J Herbmed Pharmacol. 2025; 14(1): 71-81.

http://www.herbmedpharmacol.com doi: 10.34172/jhp.2025.52718

Journal of Herbmed Pharmacology

Comprehensive analysis of bioactive compounds in *Hyssopus officinalis* L. and evaluation of its antioxidant, antibacterial, and anti-Alzheimer properties

Nour-El-Houda Hamoud[1,2*], **Zineb Maammeri**[2*]

[1]Department of Animal Biology, Faculty of Nature and Life Sciences Mentouri Brothers University Constantine 1, Algeria
[2]Laboratory of Pharmacology and Toxicology, Institute of Veterinary Sciences, Mentouri Brothers University Constantine 1, Algeria

ARTICLE INFO

Article Type:
Original Article

Article History:
Received: 1 September 2024
Accepted: 10 November 2024
epublished: 25 December 2024

Keywords:
Neurodegenerative disorder
Hyssopus officinalis
Acetylcholinesterase
Antioxidant
Antibacterial activity
Alzheimer's disease

ABSTRACT

Introduction: The persistent global challenges of oxidative stress-related diseases, bacterial infections, and neurodegenerative disorders such as Alzheimer's disease highlight the critical need for effective therapeutic solutions. *Hyssopus officinalis* has shown promise in traditional medicine for its antioxidant, antibacterial, and neuroprotective properties. This study evaluated the antioxidant, anti-Alzheimer, and antibacterial capacities of ethyl acetate extracts from the leaves (EAL) and stems (EAS) of *H. officinalis*, as well as total phenolic and flavonoid content (TPC/TFC) and metabolite profiling.

Methods: 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), and Ferric reducing antioxidant power (FRAP) techniques were used to assess the antioxidant activity. Ellman's method was used to evaluate anti-cholinesterase capacity. The agar well diffusion method was employed to assess the antibacterial effects. Folin-Ciocalteu and aluminum colorimetric techniques were used to investigate the TFC/TPC levels. The phytochemical compounds were recognized by UPLC-ESI-MS/MS.

Results: Twenty phytochemical compounds from different classes were identified. EAL exhibited high DPPH and ABTS scavenging activities comparable to butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), with a FRAP value surpassing α-tocopherol and close to ascorbic acid ($P < 0.05$). All extracts demonstrated antibacterial activity comparable to gentamicin against gram-positive bacteria. EAS possessed a strong antibacterial effect at lower concentrations against *Staphylococcus aureus* and demonstrated a moderate inhibitory action against acetylcholinesterase (AChE), comparable to galantamine ($P < 0.05$). All extracts had high levels of TPC and TFC.

Conclusion: *Hyssopus officinalis* contains a rich array of bioactive compounds with antioxidant, antibacterial activities, and anti-Alzheimer properties; however, these effects should be confirmed by clinical trials.

Implication for health policy/practice/research/medical education:
This article outlines the phytochemical composition and pharmacological properties of *Hyssopus officinalis*. The findings highlight the potential of the plant as a natural source for developing treatments targeting oxidative stress-related disorders, bacterial infections, and neurodegenerative diseases such as Alzheimer's disease. The distinct bioactive compounds identified in the extracts further support the plant's therapeutic relevance in these areas, paving the way for more focused applications in natural medicine and drug development.

Please cite this paper as: Hamoud NEH, Maammeri Z. Comprehensive analysis of bioactive compounds in *Hyssopus officinalis* L. and evaluation of its antioxidant, antibacterial, and anti-Alzheimer properties. J Herbmed Pharmacol. 2025;14(1):71-81. doi: 10.34172/jhp.2025.52718.

Introduction

Herbal medicines offer a natural and holistic approach to health and wellness, grounded in centuries of traditional knowledge and increasingly supported by scientific research. By harnessing the therapeutic properties of plants, herbal medicines provide valuable tools for preventing and treating a wide range of health conditions, contributing to the global pursuit of natural and integrative healthcare solutions (1). Medicinal plants contain a variety of bioactive compounds, which contribute to their

***Corresponding authors**: Nour-El-Houda Hamoud, Email: houdanoor31@yahoo.fr; Zineb Maammeri, Email: maameri.zineb1@gmail.com

therapeutic effects. Understanding these compounds and their mechanisms of action is essential for appreciating the therapeutic potential of medicinal herbs. The high concentration of phenolic compounds in these herbs provides significant protection against reactive oxygen species (ROS)-induced oxidative damage, inhibits the generation of free radicals, and halts the spread of radicals that damage macromolecules like lipids and proteins (2). This oxidative stress contributes to cardiovascular diseases like atherosclerosis, hypertension, and heart failure, neurodegenerative problems like Alzheimer's and Parkinson's diseases, and inflammatory diseases such as rheumatoid arthritis and cancers (3).

The human body has evolved a sophisticated defense system to protect against the harmful effects of ROS (4). These defenses are categorized into enzymatic and non-enzymatic antioxidants, which work together to neutralize ROS and repair oxidative damage. Sometimes, excessive ROS production can perturb these systems and can cause insufficient antioxidant defenses, inefficient repair mechanisms, and chronic conditions. Consequently, bioactive compounds such as polyphenols, flavonoids, carotenoids, and vitamins play an important role in reducing oxidative damage and risk of chronic diseases and exhibit a broad spectrum of therapeutic properties, including antioxidant, and anticonvulsant, antifungal, antibacterial, anti-hemolytic, antiulcer and anti-inflammatory proprieties (3,5).

Hyssopus officinalis L., also known as "Zufa" in Arabic, is one of the most frequently used medicinal herbs in traditional medicine (6). It is a member of the Lamiaceae family. This herb's usage is due to its therapeutic properties, including antioxidant, antifungal, antibacterial, antiulcer, and antispasmodic activities (1,7). In Uyghur medicine, *H. officinalis* is traditionally employed to treat conditions such as asthma, cough, fever, and rheumatism. Precedent studies have indicated that *H. officinalis* may modulate the expression of specific cytokines in asthmatic mice (8). The crude extract of dried leaves has been shown to have anti-HIV activity by inhibition of HIV reverse transcriptase (9). The extracts of *H. officinalis* also have demonstrated significant anti-leishmaniasis activity (6,10). Furthermore, in a recent study *H. officinalis* extract significantly enhanced memory and learning abilities in kindled rats, demonstrating robust neuroprotective effects. The treatment elevated brain antioxidant capacity, while markedly reducing malondialdehyde (MDA) and nitric oxide (NO) levels. Importantly, it also increased GABA receptor gene expression in pre-treated groups, indicating a robust mechanism of action via the GABAergic system, which may provide antiepileptic benefits. These findings firmly establish *H. officinalis* extract as a potent agent against hippocampal neuronal damage, offering promising therapeutic potential for cognitive enhancement (11). This study provides valuable insights that can support further research into its anticholinesterase activity; *H. officinalis* extract may exhibit complementary mechanisms enhancing cholinergic function. This opens avenues for exploring its potential in treating cognitive disorders linked to cholinergic deficits, reinforcing its role as a multifaceted therapeutic agent. The abundance of phenolic substances in this plant may prevent the production of free radicals, stop the chain reaction of radicals that damage macromolecules like lipids and proteins, and heal oxidative damage caused by ROS (1,3). Research on the antioxidant, antibacterial, and neuroprotective properties of hyssop is still quite limited. Most studies have primarily concentrated on its essential oil. Additionally, the potential anti-Alzheimer activity of *H. officinalis* has yet to be established. This study addresses the gap in the literature, by providing a detailed chemical profile and evaluating its therapeutic potential. The findings of this study offer a foundation for further preclinical and clinical investigations into the use of *H. officinalis L.* as a natural therapeutic agent. The identification of active compounds, like quercetin and esculin acid, can guide the development of new treatment options. Additionally, its antibacterial potential suggests its application in combating bacterial infections, particularly those resistant to conventional antibiotics. This research suggests the potential for an alternative treatment that is both effective and naturally sourced. As a reason, this study aims to identify and characterize the phytochemical profile of ethyl acetate (EA) extracts from leaves and stems of this aromatic and medicinal plant growing in eastern Algeria using ultra-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-ESI-MS/MS) and to determine its biological activities focusing on its antioxidant, antibacterial, and anti-Alzheimer capabilities and highlighting the pharmaceutical potential of this medicinal herb.

