan *et al.* 2004; Topçu *et al.* 2017; Adimcilar *et al.* 2019). Because of their bioactive constituents such as terpenoids, phenolics, essential oils, and fatty acids (Pitarokili *et al.* 2006; Bakoglu *et al.* 2016; Hatipoglu *et al.* 2016; Topçu *et al.* 2017) as well as their medicinal properties such as antimicrobial, antioxidant, antidiabetic, anti-inflammatory, cardiotonic, neuroprotective, antiacetylcholinesterase, anti-apoptotic, antiglycating, antitumor, and antimalarial activities (Tepe *et al.* 2006; Sezer Şenol *et al.* 2010; Bahadori *et al.* 2016; Adimcilar *et al.* 2019), various *Salvia* species have great importance in the pharmaceutical, food (e.g. food preservatives, herbal teas, spices, and flavourings), perfume, and cosmetic industries (Bayar & Genç 2018; Adimcilar *et al.* 2019).

The genus *Salvia* has schizocarp fruit. The schizocarp fruit which is known as the mericarp or/and nutlet consists of indehiscent locules separating to form four fruitlets. Each mericarp possesses a stratified pericarp including the cuticle, epicarp, mesocarp, a layer of bone cells, and the endocarp (Segura-Campos *et al.* 2013).

After Mexico, Turkey has the second largest number of *Salvia* species in the world with approximately 100 species of which almost 53 are endemic (Hatipoglu *et al.* 2016). The aromatic perennial herb, *Salvia candidissima* Vahl. subsp. *candidissima,* has ovate leaves, white bilabiate corolla with a yellow lower lip, widely elliptic to obovate nutlets, and colliculate nutlet surface sculpturing (Celep *et al.* 2020). *Salvia candidissima* subsp. *candidissima,* which is known as "galabor" in east Anatolia, has traditional uses as a stimulant, carminative, and appetiser, and infusions of the plant leaves are also used for treating the common cold (Unal *et al.* 2008; Tuzlaci & Doğan 2010; Altindag & Ozturk 2011). The presence of phenolics, terpenoids, essential oil, fatty acids and volatile organic compounds in the roots and aerial parts of the plant has been previously reported (Ulubelen *et al.* 1997; Öztürk *et al.* 2011; Bakoglu *et al.* 2016; Hatipoglu *et al.* 2016; Bayar & Genç 2018; Adimcilar *et al.* 2019). Some biological activities including antioxidant, antidiabetic, anticholinesterase and antimicrobial have also been investigated (Unal *et al.* 2008; Hatipoglu *et al.* 2016; Adimcilar *et al.* 2019). According to the literature, the phenolic (Emre *et al.* 2021), fatty acid (Azcan *et al.* 2004; Kilic *et al.* 2005; Gören *et al.* 2006), alpha-tocopherol, vitamin (Sari *et al.* 2009) and malondialdehyde contents and antimicrobial (Kursat *et al.* 2012; Emre *et al.* 2021) and antioxidant activities (Emre *et al.* 2021) of *S. candidissima* subsp. *candidissima* mericarps have been investigated. However, no study has been performed on the other chemical constituents of the mericarps. Hence, the goal of the current study is to investigate the sterol composition; the amino acid, mineral, total phenolic and flavonoid contents; and the *in vitro* antioxidant, antibacterial, antifungal, and antibiofilm activities of *S. candidissima* subsp. *candidissima* mericarps from Turkey.

MATERIAL AND METHODS

Chemicals. Fluconazole, ampicillin, 5α-cholestan-3β-ol, betulin, NaOH, sodium thiosulfate, bis(trimethylsilyl) trifluoroacetamide, trimethylchlorosilane, Folin-Ciocalteu reagent, triethylamine, gallic acid, phosphate buffered saline (PBS) and quercetin were purchased from Sigma-Aldrich (St. Louis, MO, USA); and Sabouraud dextrose agar, ethanol, hexane, HCl, dimethyl sulfoxide (DMSO), crystal violet, acetonitrile, 2,-2-diphenyl-1-picrylhydrazyl (DPPH), Mueller-hinton agar, Mueller-hinton broth (MHB), Sabouraud dextrose broth (SDB), and butylated hydroxyanisol (BHA) were purchased from Merck (Darmstadt, Germany). Solutions were prepared with distilled water and only freshly prepared solutions were used.

Plant material. Specimens of *S. candidissima* subsp. *candidissima* were collected from its natural habitat during field studies in the Bayburt province in Turkey. The collection details of this taxon are provided as follows: Bayburt, between Askale and Bayburt, about 40 km to Bayburt, near Kop Pass, rocky slopes and roadsides, at an altitude of circa 2400 m, 31.08.2019, A. Kahraman 2550. The plant was identified and confirmed by Dr. A. Kahraman. The dried voucher specimens were deposited in the Plant Systematics and Phylogenetics Research Laboratory, Uşak University.

Preparation of plant extract. *S. candidissima* subsp. *candidissima* mericarps were powdered and extracted with *n*-hexane in a Soxhlet apparatus for 6 h, then the solvent was evaporated using a vacuum evaporator. The hexane extract was placed in a brown bottle and stored at room temperature for further studies on sterol contents and antimicrobial and antibiofilm activities (Güzel *et al.* 2020). As described extensively in our previous study, powdered mericarps were also extracted with ethanol to obtain an ethanol extract (Güzel Kara *et al.* 2021). This extract was used for total phenolic and flavonoid contents as well as biological activity tests.

