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ANTIMICROBIAL POTENTIAL AND PHENOLIC COMPOSITION OF *Salvia Tomentosa* EXTRACT AGAINST SOME PATHOGENIC BACTERIA

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ABSTRACT

This study aimed to evaluate the *in vitro* antibacterial activity of ethanolic extract of *Salvia tomentosa* against *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* as well as to phenolic compounds. Phenolic compounds were analysed by HPLC-DAD. The Antibacterial activity of the extract against some bacterial strains was evaluated using the microdilution method to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), and agar well diffusion method. In the ethanolic extract of *S. tomentosa*, the major phenolic acid was rosmarinic acid and the main flavonoid was hesperidin. Maximum zones of inhibition were found in the following order: *S. aureus* (16.05 ± 0.34 mm), *E. coli* (15.07 ± 0.26 mm), and *P. aeruginosa*. No inhibition was observed against *P. aeruginosa* at all treated concentrations of the ethanolic extract (100–6.25 mg/mL). The MIC values of the extract for *S. aureus*, *E. coli*, and *P. aeruginosa* were 3.12, 6.25, and >25.00 mg/mL for 24 h, respectively. The MBC values were 25.00, 25.00, and >25.00 mg/mL, respectively. The result of this study indicates that except for *P. aeruginosa* pathogen, the ethanolic extract of *S. tomentosa* exhibited antibacterial activity against *S. aureus* and *E. coli* food spoilage pathogens at high concentrations.

KEYWORDS:
Antibacterial activity, HPLC, MIC, MBC, Phenolic content, *Salvia tomentosa*

INTRODUCTION

Food spoilage is a metabolic process that causes foods to be undesirable or unacceptable for human consumption due to the growth of spoilage microorganisms such as bacteria, yeasts, and molds [1]. To prevent microbial growth and extend the shelf-life of food, numerous synthetic chemicals with antioxidants and antimicrobial are used [2]. However, using these chemical reagents as food additives and preservatives is a sensitive issue due to their toxic effects on humans and animals [3]. In recent years, great interest has been focused on using healthy foodstuff (health-promoting foodstuffs) without synthetic preservatives. A variety of plant materials are a natural source of food preservatives due to their active compounds, including polyphenols, carotenoids, proteins, lipids, vitamins, and minerals [4-6].

Phenolic acids accounting for about a third of the polyphenolic compounds may show significant antimicrobial activities by damaging microorganism' membranes and antioxidant activities by decreasing the activity of free radicals. These natural compounds have gained great importance as natural flavouring and preservative agents to improve food quality and safety [2,7,8].

Salvia is the largest genus of the family *Lamiaceae* and consists of about 900 species distributed throughout the world [3]. From time immemorial, *Salvia* has been used in traditional medicine for the treatment of numerous diseases [9]. Currently, it has been used for several purposes like condiments, food additives, seasoning, spice, and herbal tea [10]. The essential oil obtained from various *Salvia* species has numerous effects such as antimicrobial, antioxidant, antiinflammatory, cholinesterase inhibition, anxiolytic, and sedative [10-12]. Also, due to their antioxidant effects, these species have been used for the stabilization of fat and fat-containing food [10].
Salvia tomentosa, the most consumed herbal tea, is used in many parts of Turkey for the treatment of various diseases such as colds, flu, and tonsillitis and has a wound-healing effect [9,13]. *S. tomentosa* contains abundant phenolic compounds and terpenoids, with antioxidant, insecticidal, antibacterial, herbicidal, and antifungal effects [14]. In the literature, very limited studies on the compounds and antibacterial activity of extracts of *S. tomentosa* prepared by using different organic solvents are present. This study aimed to investigate the phenolic compounds