Material and Methods

Plant sample

Aerial parts of *H. officinalis* L. (Lamiaceae) were collected from the Jijel province (Eastern Algeria). Dr. Kameleddine Bazri of the Biology and Ecology department at the Faculty of Natural and Life Sciences Mentouri Brothers University Constantine 1, Algeria, carried out the botanical authentication of the samples and a voucher specimen (Ref No; UMC1/BED/2022/085) was deposited at the University Herbarium. In order to prepare the various extracts, the leaves and stems were ground into a fine powder and preserved in the lab at room temperature until they were used.

Extraction process

With a few minor modifications, the extraction procedure was performed by the technique outlined by Boudjada et al (12). The powdered leaves and stems were macerated in a hydro-alcoholic solution (methanol/water: 70:30: v/v). This procedure was carried out three times using

a renewal solvent. After evaporation, the remaining crude extracts were diluted with distilled water to obtain aqueous solutions ready for liquid-liquid extraction using EA. The organic fraction was concentrated and weighed to determine the % yield of the soluble constituents using the following formula:

%Yield: Weight of dry extract /Weight taken or extraction ×100.

Total phenolic (TPC) and flavonoid (TFC) contents

The Folin-Ciocalteu method (13) with minor modifications was used to assess the determination of total phenols. Briefly, 20 µL of each extract was mixed with 100 µL of 1:10 diluted Folin-Ciocalteu reagent and 75 µL of sodium carbonate solution (75 g/L). During 2 hours, the mixture was maintained at ambient temperature and in darkness. In the microplate (96 wells) reader, the absorbance was measured at 740 nm. The results were expressed as µg of gallic acid equivalent per milliliter (µg/GAE/mL). The aluminum colorimetric method (14), with minor changes, was used to determine the total flavonoids. Methanol (130 µL) was placed into a micro-plate containing 50 µL of each extract, and then 10 µL of potassium acetate (1M) and 10 µL of aluminum nitrate at 10% were added. Forty minutes of the incubation were spent at room temperature. In the microplate reader, the absorbance was measured at 415 nm. The results were expressed as µg of quercetin equivalent per milliliter (µg/QE/mL),

Determination of phytochemical profile of EA extracts using UPLC-ESI-MS/MS

The procedure outlined by Akdenizl (15) was followed to get the chemical profile by UPLC-ESI-MS/MS analysis. Shimadzu 8040 Ultra-High Sensitivity with UFMS Technology and a binary bump Nexera XR LC-20AD were used in the UPLC-ESI-MS-MS analysis. In this experiment, the column Restek Ultra AQ C18 (3 µm, 150×4.6 mm) was utilized. The mobile phase was constituted of 0.1% formic acid, methanol, and water. The ESI conditions were as follows: CID gas, 230 KPs; conversion dynode, −6.00 Kv; interface temperature, 350 °C; DL temperature, 250 °C; mobilizing gas flow, 3.00 L/min; heat block, 400 °C; drying gas flow, 15.00 L/min. The standards were made in methanol at a concentration of 500 µg/L. The ion trap mass spectrometer was used in both negative and positive ions with multiple reaction monitoring mode.

Antioxidant activity assays

DPPH free radical sequestration

The method proposed by Blois (16) was used to measure the scavenging activity of the stable 1, 1-diphenyl-2-picryl-hydrazyl free radical (DPPH). A volume of 40 µL of each extract at varying concentrations was added to a 96-well microplate, followed by the addition of 160 µL of a DPPH solution, which was prepared by dissolving 6 mg of DPPH in 100 mL of methanol. The disappearance of the DPPH was measured at 517 nm after 30 minutes of incubation at room temperature. The IC_{50} value (µg/mL), which represented the concentration of 50% inhibition, was used to express the ability of the extracts of *H. officinalis* to scavenge the DPPH radical. Butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) were used as standards for comparison:

$$I\% = \frac{A_0 - A}{A}$$

I%: The percentage of inhibition;
A_0*:* The absorbance of DPPH solution without extract;
A: Absorbance in the presence of extract or standard.

ABTS•+ radical scavenging activity

The ABTS•+ (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) decolorization assay, as described by Re et al (17), was used to evaluate the antioxidant activity. In this assay, 40 µL of each extract at varying concentrations were added to a 96-well microplate. Next, 160 µL of ABTS•+ solution was added, and the absorbance was measured at 734 nm after a 6-minute incubation. The ABTS radical cation (ABTS•+) was produced by mixing two aqueous solutions 7 mM stock solution of ABTS with 2.45 mM potassium persulfate and allowing the mixture to stand in dark at room temperature (25 °C) for 12 hours; the absorbance of the solution was adjusted by ethanol or H_2O to 0.700 ± 0.020 at 734 nm before use. Using the formula provided for the DPPH assay, the scavenging rate and IC_{50} value were determined. The standards for comparison were α-tocopherol and BHA.

Ferric-reducing power assay (FRAP assay)

The FRAP was determined using the method developed by Oyaizu (18). This method allows for the evaluation of the antioxidant capacity of samples in terms of their ability to reduce Fe^{3+} to Fe^{2+}. A volume of 10 µL of each extract at varying concentrations was dispensed into a 96-well microplate. Subsequently, 40 µL of 0.2 M sodium phosphate buffer (pH 6.6) and 50 µL of 1% potassium ferricyanide [K3Fe(CN)6] were added. The mixture was incubated at 50 °C for 20 minutes. After incubation, 50 µL of 10% trichloroacetic acid, 40 µL of water, and 10 µL of 0.1% ferric chloride were transferred to the wells. The absorbance was then measured at 700 nm. The $A_{0.5}$ value is the effective concentration at which the absorbance is 0.5, obtained by the equation described for DPPH assay. Ascorbic acid and α-tocopherol were used as standards for comparison.