The yields of hexane and ethanol extracts were 127.15 mg/g dry mericarp and 250.95 mg/g dry mericarp, respectively.

Phytochemical contents

Sterol composition. The ISO 12228 (1999) standard method was used to determine the sterol compositions (Ertas *et al.* 2013). According to the procedure, the sample (1 g) and internal standards, 5α-cholestan-3β-ol (1 mL) and betulin (1,000 ppm) (1 mL) were added to a screw-capped glass tube. 1 h later, the mixture was saponified by adding NaOH (0.5 N). Following saponification, the end products were extracted with hexane (3 × 5 mL), the extract volume was reduced to 10 mL under nitrogen gas and the extract was dried with anhydrous sodium thiosulfate. The extract (0.5 mL) was silylated at 60°C for 15 min, using bis(trimethylsilyl) trifluoroacetamide/trimethylchlorosilane [4:1 (v:v)] solution (250 µL) and dry pure pyridine (250 µL). The silylated sample was analysed using a gas chromatography (Perkin Elmer, Autosystem GLX, Shelton, USA) system equipped with a flame ionisation detector. SE-54 column (5%-phenyl-1%-vinylmethylpolysiloxane, 30 m × 0.32 mm × 0.25 µm) was used. The experimental conditions were as follows carrier gas: helium, a flow rate of carrier gas: 0.8 mL/min, detector temperature: 300°C, injector temperature: 280°C, oven temperature programme: (1) initial temperature of 60°C for 2 min and (2) an increase up to 220°C at a rate of 4°C/min, (3) 1 min waiting at 220°C followed by an increase up to 310°C at a rate of 5°C/min, (4) holding at 310°C for 30 min. The sterol composition of the sample was determined by using the relative retention times of 5α-cholestan-3β-ol and betulin because all the retention times of the sterols are between 5α-cholestan-3β-ol and betulin (ISO 12228 1999; Ertas *et al.* 2013). The results were represented as the content in 100 g sterol of extract.

Amino acid content. The amino acid analyses were performed according to the method proposed by Dimova (2003), Gheshlaghi *et al.* (2008), Yust *et al.* (2004), and Zhang *et al.* (2009) with slight modifications (Güzel Kara *et al.* 2021). A Prominence ultra-fast liquid chromatography system (Shimadzu, Tokyo, Japan) equipped with a binary pump, a UV/Vis detector and a reversed phase analytical column [Shim-pact XR-ODS (75 mm × 3.0 mm i.d.)] with a fluorescence detector was used for the separation and detection. The analysis conditions are provided in our previous study (Güzel Kara *et al.* 2021). The amounts of amino acid and protein were represented as mg amino acid/100 g of dry mericarp and g protein/100g of dry mericarp, respectively.

Mineral content. The analysis was carried out using the method reported by Başgel & Erdemoğlu (2006). A CEM MARS 240/50 oven model (CEM Co., NC, USA) with a timer and variable temperature setting system was used for the microwave-assisted digestion of the samples. The analysis was conducted using a Thermo Scientific™ iCAP Q ICP-MS (Thermo Scientific, Waltham, USA). The analysis conditions are provided in our previous study (Güzel Kara *et al.* 2021). The results were expressed as µg mineral/g of dry sample.

Total phenolic content. The total phenolic content was determined using the Folin-Ciocalteu method as described by Ağ Şeleci *et al.* (2015) with minor modifications (Güzel Kara *et al.* 2021). The results were provided as µg GA (50–500 µg/mL) equivalent (GAE)/mg extract.

Total flavonoid content. The investigation of the total flavonoid content was performed according to the method proposed by Moreno *et al.* (2000) with some modifications (Güzel Kara *et al.* 2021). The results were expressed as µg Q (5–100 µg/mL) equivalent (QE)/mg extract.

DPPH assay. The DPPH free radical scavenging activity assay of ethanol extract of the mericarps was performed according to the method developed by Blois (1958) with some modifications (Güzel Kara *et al.* 2021). BHA was used as a reference. The DPPH radical scavenging activity (%) was calculated using the formula given below:

$$\text{DPPH radical scavenging activity (\%)} = 100 \times ((A_0 - A_1) / A_0)$$

where A_0 is the absorbance of the control (containing all reagents except the tested sample) and A_1 is the absorbance of the sample. Extract concentration providing 50% inhibition (IC_{50}) was also calculated.

