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Antimicrobial, Antibiofilm and Antioxidant Properties of Algerian *Satureja graeca* L. Against Human Pathogens

Mouna Menakh [1,*], Saber Boutellaa [2], Amar Zellagui[3], Ozgur Ceylan [4], Mehmet Öztürk [5], Chawki Bensouici [6]

[1] Department of Nature and Life Sciences, University Center Abdehafid Boussouf, Mila, Algeria; [2] Laboratory of Natural Sciences and Materials (LSNM), Institute of Sciences and Technology, University Center AbdehafidBoussouf, Mila, Algeria ; [3] Laboratory of Bio-molecules and Plant Breeding, Department of Nature and Life Sciences, Faculty of Exact Sciences, Oum El Bouaghi University, Oum EL Bouaghi, Algeria; [4]Apiculture Program, Ali Kocman Vocational School, Mugla Sitki Kocman University, 48640, Ula, Mugla, Turkey; [5]Department of Chemistry, Faculty of Sciences, Muğla Sıtkı Koçman University, 48000 Mugla, Turkey; [6] Biotechnology Research Center, P.B E73/UV N°03 Ali Mendjeli Nouvelle Ville, 25000 Constantine, Algeria ;

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Abstract

In this study, we evaluated the antimicrobial, antibiofilm and antioxidant properties, in addition to the chemical constituents of *Satureja graeca* L. ethyl acetate and *n*-butanol fractions using HPLC-DAD analysis, phenolic compound detection was established. Six in vitro assays (DPPH, ABTS, ß-carotene, $O_2^{\bullet-}$, CUPRAC, and Reducing power) were employed to assess the antioxidant capacities. In order to determine the minimum inhibitory concentrations (MIC) of both extracts against six bacterial strains and two fungi, the serial micro-dilution method was used. According to analysis, the extracts' main phenolic components were chlorogenic acid, rutin, ellagic acid, vanillin, and caffeic acid. Both fractions revealed high antioxidant capacities at different levels in all assays and they showed a strong antimicrobial activity against all tested strains as MIC came out to be 0.3–10 mg/ml. However, ethyl acetate fraction exerted important effect compared to that of *n*-butanol fraction on *Enterococcus faecalis* ATCC19433 (1.2 and 2.5 mg/mL), *Candida albicans* ATCC10239 (2.5 and 10 mg/mL) and *Candida tropicali*s RSKK 665 (2.5 and 5 mg/mL). Furthermore, ethyl acetate fraction showed a significant antibiofilm activity against *C. tropicalis* RSKK 665 (41.9%) and *C. albicans* ATCC 10239 (38.6%) at 2.5 mg/mL. These findings suggest that *Satureja graeca* L. can be used in food and may be a promising therapeutic agent for treating a wide range of disorders.

Keywords: Antibiofilm Activity; Microbial Inhibition; Antioxidant Properties; Phenolic profile; Greek Savory.

1. Introduction

The production of excessive free radicals in the human body, combined with the inadequacy of both endogenous and exogenous antioxidant systems, results in oxidative stress. This oxidative stress causes significant harm to cellular DNA, proteins, carbohydrates, and lipids (Sharifi-Rad *et al*., 2020). Antibiotic resistance and the ability of certain microorganisms to form biofilms pose a major threat to public health and are considered one of the most significant challenges of the 21st century (Júnior *et al*., 2018; Al-kafaween et al., 2020; Zullkiflee et al., 2023). The emergence of new pathogens, the reemergence of previously controlled ones, and the lack of effective treatments only compound the problem, making the discovery of new active molecules a continued necessity (Venkatesan, 2021).

The plant world offers a diverse array of molecules with a range of various pharmacological effects, especially phenolic substances, which have been investigated for their potential to combat diseases linked to oxidative stress and as alternatives to conventional antibiotics, which are becoming less effective (Danaei *et al*., 2021). The genus *Satureja*, part of the Lamiaceae family, the Nepetoideae subfamily, and the *Menteae* tribe, include around 200 species of fragrant plants and herbs native to the Mediterranean, Europe, Middle East, and western Asia. Algeria is home to 16 species of *Satureja*, which can be used as both a culinary and medicinal herb that can also be planted as a decorative plant. (Bensouici *et al*., 2013; Haouat *et al*., 2022). In Algeria and Morocco, *Satureja* is highly regarded for its ability to treat respiratory infections, coughs, and indigestion (Amiri, 2011). Recent studies have also shown that *Satureja* possesses vasodilatory, emmenagogue, antihyperlipidemic, bactericidal, fungicidal, antioxidant, antidiabetic characteristics (Seyedtaghiya et al., 2021).