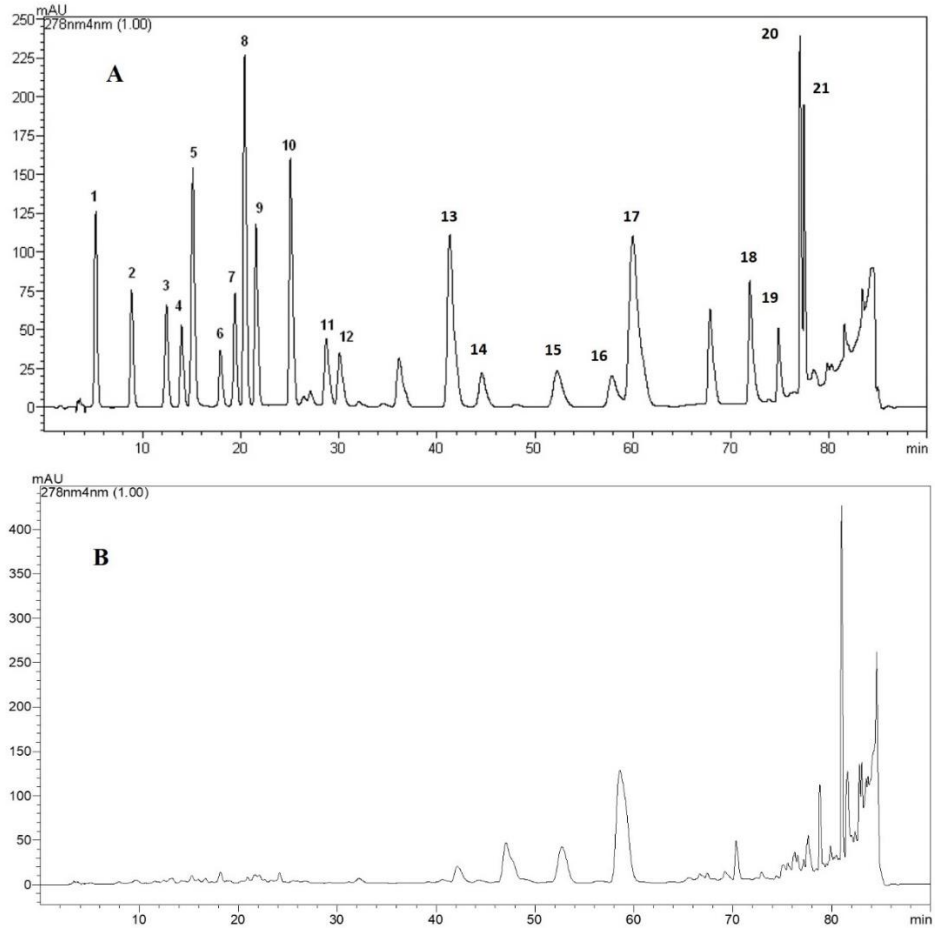

FIGURE 1
(A) Standard chromatogram. (B) Chromatogram of the *S. tomentosa* ethanolic extract

of *S. tomentosa* ethanolic extract and its' antimicrobial activity on *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*.

MATERIALS AND METHODS

Sampling. *S. tomentosa* samples were collected in Çeltikçi district of Burdur in Turkey, during the flowering stage in July 2019 (GPS Coordinates: 37°35'50.2" N, 30° 24'08.3" E). The sample was identified by Prof. Dr. Osman TUGAY from Selçuk University, Faculty of Pharmacy, Department of Pharmaceutical Botany (No: 25899). The roots and above-ground parts of the collected plants were dried in the shade at room temperature.

Extraction. An electric blender was used to obtain powder from the dried plant. Furthermore, 100 g of plant powder was suspended in 500 mL ethanol, and extraction was performed at 180 rpm and room temperature for 72 h in a Thermo shaker to ensure complete homogeneity of the solution. To remove insoluble particles, the solution was centrifuged at 5000 rpm for 15 min (Hettich Rotina 38R). After the centrifugation, the extract was filtrated through Whatman No:1 filter paper. The collected filtrates were subjected to a rotary evaporator (RV10, IKA ®, Germany) at 40 °C to remove the solvent. With a freeze dryer (Martin Christ, Alpha 1–2 LD plus, Germany), the concentrated extracts were lyophilized and kept in a sealed bottle in the dark at 4°C.