Antibacterial activity

Antibacterial potential of the EA extract using the well diffusion assay

The selected method was agar well diffusion, as described by Chandur et al (19). The bacterial strains were obtained from the National Center of Biotechnology

Research (C.R.Bt, Constantine) and comprised *Bacillus cereus* ATCC 10876, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, and *Salmonella typhi* ATCC 13076. These bacterial strains were reactivated by inoculating them on Petri dishes containing nutrient agar and incubated for 18 hours to obtain young, active cultures. The resulting bacterial colonies were transferred to tubes containing 9 mL of 0.9% saline solution and the suspension was adjusted to a turbidity equivalent to 0.5 McFarland. Samples were prepared at a concentration of 250 mg/mL in dimethyl sulfoxide (DMSO). The zones of inhibition were measured in mm. The negative control was DMSO and the positive control was gentamicin. All experiments were carried out in triplicate.

Estimation of minimal inhibitory concentration (MIC)

The MIC for a specific strain was determined as the lowest concentration at which there was no observable growth of the microorganism. The experiments for determining the MIC were carried out using the standard dilution method on the Mueller-Hinton agar medium. Each extract was subjected to the double dilution method to create a concentration range spanning 1/2, 1/4, 1/8, 1/16, 1/32, and 1/64, ranging from 125 to 3.9 mg/mL using distilled water. The inhibition zone was measured in mm. All experiments were carried out in triplicate (20).

Anti-cholinesterase activity

The spectrophotometric test described by Ellman et al (21) was used to evaluate the inhibitory measurement of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) of the samples *in vitro*. Acetylthiocholine/butyryl thiocholine was the substrate, while AChE/BChE was the enzyme that reacted with it to produce thiocholine. Ellman's reagent, 5,5′-dithio-bis-[2-nitrobenzoic acid] (DTNB), interacted with thiocholine to form the yellow-colored 2-nitrobenzoic-5-mercaptothiocholine and 5-thio2-nitrobenzoate, which could be detected at 412 nm. The standard for comparison was galantamine hydrobromide.

Statistical analysis

All samples were analyzed in triplicate. Data were expressed as means ± SD. The results were analyzed by one-way analysis of variance (ANOVA). Significant differences between means were determined by the Tukey test ($P < 0.05$).

Results

Extraction yield

Using the established formula, the methanolic extract yield from the leaves was calculated at 5.68%, while the yield from the stems was 2.49%. The EA extracts yielded 0.85% for the leaves (EAL) and 0.67% for the stems (EAS), respectively.

Total phenolic (TPC) and flavonoid (TFC) contents

The findings demonstrated the presence of TPC in both extracts, albeit at different concentrations. The EAL and EAS extracts showed substantial TPC levels (408.90 ± 1.96 and 343.02 ±1.22 µg GAE/mL, respectively). Additionally, both EAL and EAS extracts displayed significant levels of total flavonoid content, with values of 262.01 ± 1.32 µg QE/mL and 209.23 ± 0.73 µg QE/mL, respectively.

Phytochemical profile of ethyl acetate phases using UPLC-ESI-MS/MS

In this study, bioactive compounds present in the EA extracts of *H. officinalis* leaves and stems were characterized and semi-quantified using the ultrasensitive UPLC-ESI-MS/MS method, applied in both positive and negative ion modes. A total of twenty metabolites were identified from the analyzed plant parts, encompassing diverse classes such as phenolic acids, flavanones, flavonols, flavanone glycosides, flavones, vitamins, and quinones (Table 1). The analysis revealed a diverse phytochemical profile, with 20 compounds identified in the EAL extract and 16 in the EAS extract out of the 20 tested phytochemical standards. The EAL extract was particularly rich in bioactive compounds, with significant concentrations of quercetin (24.09%), esculin acid (24.37%), hesperetin acid (20.46%), and naringenin (8.63%). The EAS extract was dominated by hydroxy-coumarin acid (35.40%), hesperetin acid (24.25%), picric acid (19.7%), and folic acid (11.82%). Both extracts contained minor amounts of ascorbic acid, chlorogenic acid, caffeic acid, cinnamic acid, maleic acid, and chrysin. Notably, only the EAL extract displayed traces of vanillin acid and benzoic acid, further highlighting the distinctive chemical composition of the leaves compared to the stems. This differentiation underscores the potential for targeted extraction depending on the desired therapeutic application, as specific compounds in the leaves and stems could offer distinct health benefits.

Antioxidant activity

DPPH radical scavenging activity

The EA extract of *H. officinalis* leaves exhibited notable antioxidant activity, with an IC_{50} value of 23.19 ± 0.40 µg/mL, demonstrating a strong capacity to scavenge DPPH radicals. This performance was comparable to the well-known antioxidants BHT and BHA, which are recognized for their potent free radical scavenging properties. In contrast, the EAS extract showed significantly weaker antioxidant activity compared to the EAL extract (Table 2). This disparity suggested that the leaf extract contained higher concentrations of potent antioxidant compounds, further emphasizing the potential of *H. officinalis* leaves as a more effective source for antioxidant applications. This finding highlights the importance of specific plant parts in maximizing bioactive compound extraction for targeted therapeutic use (Figure 1).

Table 1. Ultra performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-ESI-MS/MS) analysis of *Hyssopus officinalis* ethyl acetate extract

Analytes	Retention time (min)	EAL area (%)	EAS area (%)
Naringenin	45	8.63	ND
Maleic acid	46	0.83	0.42
Lawson acid(2-hydroxy-1,4-naphthoquinone	0.95	3.91	0.25
Gallic acid	2.29	0.03	0.12
Quercetin	46.5	24.09	ND
Chrysin (5,7-Dihydroxyflavone)	4.26	0.2	0.42
Beta-carotene	43.5	0.06	0.25
Butylated hydroxytoluene	18.5	0.69	0.12
Rutin	14.85	0.3	0.42
Ascorbic acid	47.75	0.23	0.25
Chlorogenic acid	19	0.26	0.12
Caffeic acid (3,4-dihydroxy-cinnamic acid)	7.4	1.52	0.42
Cinnamic acid	11.25	0.12	0.25
Picric acid	12.1	0.04	19.7
Esculin acid	16.8	24.37	0.1
Folic acid	35.15	5.79	11.82
Hespertin acid	37.6	20.46	24.25
Hydroxy-coumarin acid	47.30	0.15	35.40
Vanillin acid	48.3	0.27	ND
Benzoic acid	47.1	8.04	ND

ND: Non detected; EAL: Leaf ethyl acetate extract; EAS: Stem ethyl acetate extract.

Table 2. Antioxidant and anticholinesterase activities of *Hyssopus officinalis* ethyl acetate extracts

Samples	Antioxidant activity			Anti-cholinesterase activity
	FRAP (µg/mL)[b]	ABTS•+radical (µg/mL)[a]	DPPH radical (µg/mL)[a]	AChE assay (µg/mL)[c]
EAL	15.71±1.13	7.47±0.24	23.19±0.40	NA
EAS	115.13±5.00	15.65±0.16	33.88±0.64	107.03±5,01
BHT[d]	NT	1.59±0.03	6.55±0.59	-
BHA[d]	NT	1.03±0.00	5.73±0.41	-
Ascorbic acid[d]	6.77±1.15	NT	NT	-
α-Tocopherol[d]	34.93±2.38	NT	NT	-
Galantamine[d]				6.27±1.15

[a] IC_{50} values representing the means ± standard error of three independent, parallel measurements, with statistical significance set at $P < 0.05$ according to the Tukey test, compared to BHT and BHA. [b] $A_{0.50}$ values ($P < 0.05$) compared to ascorbic acid and α-Tocopherol. [c] IC_{50} values compared to galantamine ($P < 0.05$). [d] Reference compounds.