This research is centered on a comprehensive analysis of the chemical profile and a thorough evaluation of biological properties of the ethyl acetate and *n*-butanol extracts derived from the aerial parts of *Satureja graeca* L. This particular species of *Satureja* is of significant medicinal and botanical interest in Algeria. The primary aim of this study is to uncover the therapeutic potential of these extracts, with a specific focus on their antioxidant

* Corresponding author. e-mail: m.menakh@centre-univ-mila.dz.

capabilities, antibacterial effects, and antibiofilm properties.

2. Material and Methods

2.1. Plant material

Satureja graeca L. aerial components were gathered in March 2020 in Mila, North Eastern Algeria (36°30'25.55"N Latitude and 6°21'43.70"E Longitude, at a height of 240 m), during the floral stage. At the Life Science and Nature Department of the University of Larbi Ben Mhidi Oum El Bouaghi in Algeria, a sample was kept in the herbarium of the Biomolecules and Plant Breeding Laboratory.

2.2. Preparation of extracts

The aerial portions of *S. graeca* L. (50 g) were pulverized and macerated for three cycles of 24 hours each with 80% ethanol and water at room temperature. The resultant hydroalcoholic extract was concentrated under decreased pressure (40°C) and twice filtered through ordinary filter paper to remove ethanol. After that, it was fractionated using progressively polar solvents. Before usage, the fractions were kept at 4°C.

2.3. Phytochemical analysis

2.3.1. Total phenolics and flavonoids quantification

The content of total soluble phenolics in the ethyle acetate (EtOAc) and butanolic extracts (*n*-but) from the aerial portions of *S. graeca* L. was evaluated using the Folin reagent by colorimetry (Singleton *et al*., 1965). 500 µL of each extract, diluted to a dose of 0.25 mg/mL, 2500 µL of F.C. reagent, diluted a tenth in water, and 2000 µL of sodium carbonate (20 g/l) made up the reaction mixture. The absorbance at 760 nm was determined after 90 minutes. The procedure utilizing aluminum trichloride was used to determine the flavonoid content of both extracts (Miliauskas et al., 2004). A 2% methanolic solution of $AlCl_3$ was combined with one milliliter of each extract (250 µg/mL) in methanol, and after incubating at room temperature for 10 minutes, the absorbance was measured at 430 nm. The total phenolics and flavonoids were expressed in µg of GA equivalent and µg of quercetin equivalent per milligram of extract, respectively, using gallic acid and quercetin as reference standards to generate calibration curves.

2.3.2. HPLC-DAD screening of phenolics

To analyze ethyle acetate and butanolic extracts as well as 27 standard phenolics, we used a Shimadzu reverse stationary phase HPLC system which is controlled by LC-solution and includes a Shimadzu model LC-20AT solvent supply unit. 35°C was chosen as the column temperature. Aqueous acetic acid 0.1% (A) and methanol served as the mobile phases for the chromatographic separation, which was carried out on an Inertsil ODS-3 guard column (4 µm, 4.0 mm x 150 mm) column (B). Elution was done in gradients ranging from 2% to 100%. Sample stock solutions were created in methanol at a concentration of 8mg.mL^{-1} and filtered through an Agilent 0.45 µm filter. 20 µL of fluid was injected. A diode array detector (DAD) operating at a wavelength of 254 nm was used to find the phenolics. The results were presented as micrograms per gram of dry weight, and their characterization was based on a comparison of the retention times (Tel-Çayan *et al*., 2015).

2.4. Antioxidant activity

Using a microplate reader (Perkin Elmer, Enspire), all experiments were carried out in 96-well microplates. The tests used to define the results were CUPRAC and Reducing power assays, where $A_{0.5}$ refers to the dose indicating 0.5 absorbance, and DPPH, ABTS, $O_2^{\bullet-}$ and ß-Carotene-linoleic acid, where IC_{50} refers to sample dose giving 50% activity.

2.4.1. DPPH assay

According to BLOIS (1958), diphenyl-picrylhydrazyl (DPPH) free radicals were used to measure the antiradical effect of ethyl acetate and *n*-butanol fractions. 160 µL of 0.1mM DPPH methanolic solution was added with triplicate samples (40 µL of 6.25-200 µg.mL^{-1}). A blank (methanol) was used as a reference during a half-hour incubation period at 25°C to quantify the mixture's absorbance. The following equation was employed to compute the proportion of DPPH scavenger activity:

$$\%I = [(A_1 - A_2)/A_1] \times 100$$

Where; A_1 and A_2 are respectively absorbencies of negative control and sample.

2.4.2. ABTS Cation Assay

To create the ABTS cation radical, A 7 mM ABTS solution in water received the addition of 2.45 mM potassium persulfate, which was then kept for 12 hours at room temperature. Later, using H_2O, the solution's absorption was brought to 0.700±0.020 at 734 nm. 40 µL of each sample and standard was diluted in triplicate with 160 µL of this solution in each well. As typical antioxidants, BHT and BHA were utilized, while methanol served as a blank. The absorbance was measured at 734 nm after ten minutes of incubation at 25°C. Using the DPPH formula the inhibition % was then determined (Re *et al*., 1999).