Antimicrobial activity. Antimicrobial susceptibility testing was performed on the ethanol and hexane extracts of *S. candidissima* subsp. *candidissima* mericarps according to Woods & Washington (1995) and Jorgensen & Ferraro (1998) with modifications. Seven bacteria: *Escherichia coli* ATCC 35150, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 29213, *Klebsiella pneumoniae* ATCC 100031, *Acinetobacter baumannii* ATCC 02026, and *Bacillus subtilis* ATCC 6633, and three yeast strains: *Candida albicans* ATCC 90028, *Candida glabrata* ATCC 15126, and *Candida parapsilosis* ATCC 90018 were used for antimicrobial activity studies. The microorganisms were obtained from the Refik Saydam Hıfzıssıhha Institute, Ankara, Turkey. The microorganism suspension concentrations were adjusted to McFarland 0.5 (5×10^5 CFU/mL) from stock cultures grown in Sabouraud dextrose agar at 28°C for 24 h and in Mueller-Hinton agar at 37°C for 24 h (for yeast and bacteria, respectively). Stock solutions of the ethanol and hexane extracts were prepared in DMSO at 1000 µg/mL. A 500–3.90 µg/mL concentration range was tested. While performing the microdilution test, 100 µL MHB for bacteria and 100 µL SDB for yeast were dispensed into each well of the 96-well microplate. A 100 µL of dissolved extract solution was added to the first well and

two-fold dilutions were made from the first well. Then, 5 µL of microorganism (bacteria or yeast) suspension was added to each well with the exception of the medium control well. Also, a microbial growth control well was prepared, containing 5 µL of a suspension of yeast or bacteria alone without the tested extracts. The microplates were incubated for 24 h at 28°C for the yeast and 37°C for the bacteria strains. The minimum inhibitory concentration (MIC) was detected using a microplate reader (BioTek Inc., USA) at 630 nm wavelength. The MIC values were evaluated as the lowest concentration at which the tested extracts inhibited growth. Fluconazole for yeast and ampicillin for bacteria were used as the reference drugs. The effect of DMSO on the growth of microorganisms included in the study was also tested and was determined to have no effect. All the tests were performed in duplicate.

Biofilm formation. The biofilm formation of *P. aeruginosa* was determined for use in biofilm assays using the crystal violet (CV) staining method according to O'Toole (2011) with modifications. Briefly, 100 µL of standardized microorganism cell suspensions (5×10^5 cells) prepared in MHB were transferred into 96-well microplates. The microplates were incubated at 37°C for 24 h to determine biofilm production. After incubation, the cell suspensions were gently removed, and the wells were rinsed three times with sterile PBS to remove non-adherent cells. Afterwards, the formed biofilms were fixed with 150 µL methanol for 15 min, then the methanol in the wells was aspirated and the microplates air-dried. 150 µL of 0.5% CV solution was added to the air-dried microplate wells for 15 min at 25°C. The CV solution was removed by washing with PBS, and after air-drying the microplate wells, 150 µL of 95% ethanol was added for 15 min to dissolve the stained CV on the biofilms. The biofilm formation was determined by measuring the absorbance at optical density (OD) at 550 nm wavelength using a microtiter plate spectrophotometer. The OD values of the wells without inoculum were used as the negative control. All the tests were performed in duplicate. The biofilm production capacity of the isolate was determined (Tekintaş *et al.* 2020).

Biofilm-prevention activity. The effect of the ethanol end hexane extracts on biofilm formation was evaluated by the CV staining assay according to Zhong *et al.* (2019) with modifications. A suspension of *P. aeruginosa* adjusted to McFarland 0.5 (5×10^5 CFU/mL) was seeded into 96-well microplates at concentrations of sub-MICs (0.5×, and 0.25× MIC) of the extracts and incubated at 37°C for 24 h. The formation of *P. aeruginosa* biofilm was then determined by means of the CV assay, as described above. PBS was used as the negative control (O'Toole 2011). The biofilm formation effects of the agents were evaluated by measuring the OD of the wells at 550 nm wavelength using a microplate reader. The lowest extract concentration at which biofilm formation was inhibited by at least 50% was defined as the minimum biofilm inhibition concentration ($MBIC_{50}$). All the tests were performed in duplicate.

Biofilm-eradication activity. The effect of the ethanol end hexane extracts on the preformed biofilm of *P. aeruginosa* was evaluated using the CV staining assay according to Zhong *et al.* (2019) with modifications. 100 µL of MHB medium and 5 µL of a suspension of *P. aeruginosa* adjusted to McFarland 0.5 (5×10^5 CFU/mL) were seeded in each well of 96-well plates at 37°C for 24 h. The supernatants were then gently aspirated and 100 µL extracts diluted to concentrations of 0.5×, 1× and 2× MIC were added to each well. The plates were then incubated at 37°C for 24 h. The formation of *P. aeruginosa* biofilm was determined by means of the CV assay, as described above. PBS was used as the negative control (O'Toole 2011). The preformed biofilm effects of the agents were evaluated by measuring the OD of the wells at 550 nm wavelength using a microplate reader. The lowest concentration of extract required to destroy at least 50% of the preformed biofilm was defined as the minimum biofilm reduction concentration ($MBRC_{50}$). All the tests were performed in duplicate.

RESULTS AND DISCUSSION

Phytochemical contents

Sterol composition. The sterol composition of the *S. candidissima* subsp. *candidissima* mericarps is summarised in Table 1. Campesterol, stigmasterol, delta 7-stigmastenol, β-sitosterol, delta 5-avenasterol, and delta 7-avenasterol were found in the mericarps. The total sterol amount was 579.04 ± 19.44 mg/kg. β-sitosterol was the most abundant (69.8 ± 0.54%). The ingestion of phytosterols prevents the intestinal absorption of cholesterol in humans, resulting in a lowering of serum cholesterol. Therefore, the use of phytosterols as enriched food ingredients in functional foods is increasing day by day (Fernández-Cuesta *et al.* 2012). β-sitosterol is one of the most common vegetable-derived phytosterols in the human diet (Paniagua-Pérez *et al.* 2005). In our previous studies, the total sterol amounts of *S. hispanica* L. mericarp, which is also known as chia seed, and *S. longipedicellata* Hedge mericarp were found to be higher than the tested mericarp (6130.97 mg/kg and 6882.44 mg/kg, respectively). Furthermore, β-sitosterol was the most abundant in both of them (65.77% and 54.46%, respectively) (Güzel 2020; Güzel *et al.* 2020). Our results indicated that the plant mericarps might be preferable as a source of β-sitosterol.