NT: Not tested; NA: No activity; -: Not applicable; EAL: Leaf ethyl acetate extract; EAS: Stem ethyl acetate extract; FRAP: Ferric-reducing antioxidant power; ABTS•+: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid; DPPH: 1-diphenyl-2-picryl-hydrazyl; AChE: Acetylcholinesterase; BHT: Butylated hydroxytoluene; BHA: butylated hydroxyanisole.

ABTS•+ radical scavenging activity

The findings from the ABTS•+ assay, which evaluated the ability of samples to neutralize the ABTS•+ radical in comparison to a standard antioxidant, revealed superior antioxidant activity compared to the DPPH method. Both the EAL and EAS extracts demonstrated significant ABTS•+ radical scavenging capabilities, with IC_{50} values ranging from 7.47 ± 0.24 to 15.65 ± 0.16 µg/mL. These results suggest that the extracts, particularly from the leaves, possessed strong electron-donating capacities, effectively neutralizing ABTS•+ radicals. The higher sensitivity of the ABTS•+ assay may reflect the broader spectrum of antioxidants present in *H. officinalis*, highlighting its potential for comprehensive antioxidant applications. These findings underscore the versatility of different antioxidant assays in fully capturing the radical-scavenging efficiency of plant extracts (Figure 1).

Reducing antioxidant capacity

The FRAP assay assessed the antioxidant's capacity to reduce the Fe^{3+}–ferricyanide complex [(K3Fe3+ (CN)6] to its Fe^{2+}–ferrocyanide form, measurable at 593 nm (22). The results in Table 2 and Figure 1 demonstrate that the EAL extract exhibited a significantly high FRAP value (A_{50}: 15.71 ± 1.13 µg/mL), surpassing that of the α-tocopherol standard and closely aligning with the ascorbic acid

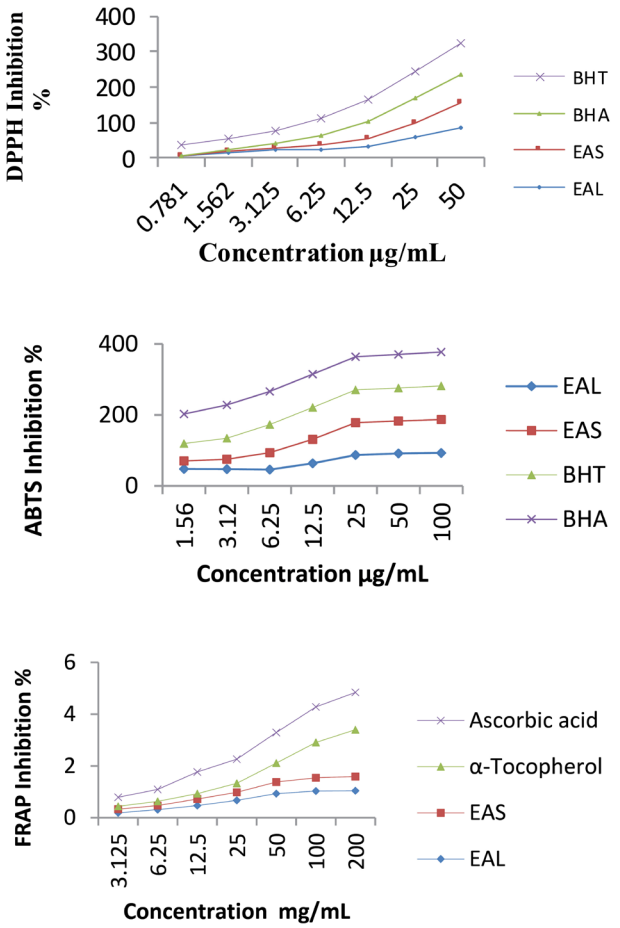

Figure 1. Kinetics of antioxidant activity of *Hyssopus officinalis* ethyl acetate extracts using DPPH (1-diphenyl-2-picryl-hydrazyl), ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) and FRAP (Ferric-reducing antioxidant power) assays. EAL: Leaf ethyl acetate extract; EAS: Stem ethyl acetate extract.

standard. Since a lower A_{50} value signifies stronger reducing power, the EAL extract's performance highlights its robust antioxidant potential in ferric ion reduction. In contrast, the EAS extract displayed negligible reducing activity, suggesting a substantially lower antioxidant capacity in this assay. These findings reinforce the idea that *H. officinalis* leaves, rather than stems, hold greater promise for applications where potent antioxidant activity, particularly reducing power, is desired (Figure 1).

Antibacterial activity

Antibacterial potential of the EA extracts using a well diffusion assay

The results presented in Table 3 indicate that gram-positive bacteria were more susceptible to the ethyl acetate extracts of *H. officinalis* compared to gram-negative strains at a concentration of 250 mg/mL. The leaf extract exhibited significant antibacterial activity, with inhibition zones ranging from 18 ± 0.01 to 20 ± 0.02 mm against *B. cereus* and *S. aureus*. Similarly, the stem extract demonstrated strong efficacy, particularly against *S. aureus*, with an inhibition zone of 20 ± 0.02 mm. However, neither EAL nor EAS extracts showed antibacterial activity against *S. typhi* or *E. coli*, suggesting selective antibacterial properties. These findings prompted further investigation into the MIC of the extracts, focusing on the more sensitive bacterial strains, *B. cereus* and *S. aureus*. This selective efficacy against gram-positive bacteria underscores the potential of *H. officinalis* extracts as natural antibacterial agents, especially for targeting specific pathogens.

Estimation of minimal inhibitory concentration

The standard dilution method was conducted on Mueller-Hinton agar to determine the MIC of the extracts. As shown in Table 4, EAS exhibited strong antibacterial activity against *S. aureus*, with an MIC of 7.81 mg/mL and an inhibition zone of 15 ± 0.03 mm, indicating effective inhibition at relatively low concentrations. In contrast, EAL required higher concentrations to achieve significant antibacterial effects. Specifically, the MIC for the EAL extract against *B. cereus* was 62.5 mg/mL, with an inhibition zone of 10 ± 0.02 mm, while against *S. aureus*, the MIC was 31.25 mg/mL, with an inhibition zone of 11 ± 0.04 mm. These results highlight that the EAS extract possesses a more potent antibacterial effect at lower concentrations, suggesting it may be a more efficient antibacterial agent compared to the EAL extract.