2.4.3. β-Carotene assay

According to Marco (1968), 25 µL of linoleic acid were combined with 0.5 mg of *β*-carotene in 1 mL of chloroform, and 200 µL of Tween 40 were used to emulsify the mixture. Chloroform was evaporated at 40°C, and the rest was recovered using 100 mL of purified water that had been infused with oxygen. With oxygenated water, the absorbance was changed to (0.8 - 0.9) at 470 nm. 40 µL of samples at concentrations ranging from 6.25 to 200 µg/mL were added to 160 µL of this solution. *β* -carotene bleaching percentage was calculated after two hours of incubation at 50 °C using an absorbance measurement at 470 nm and the result was calculated as follows:

$$R = (ln\ a_1/a_2)/t$$

a_1 and a_2 were the relative absorbencies at the beginning of the reaction (t0) and 120 minutes afterward (t120). Antioxidant capacity was computed using the following formula:

$$A\% = [(R_{control} - R_{sample})/ R_{control}] \times 100$$

2.4.4. Superoxide anion assay

The alkaline DMSO technique, with minor modifications, was used to test $O_2^{\bullet-}$ scavenging activity

(Kunchandy and Rao, 1990). This process depends on the synthesis of alkaline DMSO while changing the yellow color of Nitroblue tetrazolium (NBT) into formazan (blue hue). Following the addition of 30 µL of NBT (1 mg/mL in distilled water), 40 µL of each extract was combined with 130 µL of alkaline DMSO (created by dissolving 20 mg of NaOH in 100 mL of DMSO to create $O_2^{\bullet-}$). Tannic acid and α-tocopherol served as benchmarks. Following 10 min of incubation at ambient temperature, the absorbance was recorded at 560 nm, and the ratio of $O_2^{\bullet-}$ blockage was calculated employing the DPPH formula.

2.4.5. Reducing power assay

Using Oyaizu (1986) method of Fe^{3+} to Fe^{2+} reductive capability, the extracts' reductive capacities were evaluated. 50 µL of potassium ferricyanide (1%) (1 g of $K_3Fe(CN)_6$ in 100 mL H_2O) and 40 µL of phosphate buffer (pH 6.6) were added to 10 µL of each sample (1.562-50 µg/mL), followed by a 20 min incubation period at 50°C for the combination. Then, 10 µl of ferric chloride $FeCl_3$ (0.1%), 40 µl of water, and 50 µl of trichloroacetic acid (TCA) (10%) were added to the mixture. On a blank surface (methanol), at 700 nm, absorption was determined.

2.4.6. Cupric reducing capacity (CUPRAC)

A mixture consisting of 60 µL of ammonium acetate buffer (pH 7.0), 50 µL of 10 mM ($Cu Cl_2$, $2H_2O$), and 50 µL of 7.5 mM neocuproin was prepared. To this mixture, 40 µL of sample solutions with 6.25-200 µg/mL concentrations were then supplemented (Apak et al., 2008). After one hour of incubation, the intensity of absorption at 450 nm was obtained and contrasted with a blank solution. BHT and BHA were common antioxidants employed in this assay.

2.5. Antimicrobial activity

According to the micro-dilution method of Rota *et al*., (2004), minimal inhibitory concentration (MIC) and minimal bactericide concentration (MBC) of the extracts, which reflect the lowest concentration that gave no observable growth, were determined. *E. foecalis* ATCC19433, *L. monocytogenes* ATCC7644, *S. aureus* ATCC 25923, and *S. aureus* MU40 have been chosen as positive Gram bacteria. *C. violaceum* CV 026 and *P. fluorescens* RSKK240 have been chosen as negative Gram bacteria. All mentioned bacterial strains were put to grow in nutrient broth and incubated at 37°C for 24 h excepted *P. fluorescens* RSKK240, *L. monocytogenes* ATCC7644 and Fungi (30°C for 24-48 h). Afterwards, inocula 5×10^5 colony-forming units (CFU)/mL were prepared. The test medium was Mueller Hinton Broth (MHB) from Biolife in Milan, Italy. Each extract was produced in five final doses (0.625–10) mg/mL in DMSO solution. 10 µL of cell suspension were added to each well of a 96-well microplate containing 170 uL of sterile MHB. Then, 20 uL of sample were added in duplicate at various concentrations. Three wells were reserved for negative controls (media, media + bacteria and media + bacteria + DMSO). For 24 hours, microplates with inoculums were incubated at 37°C.