Amino acid content. The amino acid content of the *S. candidissima* subsp. *candidissima* mericarps is presented in Table 2. The presence of essential amino acids includ-

Table 1. Sterol composition of *Salvia candidissima* subsp. *candidissima* mericarps.

Sterol composition	Amount (%)
Campesterol	8.25 ± 0.57
Stigmasterol	1.48 ± 0.04
Delta 7-stigmastenol	10.59 ± 0.07
β-sitosterol	69.8 ± 0.54
Delta 5-avenasterol	8.52 ± 0.14
Delta 7-avenasterol	1.37 ± 0.07
Total sterol (mg/kg)	579.04 ± 19.44

The data are presented as mean ± SD; n = 3.

Table 2. Total amino acid content of *Salvia candidissima* subsp. *candidissima* mericarps.

Amino acid	Symbol	Amount (mg/100 g)
Essential amino acids		
Histidine	HIS	672 ± 2
Isoleucine	ILE	1133.5 ± 1.4
Leucine	LEU	1971.8 ± 13.6
Lysine	LYS	1929.7 ± 3.5
Methionine	MET	306 ± 5
Phenylalanine	PHE	1489.3 ± 6.7
Threonine	THR	621 ± 0.5
Valine	VAL	1499 ± 14
Arginine	ARG	2196.1 ± 6.2
Non-essential amino acids		
Alanine	ALA	1551 ± 19
Aspartic acid	ASP	1253.4 ± 10.5
Glycine	GLY	1607 ± 3
Glutamic acid	GLU	4895 ± 42
Proline	PRO	1235 ± 8
Serine	SER	1147 ± 10
Tyrosine	TYR	963 ± 7
Tryptophan	TRP	277 ± 6
Protein (g/100 g)		26.93 ± 0.04

The data are presented as mean ± SD, n = 3.

Table 3. Mineral content of *Salvia candidissima* subsp. *candidissima* mericarps.

Minerals	Symbol	Amount (µg/g)
Macro minerals		
Sodium	Na	920.40 ± 0.01
Magnesium	Mg	3205.20 ± 0.09
Phosphorus	P	5111.60 ± 0.06
Potassium	K	11474 ± 0.05
Calcium	Ca	1045.60 ± 0.14
Essential trace minerals		
Manganese	Mn	9.03 ± 0.08
Iron	Fe	19.02 ± 0.15
Zinc	Zn	8.44 ± 0.09
Copper	Cu	0.88 ± 0.03
Other minerals		
Aluminium	Al	2.37 ± 0.09
Strontium	Sr	4.37 ± 0.11

The data are presented as mean ± SD; n = 3

ing histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, valine, and arginine, as well as non-essential amino acids including alanine, aspartic acid, glycine, glutamic acid, proline, serine, tyrosine, and tryptophan were demonstrated in the mericarps. Glutamic acid which is a multifunctional amino acid and also a specific precursor for other amino acids such as arginine and proline (Zareian *et al.* 2012) was the most abundant (4895 ± 42 mg/100 g). The high amount of glutamic acid which is also the principal excitatory neurotransmitter in the brain and an important intermediate in metabolism (Zareian *et al.* 2012) was followed by that of arginine which serves as a precursor in the biosynthesis of an important messenger molecule, nitric oxide (2196.1 ± 6.2 mg/100 g) (Willson 2015). The protein content of the mericarps was 26.93 g/100 g (Table 2) which is higher than that of *S. virgata* Jacq. mericarps (22.10 g/100 g) (Güzel Kara *et al.* 2021) and chia seeds (varied from 15% to 23%) (Muňoz *et al.* 2013). In the literature, there is no study on the amino acid content of *Salvia* mericarps with the exception of *S. virgata* and *S. hispanica*. According to our results, the glutamic acid value of the *S. candidissima* subsp. *candidissima* mericarps was higher than that of Chia (3500 g/100 g seed) (Muňoz *et al.* 2013) and *S. virgata* (3934 mg/100 g mericarp (Güzel Kara *et al.* 2021). This is the first study on the protein and amino acid content of *S. candidissima* subsp. *candidissima* mericarps. Proteins are the most important molecules for living organisms (Blanco & Blanco 2017) and our results indicate that the tested mericarps are a good source of protein and amino acids, particularly glutamic acid.