Anti-cholinesterase activity

One of the underlying causes of Alzheimer's disease is the impaired enzymatic hydrolysis of acetylcholine. To assess the impact of extracts on this process, the Ellman's assay was employed to evaluate the inhibition of AChE and

Table 3. Antibacterial screening of *Hyssopus officinalis* ethyl acetate extracts (250 mg/mL) against selected pathogenic bacteria

Plant extracts	Inhibition zones (mm)			
	Gram-positive pathogenic bacteria		Gram-negative pathogenic bacteria	
	Bacillus cereus	*Staphylococcus aureus*	*Echerichia coli cereus*	*Salmonella typhi*
EAL	18± 0.01	17.5± 0.03	NA	NA
EAS	00	20± 0.02	NA	NA
Gentamicin (PC)	30± 0.03	31± 0.02	30± 0.04	29± 0.02

NA: No activity; EAL: Leaf ethyl acetate extract; EAS: Stem ethyl acetate extract.
Data are means of three replicates (n = 3) ± standard error with statistical significance set at $P < 0.05$ according to the Tukey test and compared to gentamicin as positive control (PC).

Table 4. Minimum inhibitory concentrations (MICs) of ethyl acetate extracts of *Hyssopus officinalis* against *Staphylococcus aureus* and *Bacillus cereus*

Plant extracts	Concentration mg/mL	Inhibition zones (mm)	
		Bacillus cereus	***Satphylococcus aureus***
EAL	125	12± 0.01	16± 0.02
	62.5	10± 0.02	15± 0.05
	31.25	00	11± 0.04
	15.63	00	5± 0.01
	7.81	00	00
	3.90	00	00
EAS	125	-	20± 0.00
	62.5	-	18± 0.01
	31.25	-	18± 0.03
	15.63	-	16± 0.05
	7.81	-	15± 0.03
	3.90	-	3± 0.01

EAL: Leaf ethyl acetate extract; EAS: Stem ethyl acetate extract; -: Not determined.
Data are presented as means of three replicates (n = 3) ± standard error.

BChE *in vitro*. The results, detailed in Table 2, indicate that EAS had moderate inhibition of AChE, with an IC_{50} value of 107.03 ± 5.01 µg/mL, suggesting a specific inhibitory effect towards AChE rather than BChE, for which no significant activity was observed. In contrast, EAL showed no notable activity against either AChE or BChE under the tested conditions. For comparative purposes, galantamine, a well-known AChE inhibitor, achieved an IC_{50} value of 6.27 ± 1.15 µg/mL. These findings highlight the selective nature of the EAS extract's activity and underscore the potential of specific extracts in targeting enzymatic pathways involved in Alzheimer's disease.

Discussion

The findings of this study highlight the rich presence of bioactive compounds in the extracts, which contribute to their important potential antioxidant, antibacterial and anticholinesterase properties. The extraction method utilized may influence the yield and profile of bioactive compounds. Traditional extraction methods often result in lower extract quality due to the degradation or loss of volatile compounds, limited extraction efficiency, prolonged processing times, and degradation of solid residues. Future research should explore alternative extraction techniques to maximize efficacy (23). Comparative research on the chemical profile of *H. officinalis* across various global regions including Poland, Spain, Turkey, Italy, Serbia, India, Iran, and Romania reveals substantial variability in TPC TFC. For instance, the Romanian subspecies *H. officinalis* L. subsp. *aristatus* exhibited notable levels of these compounds, with TPC of 77.72 ± 1.83 mg/g and TFC of 1.30 ± 0.10 mg/g. These regional differences highlight the potential for varying therapeutic applications and underscore the importance of localized studies to fully understand the medicinal value of *H. officinalis*. The significant levels of phenols and flavonoids observed reinforce the plant's potential as a source of valuable bioactive compounds with broad-spectrum health benefits (24). The chloroform fraction of *H. officinalis* subsp. *angustifolius* cultivated in Turkey was characterized by a TPC of 4.19 ± 0.03 mg/g and TFC of 1.15 ± 0.005 mg/g. The phenolic profile of this fraction prominently features chlorogenic acid and its quinic acid isomer, along with flavonoid glycosides such as isoquercitrin, rutin, and quercitrin. Additionally, two flavonoid aglycones, quercetin and luteolin, were also identified. These findings highlight the diverse array of bioactive compounds present in the Turkish *H. officinalis* subsp. *angustifolius*, contributing to its potential medicinal properties and underscoring the importance of regional variations in the chemical composition of medicinal plants (25). Furthermore, high-performance liquid chromatography (HPLC) analysis of the methanolic extract from *H. officinalis* leaves identified several key phenolic compounds, including p-coumaric acid, benzoic acid, coumaric acid, ferulic acid, and quercetin, which were significant components in the phenolic profile (26). The extract of *H. officinalis* subsp. *aristatus*, cultivated in regions spanning Albania to Kosovo, exhibited a TPC of 84.28 ± 20.18 mg/g and a TFC of 43.60 ± 6.58 mg/g. Among the caffeic acid derivatives, the extract demonstrated notably high concentrations (27). Proestos et al (28) used reversed phase high-performance liquid chromatography with UV detection and found that the most prevalent phenolic acids in *H. officinalis* were ferulic acid (13.2 mg/100 g), caffeic acid (6.5 mg/100 g) and chlorogenic acid (166.21 g/g).

Previous research on *H. officinalis* has predominantly focused on the properties of its essential oils, highlighting their antioxidant and antibacterial activities (29). However, the focus on essential oils has limited the exploration of the full phytochemical profile of the plant, particularly the potential benefits of its non-volatile extracts. This study advances the existing knowledge by investigating the biological activities of *H. officinalis* extracts, thereby addressing a significant gap in the literature.

The EA extracts showed significant antioxidant activities in DPPH, ABTS•+, and FRAP assays. These

assays measure the ability of antioxidants to scavenge free radicals or reduce ferric ions. This potency is attributed to high levels of flavonoids and phenolics in the extracts. Ebrahimzadeh et al (30) have evaluated the antioxidant activity of methanol extract of the aerial parts of *H. officinalis* L. var. *angustifolius* and their experiment conducted to potent activity in DPPH radical-scavenging. Similarly in the sample selected by Alinezhad et al (3), the acetone extracts of the aerial parts of *H. angustifolius* was characterized by a significant antioxidant activity in DPPH radical (31). Furthermore, the research carried out by Stanković et al (32) on the methanolic extract of *H. officinalis* L. from Serbia demonstrated that the antioxidant activity obtained using the FRAP technique was potent, reaching up to 0.73 mmol Fe^{2+}/g. In contrast, the work carried out by Liaqat et al (33) indicated that the crude extract of the aerial parts of *H. officinalis* L. from Pakistan exhibited low levels of total phenolic content and limited DPPH radical scavenging activity, which was significantly lower compared to extracts from *Origanum vulgare* and *Thymus vulgaris*. In addition, Vlase et al (24) showed that the ethanolic extract of *H. officinalis* had a low antioxidant capacity evaluated by the scavenging activity of DPPH• radicals with an IC_{50} of 125.44 ± 4.70 µg/mL.