2.6. Antibiofilm activity

We tested the impact of extracts in different concentrations ranging from the minimum inhibitory concentration (MIC) to one-fourth MIC on the biofilm formation ability of both bacteria and yeasts using polystyrene microplates (Ceylan and Ugur, 2015). The microorganisms were cultured overnight in 5 ml of Tryptone-Soy Broth (TSB) supplemented with glucose (1%). The microbial suspension was then diluted (5×10^5 CFU ml^{-1}), and each well received 100 µL along with 100 µL of each extract at various concentrations (MIC, MIC/2, and MIC/4) or 100 µL of the control (culture medium). Each well was rinsed with water after being incubated for 48 h at 37°C to get rid of any planktonic bacteria. The remaining bacteria were then dyed for 10 minutes at 25°C with a 0.1% crystal violet solution. To remove the crystal violet solution (which had not particularly colored the attached bacteria), the wells were washed once more. To remove any extra liquid, the microplates were flipped and firmly tapped on absorbent paper. They were then allowed to air dry. The wells containing Gram-negative and Gram-positive bacteria, respectively, received 200 l each of 95% ethanol and 33% glacial acetic acid, and the plates were automatically shaken. Spots of the biofilm dissolved at room temperature. Finally, a microplate reader was used to detect the absorbance at a wavelength of 550 nm. The biofilm formation inhibition percentages were calculated using the next equation:

$$I\% = [(Ab_{control} - Ab_{sample}) / Ab_{control}] \times 100$$

2.7. Statistical analysis

All results and findings were obtained in triplicate and reported as mean ± S.D. The data were processed by SPSS V.16. One-way analysis of variance (ANOVA) and Tukey Post Hoc test were performed to establish the differences among the means. P values < 0.05 were considered to be significant. The IC_{50} values (50% inhibition concentration) for the antioxidant activity were calculated by the linear regression method from the curve [% inhibition = f (concentrations)].

3. Results

3.1. Total phenolics and flavonoid contents

Total phenolics and flavonoid contents of ethyl acetate (EtOAc) and butanolic extracts (*n*-but) from aerial parts of *S.graeca* L. are presented in Table 1. A great richness of both extracts in total phenols was noted. However, EtOAc extract had the highest content significantly (268.4 ±1.9 µg EAG/mg E) compared to that of *n*-but extract ($P < 0.05$). In contrast, the latter contained more flavonoids (37.6 ± 0.2 EQ/mg E) than EtOAc extract (20.9 ± 0.2 EQ/mg extract) ($P < 0.05$).

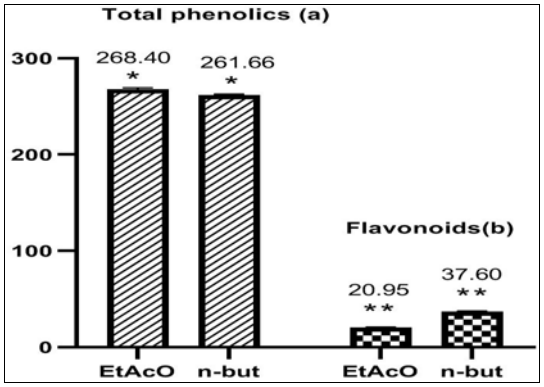

Figure 1. Total phenolics and flavonoids contents in *S. graeca* L. extracts

Results are expressed as means ± standard deviation of three measurements.

(a) Microgram of Gallic Acid Equivalent per milligram of extract,(b) Microgram of Quercetin Equivalent per milligram of extract. ***P<0.001, ** P <0.01 and * P <0.05 compared between means.

3.2. Compounds identified by HPLC-DAD

The results of HPLC analysis are given in Table 1. Only five minor compounds were identified in EtOAc fraction: chlorogenic acid (4.8µg/g), caffeic acid (1.4µg/g), vanillin (1.3µg/g), protocatechuic acid (0.8µg/g) and 4-hydroxybenzoic acid (0.2 µg/g). The majority of the detected peaks were not identified due to the absence of suitable standards (Figure 1). For *n*-but fraction, nine compounds were identified: Rutin (41.6 µg/g), chlorogenic acid (41.1 µg/g), ellagic acid (3.3 µg/g), 2,4-dihydroxybenzoic acid (0.3 µg/g), Coumarin (0.3 µg/g), p-coumaric acid (0.2 µg/g), ferulic acid (0.1 µg/g), trans-cinnamic acid (0.2 µg/g) Quercetin (0.2 µg/g) and Rosmarinic acid (0.1 µg/g).

Table 1. Bioactive phenolics obtained from of *S. graeca* L. extracts.