Phenolic compounds. Determination of phenolic compounds from ethanolic extract of *S. tomentosa* was carried out by HPLC using a chromatograph equipped with an Agilent Eclipse XDB-C18 (250 × 4.60 mm) 5-micron column and a diode array detector (SPD-M10A, Shimadzu). The column was eluted using a linear gradient of 3% acetic acid (Solvent A) and methanol (Solvent B) with a solvent flow rate of 0.8 mL/min. With the photodiode array detector, chromatograms were recorded at 278 nm. The gradient program and HPLC conditions are shown in Table 1 and Table 2. The standard solutions were prepared using methanol to dissolve the chemicals to reach concentrations ranging of 0.7–500.0 µg/mL for gallic acid (1), protocatechuic acid (2), catechin (3), p-hydroxybenzoic acid (4), chlorogenic acid (5), caffeic acid (6), epicatechin (7), syringic acid (8), vanillin (9), *p*-coumaric acid (10), ferulic acid (11), sinapinic acid (12), *o*-coumaric acid (13), rutin (14), hesperidin (15), rosmarinic

TABLE 1
Gradient program of HPLC

	Time	Action	Value
1	0.01	Start	
2	0.10	Pump B Conc.	7
3	20.00	Pump B Conc.	28
4	28.00	Pump B Conc.	25
5	35.00	Pump B Conc.	30
6	50.00	Pump B Conc.	30
7	60.00	Pump B Conc.	33
8	62.00	Pump B Conc.	42
9	70.00	Pump B Conc.	50
10	73.00	Pump B Conc.	70
11	75.00	Pump B Conc.	80
12	80.00	Pump B Conc.	100
13	81.00	Pump B Conc.	7
14	90.00	Stop	

TABLE 2
HPLC Conditions and Settings

Conditions	Settings
Instrument	Shimadzu HPLC-DAD system
Detector	SPD-M 10A vp DAD detector (Max=278nm)
System controller	SCL-10Avp
Auto sampler	SIL–10AD vp
Pump	LC-10ADvp
Degasser	DGU-14A
Column oven	CTO-10A VP
Column	Agilent Eclipse XDB-C18 (250x4.60 mm) 5 micron
Column temperature	30^0C
Mobile phase	A: 3% Acetic Acid, B: Methanol
Injection volume	20 µL
Flow rate	0.8 mL/min

acid (16), eriodictiol (17), quercetin (18), luteolin (19), kaempferol (20), and apigenin (21) (Figure 1A). In 1 mL of ethanol, 20 mg of *S. tomentosa* sample was dissolved. This solution was filtered with a polytetrafluoroethylene (PTFE) (0.45 µm) filter. Finally, 20 µL of the sample filtrate was injected into the HPLC system.

Bacterial cultures. *S. aureus* (ATCC 25923), *E. coli* (ATCC 29998), and *P. aeruginosa* (ATCC 27853) strains used in the present study were obtained from the stock culture collection of the Department of Food Hygiene and Technology Laboratory, Burdur Mehmet Akif Ersoy University. The bacterial strains were streaked on Tryptic Soy Agar (TSA, BK047HA, BIOKAR) and incubated for 18–24 h at 37°C then transferred into sterile saline (0.9%) and adjusted to 0.5 McFarland.

Agar diffusion method. The antimicrobial activity of *S. tomentosa* extract was assessed by agar well diffusion method [15]. The serial dilutions of the *S. tomentosa* ethanolic extract (100, 50, 25, 12.50, and 6.25 mg/mL) were prepared in ethanol. After streaking each bacterial culture on Mueller Hinton Agar (BK048HA, BIOKAR), wells (6 mm size and 4 mm depth) were prepared with a sterile borer. From each dilution 100 µL (100, 50, 25, 12.50, and 6.25 mg/mL) was dispensed into the wells. Enrofloxacin (64 µg/mL) and absolute ethanol were used as positive and negative controls, respectively. Following a 24-hour incubation at 37°C, the petri dishes were observed for the presence of clear inhibition zones around the wells, and the diameter of the inhibition zone was measured with a digital calliper.

Microdilution method. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of *S. tomentosa* extract were determined by using the microdilution method in 96-well microplates [16]. The bacterial strains were grown on TSA (BK047HA, BIOKAR) and incubated for 18 h at 37°C. Bacterial inoculums were adjusted to 0.5 McFarland in 0.9% sterile saline. The serial dilutions (25, 12.50, 6.25, 3.12, 1.56, 0.78, and 0.39 mg/mL) were prepared in Mueller Hinton broth (BK048HA, BIOKAR) and transferred to wells of microplates. From each bacterial inoculum, 20 µL volume was inoculated into each well, and plates

were incubated at 37°C for 24 h. Following the incubation, microbial growth was determined at 600 nm using a microplate reader (Epoch, BioTek, USA). The MBC value was determined by removing 10 µL of the suspension from each well and inoculating it on Mueller Hinton agar plates. The plates were incubated at 37°C for 24 h. The MBC was identified by determining the lowest concentration of extracts that completely inhibited the growth of bacteria [17].