Different aspects affect polyphenols capacity to scavenge free radicals including the type of the B-ring substitution, which is thought to be a driver of the antiradical efficacy of flavonoids and phenolic acids, and the hydroxyl group ensures a significant amount of scavenging ability, particularly if it is in position C4'. Also, the radical quenching capacity of unsaturated flavonoids was higher than in saturated compounds such as flavanones. Moreover, with an ortho-dihydroxy structure on the B ring and -OH group at position 3, as is the case with quercetin, flavonols have high scavenging capability (34,35). The EAL extract had a considerably higher FRAP value (15.71±1.13 µg/mL) than the EAS extract, which had a feeble activity measured at 115 ±5.00 µg/mL. The atomic hydrogen in phenolic compounds, which acts as a powerful reducing agent, may be responsible for their capacity to have a reducing potential. The structural characteristics of phenolic compounds, especially the number and position of hydroxyl groups, are critical determinants of their antioxidant capacity. Flavonols, with their specific hydroxyl group arrangements, stand out as the most potent reducing agents among phenolic compounds (36).

The outcomes of this investigation reveal the significant antibacterial potential of *H. officinalis* aerial parts extracts against gram positive bacteria *B. cereus* and *S. aureus*, which aligns with the findings observed by Vlase et al (24). Also, in a subsequent study, *H. officinalis* extract exhibited a potent inhibition of some dangerous bacteria's growth and biofilm formation (37). The essential oil of *H. officinalis* ssp. *angustifolius* has been shown significant in vitro antibacterial activity against eight species of bacteria, including *S. aureus* and *Bacillus subtilis*, with an MIC ranging from 15.62 to 62.5 mg/mL. However, the methanolic extract was inactive (38). Further, in vitro studies conducted by Fathiazad and Hamedeyazdan (39) indicated that the extracts of *H. officinalis* exhibited moderate antibacterial effects against both gram-positive and gram-negative bacteria. This suggests a broad-spectrum antibacterial potential, though the efficacy might vary depending on the type of bacteria and the specific extract used.

Gram-positive bacteria, with their simpler cell wall structure, are often more susceptible to phenolic compounds compared to gram-negative bacteria, which have an additional outer membrane. The phenolic compounds contained in medicinal plants are the primary factor affecting the bacterial activity (40), which is in accordance with the results of this study. EAL extract is characterized by the higher amount of flavonoids with different subclasses such as quercetin, hesperetin acid, esculin and naringenin. The antibacterial ability of quercetin and naringenin is associated with their solubility and their interaction with the bacterial cell membrane. Additionally, quercetin inhibits bacterial gyrase and causes DNA cleavage, and gram-positive bacteria are particularly susceptible to the bactericidal effects of this flavonoid than gram-negative bacteria (41), which is in line with this study's results. As per the findings, the hydroxyl coumarin constituted 35.40% of the overall composition of the EAS extracts and the outcomes of this investigation are consistent with a previous research showing the potent antibacterial activity of coumarin derivatives, especially against gram-positive bacteria like *S. aureus* (42) because these chemicals have the ability to inhibit the efflux pump in strains of *S. aureus*, as shown by de Araújo et al (43). Moreover, the EAS extract included large concentrations of hesperetin, which has been shown to have important antibacterial properties resulting from a range of processes, including the rupture of bacterial membranes and the inactivation of microbial enzymes (44).

The findings showed that EAS had a moderate anticholinesterase activity against AChE, consistent with findings of Wszelaki et al (45). Using Ellman's colorimetric method, they tested the methanolic and hexane extracts of *H. officinalis* for the symptomatic treatment of Alzheimer's disease. The results showed a moderate activity with values of 5.2 ± 8.2 and 29.6 ± 2.3 AChE inhibition (%) and 11.5 ± 0.5 and 23.2 ± 2.0 BChE inhibitions (%) at the concentrations of 100 µg/mL, respectively. In contrast, the essential oil of anise hyssop from Bulgaria showed a substantial anticholinesterase capacity similar to the reference inhibitor galantamine (46). Despite the known medicinal benefits of *H. officinalis*, specific data on its AChE inhibitory activity was relatively rare in the scientific literature.

The presence of bioactive compounds such as quercetin,

hesperetin, and hydroxy-coumarin plays a significant role in mediating these effects. EAS extract contained a big ratio of hydroxy-coumarin acid, as determined by the UPLC-ESI-MS-MS study (35.40%). This phenolic substance has a potent inhibitory effect on AChE action (47). Basically, the Peripheral Anionic Site (PAS) of AChE is known to interact with coumarins, and the efficacy of AChE inhibition is increased by substituting the coumarin template, in particular at positions 3 and 4, with an amine functional moiety, such as a benzylamino, phenylpiperazine, or aniline (48). Furthermore, a significant number of coumarin derivatives have the high inhibitory activity because they combine well with the peripheral active site of AChE. In their hunt for AChE inhibitors, Rollinger et al (49) hypothesize that coumarins' ability to raise ACh concentrations might be due to their ability to block AChE activity.

Conclusion

The current study investigated the biochemical profile of *H. officinalis* L. aerial parts extracts and evaluated their antioxidant, antibacterial, and anti-Alzheimer effects. The findings highlight the pharmacological potential of this aromatic and therapeutic herb, suggesting its valuable contributions to the pharmaceutical sector. Both EAL and EAS extracts were found to be abundant in phenolics and flavonoids. The extracts contained twenty metabolites from various classes, including flavonones, flavonols, flavanone glycoside, flavones, vitamins, and quinones. The high levels of TPC and TFC indicate strong antioxidant properties. The extracts demonstrated remarkable activities in DPPH, ABTS•+, and FRAP assays and showed significant antibacterial potential, particularly against gram-positive bacteria like *B. cereus* and *S. aureus*. Phenolic compounds such as quercetin and coumarins probably contribute to this antibacterial efficacy. The EAS extract exhibited moderate AChE inhibitory activity. The pharmacological properties of *H. officinalis*, particularly its antioxidant, antibacterial, and anti-Alzheimer effects, emphasize its potential as a source for developing natural therapeutic agents. The presence of phenolic compounds such as coumarins, quercetin, and hesperetin position them as promising scaffolds for the creation of novel pharmacological agents targeting critical molecular pathways, including NF-κB and MAPK signaling. Future research should prioritize elucidating their mechanisms of action through comprehensive structure-activity relationship studies and *in vivo* models, alongside optimizing formulation strategies to enhance bioavailability and therapeutic efficacy in clinical applications. This focused approach could substantially advance the field of natural therapeutics.

Acknowledgement

We would like to express our deep appreciation to the members of Bacteriology laboratory and Biochemistry laboratory at the Biotechnology Research Center in Constantine, Algeria for their outstanding professionalism and crucial contributions to this research. Another extension of gratitude to Boussebaa Walid from the Science and Technological Research Center in Physico-Chemical Analysis- Ouargla, Algeria for his invaluable assistance.