N°	Compounds	Rt (min)	EtOAc (µg/g)	*n*-but (µg/g)
1	Protocatechuic acid	14.0	0.8	-
2	4-hydroxybenzoic acid	19.5	0.2	-
3	Caffeic acid	24.1	1.4	-
4	Vanillin	24.8	1.3	-
5	2,4,-dihydroxybenzoic acid	25.2	-	0.3
6	Chlorogenic acid	27.3	4.8	41.1
7	*p*-coumaric acid	29.8	-	0.2
8	Ferrulic acid	31.0	-	0.1
9	Coumarin	32.5	-	0.2
10	Rutin	34.2	-	41.6
11	Ellagic acid	38.1	-	3.3
12	*trans*-cinnamic acid	43.2	-	0.2
13	Quercetin	44.0	-	0.2
14	Rosmarinic acid	45.3	-	0.1

(-): not detected

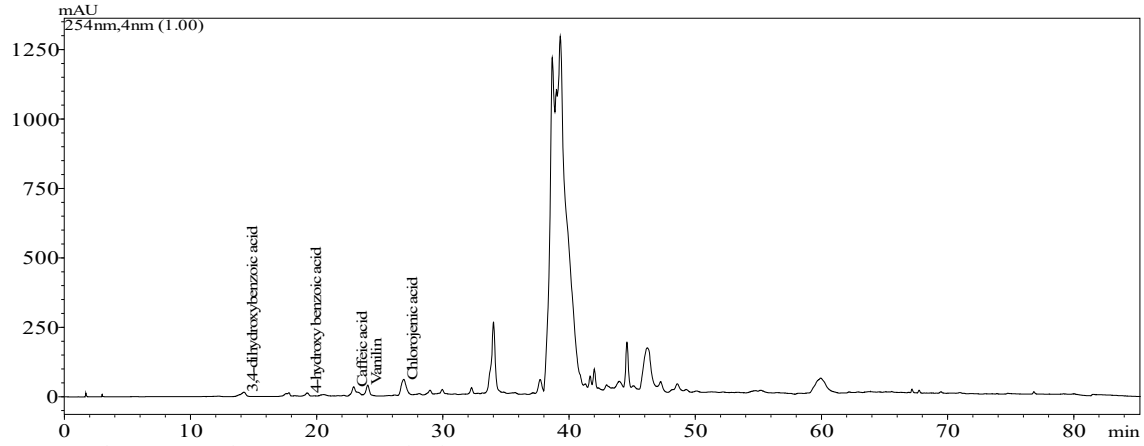

Figure 2. Chromatogram of *S. graeca* L. EtOAc fraction at 254 nm.

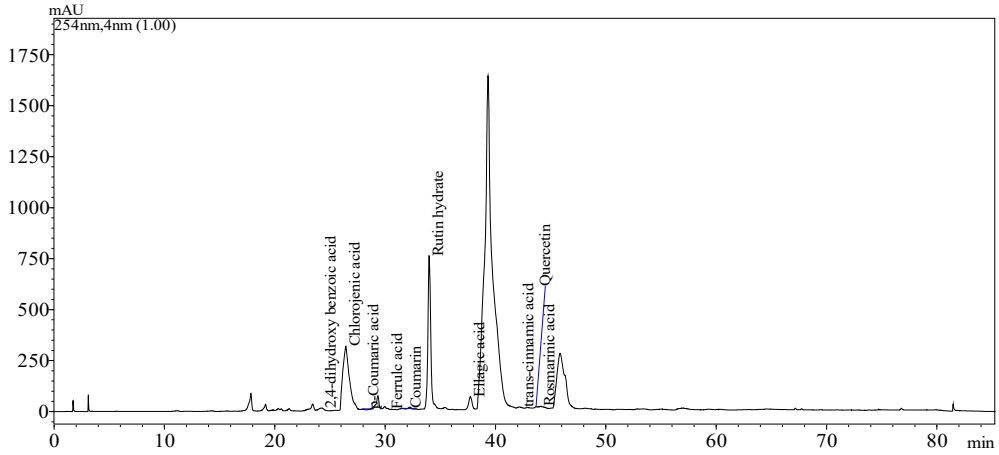

Figure 3. Chromatogram of *S. graeca* L. *n*-but t fraction at 254 nm.

3.3. Antioxidant activity

Results of antioxidant activities are represented as percentage inhibition for each concentration as well as 50% radical scavenging concentration (IC_{50}) values for the DPPH, ABTS, $O_2^{\bullet-}$ and β-Carotene tests, and $A_{0.5}$ values, which correspond to the concentrations indicating 0.5 absorbance for CUPRAC and reducing power (RP) tests. According to data mentioned in Tables 2 and 3, and based on the values of CI_{50} and $A_{0.5}$, we have found that both extracts exhibited a very significant activity in comparison to standard antioxidants, as we have recorded CI_{50} values very near to those of BHA in the ABTS and β-carotene tests (P <0.05), significantly better than those of BHT in DPPH (CI_{50}: 10.6 ± 0.4 µg/mL and 11.4 ± 0.5 µg/mL) and CUPRAC ($A_{0.5}$: 4.4 ± 0.4 µg/mL and 6.2 ± 0.3 µg/mL) tests (P <0.01) and significantly better than that of α-tocopherol in the RP test ($A_{0.5}$: 6.9 ± 0.6 µg/mL and 11.5 ± 0.7 µg/mL) for EtOAc and *n*-but, respectively (P<0.001).