Mineral content. The mineral content of the *S. candidissima* subsp. *candidissima* mericarps is shown in Table 3. The presence of 11 different minerals (Mg, Na, P, K, Fe, Ca, Mn, Zn, Cu, Sr, and Al) was reported in the mericarps. Among the macro minerals, K (11474 ± 0.05 µg/g), P (5111.60 ± 0.06 µg/g), and Mg (3205.20 ± 0.09 µg/g) were determined as the most abundant. A comparison of the amounts of essential trace minerals showed that

Fe was the highest, followed by Mn and Zn (19.02 ± 0.15µg/g; 9.03 ± 0.08 µg/g; and 8.44 ± 0.09 µg/g, respectively). In the literature, K, Ca, Mg, Na, Zn, Cu, Fe, Ni, Mn, Se, Al, Co, P, and Sr were reported as the minerals found in different *Salvia* mericarps. K, Mg, Ca, and P were the most abundant minerals (Ding *et al.* 2018; Güzel *et al.* 2019, 2020; Güzel 2020; Güzel Kara *et al.* 2021). This is the first study on the mineral content of *S. candidissima* subsp. *candidissima* mericarps and the results of the current study are consistent with the literature data. Adequate mineral intake is important for human health (Verkaik-Kloosterman *et al.* 2012). *Salvia candidissima* subsp. *candidissima* mericarps which, according to the presented results, abound in minerals, could be recommended as a mineral source.

Total phenolic content. The total phenolic content of the ethanol extract of the tested mericarps was 3.47 ± 0.81 µg GAE/mg extract. In the study conducted by Emre *et al.* (2021), the total phenolic content of the same part of *S. candidissima* subsp. *candidissima* was 76.49 µg GAE/mg. In the literature, the total phenolic content of the aerial parts of *S. candidissima* was 59.19 for the hexane extract, 49.15 for ethyl acetate, 63.27 µg (Pirokatechol mg^{-1} extract) for the ethanol extract, and 83.53 mg GAE/g extract for the methanol extract (Bayar & Genç 2018). Furthermore, the total phenolic content of the methanol extract obtained from the leaves of *S. candidissima* subsp. *candidissima* was 94.5 mg GAE g^{-1} DW (Adimcilar *et al.* 2019). Ethanol, acetone, and water extracts obtained from the aerial parts of the plant were investigated for their total phenolic contents and the acetone extract showed the highest total phenolic content (Unal *et al.* 2008). In our previous studies, the total phenolic contents of ethanol extracts of *S. hispanica*, *S. longipedicellata*, and *S. virgata* mericarps were 0.93 µg GAE/mg extract (Güzel 2020), 1.04 µg GAE/mg extract (Güzel *et al.* 2020), and 2.50 µg GAE/mg extract (Güzel Kara *et al.* 2021), respectively. According to our current results, the total phenolic content of the tested mericarps was found to be higher than *S. hispanica*, *S. longipedicellata*, and *S. virgata* mericarps.

Total flavonoid content. The total flavonoid content of the ethanol extract obtained from *S. candidissima* subsp. *candidissima* mericarps was 0.35 ± 0.03 µg QE/mg extract. Rutin, myricetin, morin, quercetin, kaempferol, naringin (the major one: 9.27 µg/mg), and naringenin were previously reported as the flavonoids present in the same part of the plant (Emre *et al.* 2021). The total flavonoid content of the methanol extract of the aerial parts of *S. candidissima* was also examined (59.02 mg QE/g extract) (Bayar & Genç 2018). In our previous studies, the total flavonoid contents for the ethanol extracts of *S. hispanica*, *S. longipedicellata*, and *S. virgata* mericarps were determined as 0.17 µg QE/mg extract (Güzel 2020), 0.32 µg QE/mg extract (Güzel *et al.* 2020), and 0.34 µg QE/mg extract (Güzel Kara *et al.* 2021), respectively. In the current study, the total flavonoid content of *S. candidissima* subsp. *candidissima* mericarps was found to be higher than that of the *S. hispanica*, *S. longipedicellata*, and *S. virgata* mericarps.

Antioxidant activity. The results of the DPPH radical scavenging activity of the ethanol extract of the mericarps are shown in Fig. 1. The highest DPPH radical scavenging activity was determined at a concentration of 10 mg/mL with a value of 79.79%. As seen in Fig. 1, the tested extract neutralised the DPPH radicals in a concentration-dependent manner. The IC_{50} value of the tested extract was determined as 2.15 ± 0.08 mg/mL. The DPPH radical scavenging activities of ethanol extracts of the same concentration obtained from the mericarps of *S. hispanica* (Güzel 2020), *S. longipedicellata* (Güzel *et al.* 2020), and *S. virgata* (Güzel Kara *et al.* 2021) were 74.54%, 53.34%, and 92.44%, respectively. In the study conducted by Emre *et al.* (2021), the highest DPPH radical scavenging activity of the same part of the plant was 82.14% for 25 µL of extract. The DPPH radical scavenging activity of the dichloromethane extract obtained from the aerial parts of the plant was 12.75% at a concentration of 25 µg/mL, 17.12% at a concentration of 50 µg/mL, and 25.06% at a concentration of 100 µg/mL (Sezer Şenol *et al.* 2010). Tepe *et al.* (2006) reported that the methanol extract of *S. candidissima* subsp. *candidissima* showed DPPH radical scavenging activity with the value of IC_{50} 49.7 µg/mL. Unal *et al.* (2008) investigated ethanol, acetone, and water extracts obtained from the aerial parts of *S. candidissima* subsp. *candidissima* for their DPPH radical scavenging activities and according