Authors' contribution

Conceptualization: Nour-El-Houda Hamoud**.**
Data curation: Nour-El-Houda Hamoud**.**
Formal analysis: Nour-El-Houda Hamoud and Zineb Maammeri.
Investigation: Nour-El-Houda Hamoud**.**
Methodology: Zineb Maammeri.
Project administration: Zineb Maammeri.
Resources: Nour-El-Houda Hamoud**.**
Software: Nour-El-Houda Hamoud**.**
Supervision: Zineb Maammeri.
Validation: Zineb Maammeri.
Visualization: Nour-El-Houda Hamoud**.**
Writing–original draft: Nour-El-Houda Hamoud**.**
Writing–review & editing: Nour-El-Houda Hamoud**.**

Conflicts of interests

The authors declare no conflicts of interest.

Ethical considerations

The authors of this study confirm that all ethical issues such as copyright infringement, plagiarism, data creation, duplicate publication, and redundancies have been considered and resolved. No animal or human subjects have been utilized in the present study.

Funding/Support

The study had no funding. The authors covered the expenses.

References

1. Kaur C, Kapoor HC. Anti-oxidant activity and total phenolic content of some Asian vegetables. Int J Food Sci Technol. 2002;37(2):153-61. doi: 10.1046/j.1365-2621.2002.00552.x.
2. Simon HU, Haj-Yehia A, Levi-Schaffer F. Role of reactive oxygen species (ROS) in apoptosis induction. Apoptosis. 2000;5(5):415-8. doi: 10.1023/a:1009616228304.
3. Alinezhad H, Azimi R, Zare M, Ebrahimzadeh MA, Eslami S, Nabavi SF, et al. Antioxidant and antihemolytic activities of ethanolic extract of flowers, leaves, and stems of *Hyssopus officinalis* L. Var. *angustifolius*. Int J Food Prop. 2013;16(5):1169-78. doi: 10.1080/10942912.2011.578319.
4. Zheng W, Wang SY. Antioxidant activity and phenolic compounds in selected herbs. J Agric Food Chem. 2001;49(11):5165-70. doi: 10.1021/jf010697n.
5. Akhlaghi L, Yeganeh M, Maleki F, Golestani M, Noori M, Ghelman M, et al. Antileishmanial activity of *Hyssopus officinalis*, *Tussilage farfara*, *Carum copticum* extracts in mice infected with *Leishmania major*. Int J Herb Med.

2014;2(2):142-5.
6. Moumni S, Elaissi A, Trabelsi A, Merghni A, Chraief I, Jelassi B, et al. Correlation between chemical composition and antibacterial activity of some Lamiaceae species essential oils from Tunisia. BMC Complement Med Ther. 2020;20(1):103. doi: 10.1186/s12906-020-02888-6.
7. Hikal WM, Said-Al Ahl HA. Anti-leishmanial activity of *Hyssopus officinalis*: a review. International Journal of Environmental Planning and Management. 2017;3(2):10-5.
8. Ma X, Ma X, Ma Z, Sun Z, Yu W, Wang J, et al. The effects of Uygur herb *Hyssopus officinalis* L. on the process of airway remodeling in asthmatic mice. Evid Based Complement Alternat Med. 2014;2014:710870. doi: 10.1155/2014/710870.
9. Kreis W, Kaplan MH, Freeman J, Sun DK, Sarin PS. Inhibition of HIV replication by *Hyssop officinalis* extracts. Antiviral Res. 1990;14(6):323-37. doi: 10.1016/0166-3542(90)90051-8.
10. Hammer KA, Carson CF. Antibacterial and antifungal activities of essential oils. In: Lipids and Essential Oils as Antimicrobial Agents. Chichester: Wiley; 2011. p. 255-306.
11. Fatahinezhad N, Lorigooini Z, Arabi M, Rabiei Z, Kazemi Sheykhshabani S, Rafieian-Kopaei M. Effects of *Hyssopus officinalis* hydroalcoholic extract on pentylenetetrazol-induced convulsive seizures in rat. Neurochem Res. 2022;47(12):3792-804. doi: 10.1007/s11064-022-03759-x.
12. Boudjada A, Touil A, Bendif H, Bensouici C, Rhouati S. Phytochemical constituents, phenolic contents, and antioxidant activity of *Crataegus azarolus* extracts. Asian J Pharm Clin Res. 2018;11(4):133-7. doi: 10.22159/ajpcr.2018.v11i4.23724.
13. Singleton VL, Rossi JA. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am J Enol Vitic. 1965;16(3):144-58. doi: 10.5344/ajev.1965.16.3.144.
14. Topçu G, Ay M, Bilici A, Sarıkürkcü C, Öztürk M, Ulubelen A. A new flavone from antioxidant extracts of *Pistacia terebinthus*. Food Chem. 2007;103(3):816-22. doi: 10.1016/j.foodchem.2006.09.028.
15. Akdeniz M. Screening of Chemical Content Specific to *Hyperıcum* species Growing in Different Parts of Turkey by LC-MS/MS and Method Validation; Investigation of Their Biological Activities and Chemometric Evaluation [dissertation]. Diyarbakır: Dicle University Institute of Science Department of Chemistry; 2018.
16. Blois MS. Antioxidant determinations by the use of a stable free radical. Nature. 1958;181(4617):1199-200. doi: 10.1038/1811199a0.
17. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med. 1999;26(9-10):1231-7. doi: 10.1016/s0891-5849(98)00315-3.
18. Oyaizu M. Studies on products of browning reaction antioxidative activities of products of browning reaction prepared from glucosamine. The Japanese Journal of Nutrition and Dietetics. 1986;44(6):307-15. doi: 10.5264/eiyogakuzashi.44.307.
19. Chandur U, Rao BG, Ramadevi D, Kalyani AL. Evaluation of the anti-bacterial and anti-fungal activity of Ipomoea hederacea. Acta Biomed Sci. 2015;2(3):134-6.
20. Wiegand I, Hilpert K, Hancock RE. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc. 2008;3(2):163-75. doi: 10.1038/nprot.2007.521.
21. Ellman GL, Courtney KD, Andres V Jr, Feather-Stone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol. 1961;7:88-95. doi: 10.1016/0006-2952(61)90145-9.
22. Benzie IF, Strain JJ. Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. Methods Enzymol. 1999;299:15-27. doi: 10.1016/s0076-6879(99)99005-5.
23. Khajenoori M, Haghighi-Asl A, Hormozi F, Eikani MH, Noori Bidgoli H. Subcritical water extraction of essential oils from *Zataria multiflora* Boiss. J Food Process Eng. 2009;32(6):804-16. doi: 10.1111/j.1745-4530.2008.00245.x.
24. Vlase L, Benedec D, Hanganu D, Damian G, Csillag I, Sevastre B, et al. Evaluation of antioxidant and antimicrobial activities and phenolic profile for *Hyssopus officinalis*, *Ocimum basilicum* and *Teucrium chamaedrys*. Molecules. 2014;19(5):5490-507. doi: 10.3390/molecules19055490.
25. Han H, Yılmaz H, Gülçin I. Antioxidant activity of flaxseed (*Linum usitatissimum* L.) shell and analysis of its polyphenol contents by LC-MS/MS. Rec Nat Prod. 2018;12(4):397-402.
26. Hatipoğlu G, Sökmen M, Bektaş E, Daferera D, Sökmen A, Demir E, et al. Automated and standard extraction of antioxidant phenolic compounds of *Hyssopus officinalis* L. ssp. *angustifolius*. Ind Crops Prod. 2013;43:427-33. doi: 10.1016/j.indcrop.2012.07.028.
27. Hajdari A, Giorgi A, Beretta G, Gelmini F, Buratti S, Benedetti S, et al. Phytochemical and sensorial characterization of *Hyssopus officinalis* subsp. *aristatus* (Godr.) Nyman (Lamiaceae) by GC–MS, HPLC–UV–DAD, spectrophotometric assays and e-nose with aid of chemometric techniques. Eur Food Res Technol. 2018;244(7):1313-27. doi: 10.1007/s00217-018-3046-z.
28. Proestos C, Chorianopoulos N, Nychas GJ, Komaitis M. RP-HPLC analysis of the phenolic compounds of plant extracts. investigation of their antioxidant capacity and antimicrobial activity. J Agric Food Chem. 2005;53(4):1190-5. doi: 10.1021/jf040083t.
29. Moulodi F, Khezerlou A, Zolfaghari H, Mohamadzadeh A, Alimoradi F. Chemical composition and antioxidant and antimicrobial properties of the essential oil of *Hyssopus officinalis* L. J Kermanshah Univ Med Sci. 2019;22(4):e85256. doi: 10.5812/jkums.85256.
30. Ebrahimzadeh MA, Nabavi SM, Nabavi SF, Bahramian F, Bekhradnia AR. Antioxidant and free radical scavenging activity of *H. officinalis* L. var. *angustifolius*, *V. odorata*, *B. hyrcana* and *C. speciosum*. Pak J Pharm Sci. 2010;23(1):29-34.
31. Shomirzoeva O, Li J, Numonov S, Mamat N, Shataer D, Lu X, et al. Chemical components of *Hyssopus seravshanicus*: antioxidant activity, activations of melanogenesis and tyrosinase, and quantitative determination by UPLC-DAD. Nat Prod Res. 2019;33(6):866-70. doi: 10.1080/14786419.2017.1408105.
32. Stanković N, Mihajilov-Krstev T, Zlatković B, Stankov-Jovanović V, Mitić V, Jović J, et al. Antibacterial and antioxidant activity of traditional medicinal plants from the