Table 2. Antioxidant activity of *S. graeca* L. extracts by DPPH•, $ABTS^{\bullet+}$, $O_2^{\bullet-}$, *β*-carotene, inhibition % at minimum concentration and IC_{50}.

	DPPH• assay		$ABTS^{\bullet+}$ assay		*β*-carotene-linoleic acid assay		$O_2^{\bullet-}$ assay	
	Inhibition (%) at 6.2µg/mL	IC_{50} µg/mL	Inhibition (%) at 6.2µg/mL	IC_{50} µg/mL	Inhibition (%) at 6.2µg/mL	IC_{50} µg/mL	Inhibition (%) at 6.2µg/mL	IC_{50} µg/mL
EtOAc	28.9±0.1*	10.6 ±0.4**	89.5±0.3*	3.3±0.1*	66.4±2.5*	2.4±0.1*	82.8±0.4	1.2±0.1*
n-but	26.5±1.3*	11.4 ±0.5**	65.2±1.8	4.6±0.3*	61.1±0.4*	2.6±0.1*	78.1±1.4	7.3±0.2***
BHA	54.3±1.6**	5.7 ±0.4***	93.5±0.1*	1.8±0.1*	90.1±0.6**	0.9±0.1*	nt	nt
BHT	22.2±1.3*	13.0 ±0.4**	78.5±3.4*	1.2±0.3**	86.1±1.0**	1.0±0.1*	nt	nt
Tannic ac	nt	nt	nt	nt	Nt	nt	78.8±0.9	1.6±0.1*
α-Tocopherol	nt	nt	nt	nt	Nt	nt	64.9±1.0*	3.1±0.4*

Results are expressed as means ± standard deviation of three measurements

(-) : no inhibition; nt : not tested. ***P<0.001, ** P <0.01 and * P <0.05 compared between means.

Table 3. Antioxidant activity of *S. graeca* L. extracts py CUPRAC, Ferric reducing power, inhibition % at minimum concentration and $A_{0.5}$.

	CUPRAC		Ferric reducing power	
	Inhibition (%) at 6.2µg/mL	$A_{0.50}$ µg/mL	Inhibition (%) at 1.5µg/mL	$A_{0.50}$ µg/mL
EtOAc	0.6±0.1**	4.4±0.4**	0.3±0.00***	6.9±0.6***
n-but	0.5±0.1**	6.1±0.3**	0.3±0.01***	11.4±0.7***
BHA	0.8±0.1**	3.6±0.1**	0.1±0.01***	8.4±0.6***
BHT	0.3±0.2**	9.6±0.8**	0.1±0.00***	>50***
Tannic ac	nt	nt	Nt	5.3±0.9***
α-Tocopherol	nt	nt	Nt	34.9±2.3***
Ascorbic ac	nt	nt	0.1±0.00***	6.7±1.1***

Results are expressed as means ± standard deviation of three measurements

(-) : no inhibition; nt : not tested. ***P<0.001, ** P <0.01 and * P <0.05 compared between means.

3.4. Antimicrobial and Antibiofilm activities

Antimicrobial activity of both *S. graeca* L. extracts against 9 reference microbial strains tested in this study was qualitatively evaluated by the minimum inhibitory concentrations (MICs) and results were represented in Table 4. Our data showed that EtOAc fraction of *S. graeca* L. possesses a strong effect and a wide range of action covering Gram positive, Gram negative bacteria and *Candida* genus, as MIC came out to be 0.3–10 mg/ml (mean range). *n*-but fraction had the same antibacterial

effect (MIC) against *S.aureus* ATCC25923 (0.6 mg/ml), *L. monocytogenes* ATCC7644 (1.2 mg/ml) and *P. fluorescens* RSKK240 (0.3 mg/ml). However, EtOAc fraction exerted a significant effect compared to that of *n*-but on *E. faecalis* ATCC19433 (1.2 and 2.5 mg/mL), *C. albicans* ATCC10239 (2.5 and 10 mg/mL) and *C. tropicalis* RSKK 665 (2.5 and 5 mg/mL).

Results of antibiofilm activity presented in Table 4 as inhibition percentages showed that EtOAc extract exerted a moderate effect of inhibition of the biofilm formed by only three strains: *L. monocytogenes* (23.4%), *C. albicans* (38.6%) and *C. tropicalis* (42.1%). However, there was no effect with the *n*-but fraction against the biofilm produced by the major strains examined.

Table 4. Antimicrobial and antibiofilm activities of *S. graeca* L. extracts values.