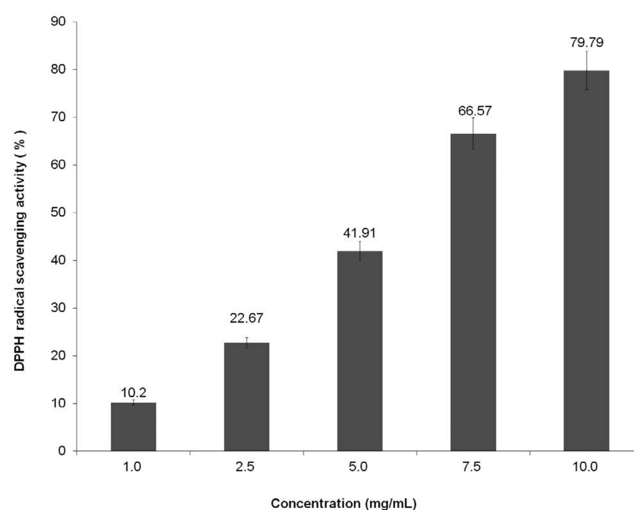

Fig. 1. Free radical scavenging activity of the ethanol extract obtained from *Salvia candidissima* subsp. *candidissima* mericarps by DPPH assay (Values are the mean ± SD of the data; n = 3).

Table 4. MIC values of the ethanol and hexane extracts of *S. candidissima* subsp. *candidissima* mericarps, and reference drugs against microbial strains.

Microorganism	MIC values of tested materials (µg/mL)			
	Tested Extract		Reference Drug	
	Ethanol	Hexane	Ampicillin	Fluconazole
Bacterial strains				
Staphylococcus aureus ATCC 29213	125	125	0.48	-
Bacillus subtilis ATCC 6633	125	62.5	1.95	-
Escherichia coli ATCC 35150	125	125	3.90	-
Pseudomonas aeruginosa ATCC 27853	125	125	31.25	-
Enterococcus faecalis ATCC 29212	62.5	125	0.97	-
Klebsiella pneumoniae ATCC 100031	125	125	1.95	-
Acinetobacter baumannii ATCC 02026	125	125	31.25	-
Fungal strains				
Candida albicans ATCC 90028	125	125	-	0.12
Candida parapsilosis ATCC 90018	62.5	62.5	-	0.24
Candida glabrata ATCC 15126	62.5	62.5	-	8

The MICs are determined in duplicate with deviations within one two-fold dilution. -: not tested

to their results, the water extract showed the highest activity at a concentration of 100 µg/mL, followed by the acetone and ethanol extracts, respectively. Literature screening indicates that free radicals play crucial roles in various pathological situations and also provoke food spoilage. Natural phenolics with antioxidant effects are frequently preferred to protect food quality (Karatoprak *et al.* 2016). Water and ethanol are the most preferred solvents in phenolic extraction for food safety (Alcântara *et al.* 2019). For this reason, in the current study ethanol was chosen as an extraction solvent to evaluate the total phenolic and total flavonoid contents, and the DPPH radical scavenging activity. In Europe and Asia, various products obtained from sage have been used as protective agents in the food industry. Moreover, oral solutions have been used in oral hygiene and the treatment of dental plaque due to their antioxidant, antiseptic, and antimicrobial properties (Topçu *et al.* 2017). In this respect, the mericarps of *S. candidissima* subsp. *candidissima* can be considered as a potential resource for the food industry due to their antioxidant potential with antimicrobial and antibiofilm effectiveness.

Antimicrobial activity. The antimicrobial activity results of the ethanol and hexane extracts are shown in Table 4. When compared with the reference antibacterial agent ampicillin both the tested extracts showed antimicrobial activity against all the used bacterial strains with MIC values in the range of 62.5–125 µg/mL. The ethanol extract was more effective against *E. faecalis* and the hexane extract was more effective against *B. subtilis* than the other bacterial strains (MIC 62.5 µg/mL). When compared with the reference antifungal drug fluconazole both the tested extracts showed antifungal activity against all the tested fungal strains with MIC values in the range of 62.5–125 µg/mL. The antifungal activity of both the tested extracts against *C. albicans* was lower than that against *C. glabrata* and *C. parapsilosis* (MIC values: 125 µg/mL, 62.5 µg/mL, and 62.5 µg/mL, respectively). According to our results, both the tested extracts exhibited antimicrobial activity against all the tested microorganisms, however, the efficiency of the extracts was not as high as that of ampicillin and fluconazole. The phenolic content of the same part of the plant was investigated for its antimicrobial activity against various microbial strains (including *E. coli*, *K. pneumoniae*, *S. aureus*, *Bacillus megaterium*, *Trichophyton* sp., *Epidermophyton* sp., *C. albicans*, and *C. glabrata*) using the agar well diffusion method and the results indicated that with the exception of *E. coli*, *Trichophyton* sp., and *Epidermophyton* sp., the plant exhibited antimicrobial effectiveness with different zone inhibition values ranging from 8.33 mm to 14.33 mm (Emre *et al.* 2021). The hexane extract of the same part was tested against *S. aureus*, *E. coli*, *Mycobacterium smegmatis,* and *C. albicans,* showing antimicrobial activity only against *M. smegmatis* (MIC 0.25 mg/mL) (Kilic *et al.* 2005). The antimicrobial activity of the hexane extract of the same part was also tested against *S. aureus*, *B. megaterium*, *K. pneumoniae*, *E. coli*, *C. albicans*, *C. glabrata*, *Trichophyton* sp., and *Epidermophyton* sp. using the well agar method and with the exception of *C. albicans, C. glabrata,* and *Trichophyton* sp., the tested extract showed changeable antimicrobial