Balkan Peninsula. NJAS Wageningen J Life Sci. 2016;78:21-8. doi: 10.1016/j.njas.2015.12.006.
33. Liaqat I, Bibi U, Arshad M, Arshad N. Antioxidant activity of six selected medicinal plants, Pakistan. J Innov Sci. 2022;8(1):13-9. doi: 10.17582/journal.jis/2022/8.1.13.19.
34. Rice-Evans CA, Miller NJ, Paganga G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radic Biol Med. 1996;20(7):933-56. doi: 10.1016/0891-5849(95)02227-9.
35. Milenković D, Dimitrić Marković, Dimić D, Jeremić S, Amić D, Pirković MS, et al. Structural characterization of kaempferol: a spectroscopic and computational study. Maced J Chem Chem Eng. 2019;38(1):49-62. doi: 10.20450/mjcce.2019.1333.
36. Dutta MS, Mahapatra P, Ghosh A, Basu S. Estimation of the reducing power and electrochemical behavior of few flavonoids and polyhydroxybenzophenones substantiated by bond dissociation energy: a comparative analysis. Mol Divers. 2022;26(2):1101-13. doi: 10.1007/s11030-021-10232-4.
37. Hassanshahiyan M, Saadatfar A, Masoumi F. Antimicrobial properties of *Hyssopus officinalis* extract against antibiotic-resistant bacteria in planktonic and biofilm form. Biological Journal of Microorganism. 2018;7(28):91-101.
38. Özer H, Sökmen M, Güllüce M, Adigüzel A, Kilic H, Şahin F, et al. In vitro antimicrobial and antioxidant activities of the essential oils and methanol extracts of *Hyssopus officinalis* L. ssp. *angustifolius*. Ital J Food Sci. 2006;18(1):73-83.
39. Fathiazad F, Hamedeyazdan S. A review on *Hyssopus officinalis* L.: composition and biological activities. Afr J Pharm Pharmacol. 2011;5(17):1959-66. doi: 10.5897/ajpp11.527.
40. Biharee A, Sharma A, Kumar A, Jaitak V. Antimicrobial flavonoids as a potential substitute for overcoming antimicrobial resistance. Fitoterapia. 2020;146:104720. doi: 10.1016/j.fitote.2020.104720.
41. Wang Y, Xie M, Ma G, Fang Y, Yang W, Ma N, et al. The antioxidant and antimicrobial activities of different phenolic acids grafted onto chitosan. Carbohydr Polym. 2019;225:115238. doi: 10.1016/j.carbpol.2019.115238.
42. Lin PY, Yeh KS, Su CL, Sheu SY, Chen T, Ou KL, et al. Synthesis and antibacterial activities of novel 4-hydroxy-7-hydroxy- and 3-carboxycoumarin derivatives. Molecules. 2012;17(9):10846-63. doi: 10.3390/molecules170910846.
43. de Araújo RS, Barbosa-Filho JM, Scotti MT, Scotti L, da Cruz RM, dos Santos Falcão-Silva V, et al. Modulation of drug resistance in *Staphylococcus aureus* with coumarin derivatives. Scientifica (Cairo). 2016;2016:6894758. doi: 10.1155/2016/6894758.
44. Yuan G, Guan Y, Yi H, Lai S, Sun Y, Cao S. Antibacterial activity and mechanism of plant flavonoids to gram-positive bacteria predicted from their lipophilicities. Sci Rep. 2021;11(1):10471. doi: 10.1038/s41598-021-90035-7.
45. Wszelaki N, Kuciun A, Kiss AK. Screening of traditional European herbal medicines for acetylcholinesterase and butyrylcholinesterase inhibitory activity. Acta Pharm. 2010;60(1):119-28. doi: 10.2478/v10007-010-0006-y.
46. Ivanov IG, Vrancheva RZ, Petkova NT, Tumbarski Y, Dincheva IN, Badjakov IK. Phytochemical compounds of anise hyssop (*Agastache foeniculum*) and antibacterial, antioxidant, and acetylcholinesterase inhibitory properties of its essential oil. J Appl Pharm Sci. 2019;9(2):72-8. doi: 10.7324/japs.2019.90210.
47. Detsi A, Kontogiorgis C, Hadjipavlou-Litina D. Coumarin derivatives: an updated patent review (2015-2016). Expert Opin Ther Pat. 2017;27(11):1201-26. doi: 10.1080/13543776.2017.1360284.
48. Shen Q, Peng Q, Shao J, Liu X, Huang Z, Pu X, et al. Synthesis and biological evaluation of functionalized coumarins as acetylcholinesterase inhibitors. Eur J Med Chem. 2005;40(12):1307-15. doi: 10.1016/j.ejmech.2005.07.014.
49. Rollinger JM, Hornick A, Langer T, Stuppner H, Prast H. Acetylcholinesterase inhibitory activity of scopolin and scopoletin discovered by virtual screening of natural products. J Med Chem. 2004;47(25):6248-54. doi: 10.1021/jm049655r.