Strains		*n*-but				EtOAc			
		MIC	% inhibition on biofilm formation			MIC	% inhibition on biofilm formation		
		mg/mL	MIC	MIC/2	MIC/4	Mg/mL	MIC	MIC/2	MIC/4
Gram-Positive bacteria	*S.aureus* MU40	0.62	-	-	-	0.6	-	-	-
	E. foecalis ATCC19433	2.5	-	-	-	1.2	-	-	-
	L. monocytogenes ATCC7644	1.2	-	-	-	1.2	23.4±0.4	18.7±0.8	12.4±1.2
	B. subtilis ATCC6633	10	nt	nt	nt	5	nt	Nt	nt
	S.aureus ATCC25923	> 10	-	-	-	5	-	-	-
Gram-Negative bacteria	*C. violaceum* CV 026	1.2	-	-	-	1.2	-	-	-
	P. fluorescens RSKK240	0.3	-	-	-	0.3	-	-	-
Fungi	*C. albicans ATCC 10239*	10	19.3±3.2*	-	-	2.5	38.6±2.2*	16.9±2.7*	-
	C. tropicalis RSKK 665	5	-	-	-	2.5	42.1.±0.2	-	-

Results are expressed as means ± standard deviation of three measurements.

MIC: minimal inhibitory concentration (mg. mL^{-1})

(-): no inhibition; nt : not tested. * P <0.05 compared between means.

4. Discussion

Infectious diseases brought on by bacteria, viruses, and fungi continue to pose a significant threat to public health (Al-kafaween *et al*., 2020; Shidiki and Vyas, 2022). Finding alternate methods to combat bacterial infection has become urgently necessary due to the rise in multidrug-resistant pathogenic microorganisms (Zullkiflee *et al*., 2023).

In the current study, rutin, ellagic acid, vanillin, caffeic and chlorogenic acids, were shown to be the major phenolic components in *Satureja graeca* L. extracts. According to several studies, phenolic chemicals are commonly found in species of *Satureja*. For example, *p*-coumaric, ferulic, caffeic and protocatechuic acids have been detected in ethyle acetate and *n*-butanol extracts of *S. montana* L. which are analyzed qualitatively by HPLC-DAD, as well as in the methanolic extract of *S. montana* subsp. *Kitaibelii* of Serbia (López-Cobo *et al*., 2015). HPLC analysis of *S. hortensis* (Georgia) ethanol extract confirmed the presence of several phenolic compounds such as caffeic, *p*-coumaric acids, rutin, hesperidin and 7-glucoside (Boroja *et al*., 2018).

Antioxidant activity can be determined using a variety of techniques. Depending on the test employed, the chemical structure of extracts, which sometimes consists of several of compounds mixed together with different functional groups, polarity, and chemical behaviors, may produce dispersed results (Boutellaa *et al*., 2019; Menakh *et al*., 2020). Therefore, it would be more beneficial and even required to utilize a method that involves many tests to estimate the antioxidant capability of extracts. In this investigation, six methods were used to evaluate in *vitro* antioxidant activity of ethyl acetate (AcOEt) and *n*-butanol (*n*-but) fractions of *S. graeca* L. based on different mechanisms of action such as radical scavenging properties ($DPPH^{\bullet}$, $ABTS^{\bullet+}$ and $O_2^{\bullet-}$), ability to prevent lipid peroxidation (*β*-carotene) and ability to reduce copper and ferric ions (CUPRAC and RP). Our findings consistently demonstrated that both the AcOEt and *n*-but extracts exhibited similar antioxidant activities across most of these tests. Notably, the AcOEt extract displayed a slightly higher level of activity compared to the *n*-but extract. This observation underscores the robust antioxidant potential of both extracts.

Additionally, our analysis of the extracts' composition revealed high and closely matched contents of total phenolic compounds and flavonoids in both fractions. These results suggest that the extracts' antioxidant activities can be attributed to their rich content of these bioactive compounds, further supporting their potential utility in medicinal and therapeutic applications.

Our antioxidant properties investigation of *S.graeca* L. extracts are consistent with those reported by previous studies for aqueous and ethanolic extracts of *S. hispidula* (Algeria), for wild and cultivated *S. bachtiarica* (Iran), for methanolic extract of *S. rechingeri* (Iran) depending on the phenological stage and for *S. montana* (Serbia) who have all found similar relationships between these species' total phenolic compound concentration and antioxidant capacity (Alizadeh, 2015; Veličković *et al*., 2018; Haouat *et al*., 2022). Thus, HPLC analyses have proven a diversity of phenolic compounds in both extracts. The presence of Chlorogenic acid and Rutin at high concentrations and Ellagic acid in *n*-but extract, on one hand, and the presence of Caffeic acid, Vanillin and Protocatechuic acids in AcOEt extract could be the reason for their most powerful antioxidant potential. Moreover, the proportion of

unidentified compounds in both fractions is generally non-negligible.