RESULTS

In the present study, the phenolic components in *S. tomentosa* ethanolic extract were studied by using HPLC-DAD. The phenolic standard mixture and the phenolic profile of *S. tomentosa* ethanolic extract chromatograms are shown in Figures 1A and 1B, respectively. The phenolic components (µg/g) of the extract are shown in Table 3. Rosmarinic acid (9920.6 µg/g) and hesperidin (3606.0 µg/g) were the most abundant components in the extract, followed by chlorogenic acid, apigenin, kaempferol, o-coumaric acid, caffeic acid, and luteolin. Catechin, epicatechin, syringic acid, rutin, and eriodyctiol were not found in the extract.

In this study, agar well dilution and microdilution methods were used to determine the antibacterial activity of ethanolic extract of *S. tomentosa* against *S. aureus*, *E. coli*, and *P. aeruginosa* (Table 4). In the agar diffusion test, all tested concentrations (from 6.25–100.00 mg/mL) of *S. tomentosa* ethanolic extract did not show antibacterial effect against *P. aeruginosa*. The extract exhibited antibacterial activity on *S. aureus* and *E. coli* with an inhibition zone from 10.7 ± 0.84 to 16.05 ± 0.34 mm and from 11.15 ± 0.49 to 15.07 ± 0.26 mm, respectively.

While the MIC values of the *S. tomentosa* ethanolic extracts on *S. aureus* and *E. coli* were 3.12 mg/mL and 6.25 mg/mL, respectively, the MBC values detected for bacteria were 25 mg/mL. The MIC and MBC values were >25 mg/mL against *P. aeruginosa*. The ethanolic extract showed no detectable antibacterial activity against *P. aeruginosa* in the concentrations used. Consequently, the extract was more potent in inhibiting *S. aureus* and *E. coli* than *P. aeruginosa*.

DISCUSSION

Phenolic acids, ubiquitous secondary metabolites in plants, show strong antioxidant activity due to the presence of hydroxyl groups in their structure [18]. The most abundant phenolic compounds in the methanolic extracts of *S. tomentosa* [9,19] were rosmarinic acid, *p*-coumaric acids, and catechin. In the present study, rosmarinic acid was the most abundant in the ethanolic extract, followed by hesperidin. Compared with other studies, the phenolic profile of the ethanolic extract obtained in this study was largely similar. The reason for the observed differences may be related to geographical origin, harvest period, and extraction method [9].

TABLE 3
Phenolic compounds of *S. tomentosa* ethanolic extract

Phenolic Compounds	Concentration (µg/g)
Gallic Acid	19.3
Protocatechic Acid	22.6
Catechin	*
P-Hydroxy Benzoic Acid	79.6
Chlorogenic Acid	447.3
Caffeic Acid	222.8
Epicatechin	*
Syringic Acid	*
Vanilin	91.5
P-Coumaric Acid	18.5
Ferulic Acid	20.5
Sinapinic Acid	94.8
O-Coumaric Acid	226.9
Rutin	*
Hesperidin	3606.0
Rosmarinic Acid	9920.6
Eriodictiol	*
Quercetin	114.2
Luteolin	216.5
Kamferol	205.6
Apigenin	326.0

*: Not Detected

TABLE 4
Antimicrobial activity, minimum inhibitory and bactericidal concentrations of *S. tomentosa* extract against selected pathogens

Method	Concentration (mg/mL)	*S. aureus*	*E. coli*	*P.aeruginosa*
Agar well diffusion (mm)	100	16.05±0.34	15.07±0.26	ND
	50	14.85±0.21	14.55±0.07	ND
	25	13.45±0.21	13.35±0.35	ND
	12.5	12.65±0.07	12.6±0.14	ND
	6.25	10.7±0.84	11.15±0.49	ND
	Enrofloxacin	32.65±0.63	30.15±0.21	19.6±0.14
MIC (mg/mL)		3.12	6.25	>25
MBC (mg/mL)		25	25	>25