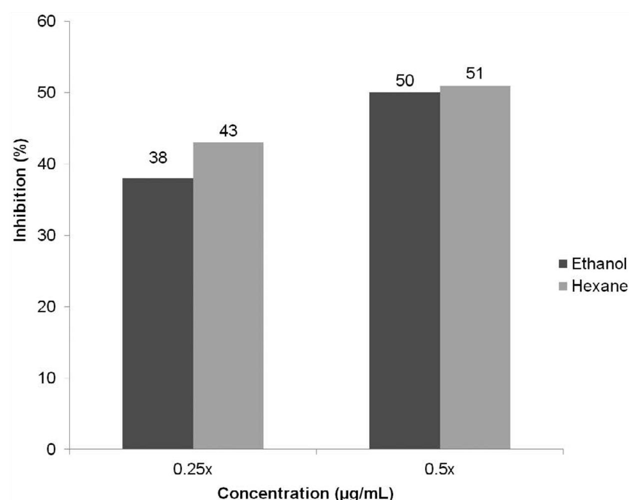

Fig. 2. Prevention of biofilm formation for the ethanol and hexane extracts of *Salvia candidissima* subsp. *candidissima* mericarps. Inhibition (%) of biofilm formation by the extracts at 0.5× and 0.25× concentrations. $MBIC_{50}$ 62.5 (0.5×) for both extracts.

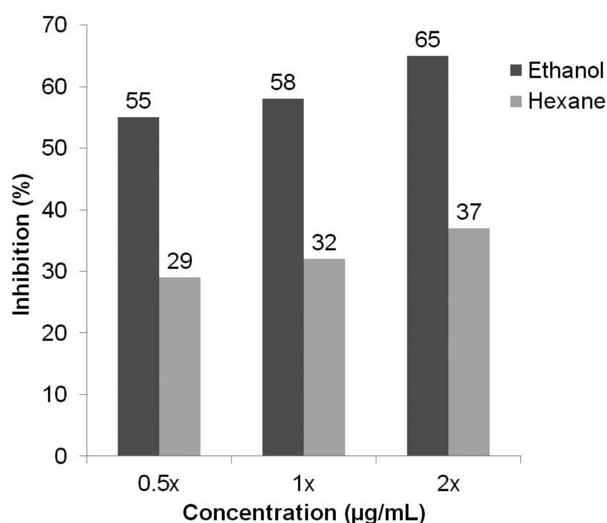

Fig. 3. Eradication of biofilm formation for the ethanol and hexane extracts of *S. candidissima* subsp. *candidissima* mericarps. Preformed biofilm inhibition (%) of the extracts at 0.5×, 1×, and 2× concentrations. While $MBRC_{50}$ for the ethanol extract is 62.5 (0.5×), there is no $MBRC_{50}$ value for the hexane extract at the applied concentrations.

activity against all the tested microorganisms (diameter of inhibition range: 8–33 mm/10 µL) (Kursat *et al.* 2012). Chloroform, ethanol, acetone, and water extracts of the aerial parts of the plant were also examined for their antimicrobial activity against certain bacterial and fungal strains (*B. megaterium*, *B. subtilis*, *E. coli*, *E. cloacae*, *K. pneumonia*, *P. mirabilis*, *P. vulgaris*, *S. enteritidis*, *S. pyogenes*, *S. aureus*, and *C. albicans*) using both the microwell dilution and disk diffusion methods, however, no activity was determined against the tested microorganisms (Unal *et al.* 2008). Due to its low boiling point and easy recovery from the extract, hexane is the most commonly used extraction solvent for oils from plant sources such as seeds. Ethanol which is less toxic and renewable, is more preferable as an extraction solvent in terms of environmental health (Keneni *et al.* 2021). In the current study, the antimicrobial activity of ethanol and hexane extracts of the mericarps was investigated. According to the literature, this is the first comparative study on two different polarity extracts obtained from *S. candidissima* subsp. *candidissima* mericarps.