The creation of bioactive secondary metabolites, which leads to the discovery of antibiotics, has attracted the attention of researchers who have been mining the understudied sources to find novel secondary metabolites that are of considerable interest in the present (Abdolhosseini *et al*., 2019; Abdel-Mawgoud *et al*., 2019). Our funding demonstrated that *S. graeca* L. extracts possess a strong antimicrobial activity because they are abundant in phenolic and flavonoid chemicals, which can be used as an alternative to antibiotics. Besides, chlorogenic acid and rutin, known for their antimicrobial potential, were detected as major phenolic compounds, which could explain the observed effect (Kabir *et al*., 2014; Stojković *et al*., 2013). According to the literature review, there was no work on the antimicrobial activity of *S. graeca* L. extracts. Previous studies have examined the antimicrobial properties of extracts from other species within the *Satureja* genus. For instance, research on *S. kitaibelii* explored the antimicrobial potential of chloroform, ethyl acetate, and *n*-butanol extracts. The results indicated that the ethyl acetate extract exhibited the highest activity, with a minimum inhibitory concentration (MIC) of 10 mg/ml against *P. aeruginosa* and *S. aureus*, while the chloroform extract was particularly effective against *P. aeruginosa*. However, the *n*-butanol extract showed weaker activity with an MIC of 50 mg/ml against *S. aureus* (López-Cobo *et al*., 2015). In another study, aqueous and EtOH extracts of *S. boissieri* (Turkey), rich in hesperidin and ac rosmarinique, showed weak activity against *S. aureus* and *P. aeruginosa*, and were not effective against *C. albicans (Aras et al*., 2018). Our results are similar with those of previous funding who reported a strong antimicrobial activity of *S. kitaibelii* MeOH extract from Serbia against *S. aureus* (0.6 mg/mL), *L. monocytogenes* (1.25 mg/mL), *P. aeruginosa* (1.2 mg/mL) and *C. albicans* (0.6 mg/mL) (Stanojković *et al*., 2013) . Moreover, with MICs varying from 0.02 to 0.4 mg/mL, the antibacterial activity of *S. hortensis* (Turkey) extracts in EtOH, MeOH, and dichloromethane was significant against *S. aureus*, *E. faecalis*, *P. aeruginosa*, and *C. albicans* (Sharifi *et al*., 2018).

Bacterial survival in challenging environments is supported by biofilms, which are essential cell colonies that bind to biotic or abiotic environments and produce an extracellular matrix. Different degrees of biofilm formation are capable of occurring in both Gram-positive and Gram-negative bacteria (Al-kafaween *et al*., 2020; Seyedtaghiya *et al*., 2021).

The results of the current study's evaluation of the antibiofilm qualities of *S. graeca* L. extracts revealed that AcOEt extract reduced the development of biofilms at concentrations lower than MIC. This finding holds significant promise for the potential application of S. graeca L. extracts in combating biofilm-related issues, a subject for which data have been notably scarce in the existing literature. Currently, there is a dearth of data on the antibiofilm activity of S. graeca L. extracts, making our study one of the pioneering works in this regard. While previous research has focused on related species like *S. hortensis* and its essential oil (Seyedtaghiya et al., 2021; Sharifi et al., 2018), few studies have specifically delved into the antibiofilm qualities of *S. graeca* L. extracts. The antibiofilm effects of the plant extract may be caused by a reduction in structure development, such as a decrease in exopolysaccharide production, or an alteration in the transcription of genes linked to biofilms (Swamy *et al*., 2016).

In summary, our research not only highlights the promising antibiofilm properties of *S. graeca* L. extracts but also underscores the need for more extensive studies to elucidate the mechanisms underlying these properties and their suitability for practical applications in areas such as medicine, agriculture, and biotechnology.

5. Conclusion

In conclusion, our study has revealed that *Satureja graeca* extracts, characterized by their rich composition of phenolic compounds and flavonoids, have the potential to serve as a valuable source of bioactive phenolic compounds. These compounds hold promise in the fields of medicine and therapy due to their demonstrated antioxidant and antimicrobial properties. Furthermore, this research has contributed to a deeper understanding of the antibiofilm properties of *Satureja graeca* L. extracts, showing their effectiveness in inhibiting biofilm formation by bacterial and yeast species. This discovery is particularly significant, as biofilm inhibition has wide-ranging implications in various industries, including healthcare and agriculture. While our findings are promising, it is important to note that further research is necessary to validate the applicability of these extracts, both as feed additives and in clinical settings. Future in vivo studies are warranted to confirm the efficacy and safety of these extracts for potential use in practical applications.

Disclosure statement

The authors report no conflicts of interest.

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