ND: Not detected

There are limited studies on the antibacterial activity of *S. tomentosa* extracts obtained by using different solvents. In these studies, the antibacterial activities of *S. tomentosa* extracts against many bacteria were assessed by using agar well diffusion and disk diffusion methods. While water, ethanol, and methanol extracts of *S. tomentosa* did not show any antibacterial effect against *S. aureus*, *E. coli*, and *P. aeruginosa*, they were effective against *Salmonella typhimurium*, *Proteus vulgaris*, and *E. cloacae*. Hexane and deodorized methanol chloroformic extract of *S. tomentosa* showed antibacterial activities against *S. aureus* [3,20]. Its essential oil showed inhibitory activity against *E. coli* and *S. aureus* [21]. According to agar well diffusion assay, except for *P. aeruginosa*, the ethanolic extract presented inhibitory activity against *S. aureus* and *E. coli*.

Several studies have analysed the antibacterial activities of different extracts of *S. tomentosa* using the microdilution method. It has been reported that MIC values of methanolic extract of *S. tomentosa* against *S. aureus* and *E. coli* were 5.12 mg/mL and 0.64 mg/mL, respectively [19]. Its essential oil possesses antibacterial activity against *S. aureus*, *E. coli*, and *P. aeruginosa*, and the MIC values vary between 0.30–18.00 mg/mL, 1.25–92.34 mg/mL, and 1.25–369.38 mg/mL, respectively [3,22,23]. However, Tepe et al. [3] reported that *S. tomentosa* essential oil was not effective against *E. coli* and *P. aeruginosa*. It has been reported that the MBC values of the essential oil against *S. aureus* and *E. coli* were ranged between 1.25–46.17 mg/mL and 1.25–369.38 mg/mL, respectively, and MBC values could not be determined against *P. aeruginosa* at the tested concentrations of the essential oil (>369.38 mg/mL and >2.5 mg/mL) [22,23]. In the current study, we found that MIC/MBC values of *S. tomentosa* ethanolic extract for *S. aureus*, *E. coli*, and *P. aeruginosa* were 3.12/25.00 mg/mL, 6.25/25.00 mg/mL, and >25.00/>25.00 mg/mL, respectively. At all tested concentrations, the ethanolic extract presented no detectable antibacterial activity against *P. aeruginosa*. The extract was more potent in inhibiting *S. aureus* and *E. coli* than *P. aeruginosa*. The MIC and MBC values for these bacteria reported in the current work were similar to those obtained for other *S. tomentosa* extracts obtained with different methods.

In this study, rosmarinic acid was the most abundant phenolic compound among all the phenolic acids in the extract, followed by hesperidin. Rosmarinic acid is a phenolic compound which is naturally present in many medicinal plant species belonging to the *Lamiaceae* and *Boraginaceae*, including basil, sage, rosemary, and peppermint [24]. Rosmarinic acid and its derivatives have positive effects on preventing and/or treating cognitive disorders, heart disorders, Alzheimer's disease, and kidney disorders. In addition, it showed remarkable antibacterial activity against *Bacillus subtilis*, *Micrococcus luteus*, and *E. coli* [25-27]. Hesperidin is a naturally occurring flavanone glycoside, which is abundant in citrus fruits such as oranges and lemons. It possesses various effects, including antiaging, antiproliferative, lipid lowering, cardioprotective, hepatoprotective, antimicrobial, antioxidant, and antiinflammatory [28-32]. The antibacterial activity observed in the current study may be related to phenolic acids such as rosmarinic acid and hesperidin, which have antibacterial activity and are abundant in the ethanolic extract of *S. tomentosa*.

CONCLUSIONS

This study indicates that except for *P. aeruginosa*, the ethanolic extracts of *S. tomentosa* present antibacterial activity against *S. aureus* and *E. coli* and also contain mostly rosmarinic acid and hesperidin. This antibacterial activity can be attributed due to the presence of abundant phenolic acids in the extract. Therefore, *S. tomentosa* could be used as a natural antibacterial agent.

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