Antibiofilm activity. The antibiofilm activity results of the ethanol and hexane extracts of *S. candidissima* subsp. *candidissima* mericarps are presented in Figs. 2 & 3. The biofilm prevention test determined that both the ethanol and hexane extracts inhibited biofilm formation by 50%, 38%, and 51%, 43% at 0.5× and 0.25× MIC, respectively (Fig. 2). The $MBIC_{50}$ value of both the tested extracts is 62.5 µg/mL. In addition, the biofilm eradication test determined that both the ethanol and hexane extracts inhibited biofilm formation by 55%, 58%, 65% and 29%, 32%, 37% at 0.5×, 1× and 2× MIC, respectively (Fig. 3). According to the data, while the $MBRC_{50}$ value of the ethanol extract is 62.5 µg/mL, the hexane extract did not reduce the biofilm formed by 50% at the tested concentrations (62.5, 125, and 250 µg/mL). In general, when we compare the two tested extracts in terms of preventing biofilm formation, there is not much difference, but when we compare their effectiveness on the formed biofilm, it was seen that the ethanol extract of the mericarps was more effective than the hexane extract. The antibiofilm properties of different parts (fresh leaves, aerial parts, and seeds) of some *Salvia* species have been reported in a few studies (Al-Bakri *et al.* 2010; Menjivar & Bendaoud 2020), however, except for chia seeds, there is no study on the antibiofilm activity of mericarps of *Salvia* species (Menjivar & Bendaoud 2020). The fresh leaves and aerial parts of seven *Salvia* species were investigated for their antibiofilm activity and *S. triloba* extract and volatile oil were found to be successful in preventing and controlling the biofilm (Al-Bakri *et al.* 2010). The chia seed oil extract showed the most effective antibiofilm properties against *P. aeruginosa* and *Staphylococcus epidermidis* (Menjivar & Bendaoud 2020). The increasing incidence of antibiotic-resistant strains has become one of the crucial problems in the antimicrobial area with phenotypic resistance caused by biofilm activity (Al-Bakri *et al.* 2010). Biofilms are extremely competitive communities and some microorganisms have antibiofilm properties including bacterial growth inhibition, exclusion or/and competition, which enable them to gain the advantage and thus become dominant. According to the National Institutes of Health, biofilms are responsible for more than 75% of

human microbial infections (Miquel *et al.* 2016). In the current study, the antibiofilm activity of the ethanol and hexane extracts of the mericarps against *P. aeruginosa* biofilms was studied for the first time.

CONCLUSIONS

This is the first report describing the amino acid, sterol, and mineral contents, as well as the antibiofilm activity of *S. candidissima* subsp. *candidissima* mericarps. The antimicrobial efficiency of the ethanol and hexane extracts obtained from the mericarps was compared for the first time. According to our results, the mericarps provide good nutritional potential. With the increasing uses of natural ingredients as antioxidants, antimicrobial agents, and food stabilisers, *S. candidissima* subsp. *candidissima* mericarps, abundant in nature with rich chemical content, antioxidant potential, antimicrobial and antibiofilm efficiency, appear to be a promising resource for the development of new therapeutics and food additives in different industries.

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REZIME

Fitohemijska analiza i biološke aktivnosti merikarpa *Salvia candidissima* subsp. *candidissima*

Sevda Güzel Kara, Zehra Öksüz, Şerife Selma Uras Güngör, Samet Belveren i Ahmet Kahraman

U ovoj studiji su istraživani sadržaj sterola, aminokiselina, minerala, ukupnih fenola i ukupnih flavonoida; kao i antioksidativne, antimikrobne i antibiofilmske aktivnosti merikarpa *Salvia candidissima* subsp. *candidissima*. Merikarpi su sakupljeni u provinciji Bayburt, Turska. Za analizu sterola korišćena je gasna hromatografija, za analizu aminokiselina ultra-brza tečna hromatografija, a za analizu minerala ICP-MS. Folin-Ciocalteu, $Al(NO_3)_3$ i DPPH testovi aktivnosti uklanjanja radikala su izvedeni na etanolnom ekstraktu merikarpa da bi se procenio ukupni sadržaj fenola i flavonoida, odnosno antioksidativni potencijal. Ekstrakti etanola i heksana merikarpa su testirani s aspekta postojanja antimikrobne aktivnosti protiv sedam bakterijskih i tri soja gljivica metodom mikrodilucije i antibiofilmske aktivnosti protiv biofilma *Pseudomonas aeruginosa* metodom kristalno ljubičastog bojenja. Sadržaj sterola, aminokiselina, mineralni sadržaj i antibiofilmska aktivnost merikarpa su proučavani prvi put. Najčešće komponente merikarpa su β-sitosterol (69.8%), glutaminska kiselina (4895 mg/100 g), i kalijum (11474 µg/g). Rezultati ukupnog sadržaja fenola i flavonoida i aktivnosti uklanjanja slobodnih radikala DPPH bili su 3,47 µg GAE/mg ekstrakta, 0,35 µg KE/mg ekstrakta i 79,79%, respektivno. Oba testirana ekstrakta su pokazala antimikrobnu aktivnost prema svim ispitivanim mikroorganizmima, međutim, efikasnost ekstrakta nije bila tako jaka kao referentni lekovi ampicilin i flukonazol. Dok su oba ekstrakta bila efikasna u sprečavanju formiranja biofilma, otkriveno je da je etanolni ekstrakt efikasniji od ekstrakta heksana u inhibiciji prethodno formiranog biofilma. Kao zaključak, merikarpi *S. candidissima* subsp. *candidissima* imaju dobar nutritivni potencijal sa visokim količinama aminokiselina, sterola (posebno β-sitosterola), minerala, fenola i flavonoida. Pored toga, prezentovani nalazi pružaju važne preliminarne podatke u pogledu antibiofilmske aktivnosti merikarpa *Salvia candidissima* subsp. *candidissima*.

Ključne reči: *Salvia candidissima* subsp. *candidissima*, merikarp, hemijski sadržaj, DPPH, antimikrobna aktivnost, antibiofilmska aktivnost