Original Research Article

Determination of Phenolic Contents by HPLC, and Antioxidant, Antimicrobial, Antityrosinase, and Anticholinesterase Activities of *Psephellus huber-morathii*

Running Title: Biological Activity of ***Psephellus huber-morathii***

ABSTRACT

The goal of our study was to examine of antioxidant, antimicrobial, anticholinesterase activities, and phenolic composition of *Psephellus huber-morathii.* The antioxidant activities of extracts have been assessed by Ferric reducing antioxidant power (FRAP), cupric reducing antioxidant capacity (CUPRAC), and 2,2-diphenylpicrylhydrazyl (DPPH) radical scavenging. Phenolic constituents were measured using reverse phase-high performance liquid chromatography (RP-HPLC), and antimicrobial activity was investigated using the agar well diffusion method. Total phenolic content, FRAP, and CUPRAC results of aqueous extract have been better than methanolic extract, except for DPPH activity. Benzoic acid, and *p*-coumaric acid as major phenolic compounds were specified. Methanolic extract was especially effective against all microorganisms tested except for *Yersinia pseudotuberculosis.* The methanolic extract have been displayed inhibitory effect on tyrosinase. All extracts have been exhibited lower acetylcholinesterase, and butyrylcholinesterase inhibitory activities than galantamine. *P. huber-morathii* can be considered in the food, and drug industries due to antioxidant capacity and antimicrobial activities of the species. It can be potential source as anti-browning agents because of its average tyrosinase inhibitory activity.

Keywords: *Antioxidant, Antimicrobial, anticholinesterase, Psephellus huber-morathii*

1. INTRODUCTION

Free radicals, particularly oxygen free radicals (OFRs) or reactive oxygen species (ROS) (such as superoxide, hydroxyl and hydrogen peroxide), are active oxygen compounds produced by the oxidation reactions of external factors [1]. These reactive species are liable for oxidizing proteins, lipids and DNA, and of triggering various degenerative and chronic disorders [2-5]. Antioxidants can suppress or delay oxidation when present at lower levels than oxidizable substrates [6]. They are prominent to preserve human health and averting free radical-induced disease. The health benefits of antioxidants are so great that foodstuffs and pharmaceutical products are routinely reinforced with synthetic antioxidant supplements, including BHA, BHT and PG. However, synthetical antioxidants may have carcinogenic and other toxic side-effects [7]. Natural antioxidants are for that reason currently preferred to synthetic equivalents, and limitations on the use of the latter have been recommended.

Alzheimer's disease (AD) knowed by memory disturbance is a widespread neurodegenerative disease. The most prominent biochemical change in the disease is a decrease in cerebral acetylcholine levels [8]. Raising acetylcholine levels, by means of suppression of the two principal form of cholinesterase, acetylcholinesterase (AChE) and butrylcholinesterase, can therefore be adopted as a therapeutic approach in AD (BChE) [9]. Agents used to inhibit cholinesterase in the treatment of AD include tacrine, rivastigmine and galantamine. However, side-effects have also been observed with these compounds, particularly hepatotoxicity and gastrointestinal disturbances [10,11]. There has therefore been growing focus on safe and effective AChE inhibitors obtained from natural products.

Psephellus huber-morathii (Wagenitz) Wagenitz, otherwise known as *Centaurea huber-morathii* Wagenitz, is a member of the Asteraceae family. The genus *Centaurea* (Asteraceae) consists of some 500 species distributed in the Old World [12]. On the Anatolian peninsula, the genus is represented by approximately 190 species, more than 100 of which are endemic [13]. Some Centaurea species are employed as herbal therapies for fever, diabetes, hemorrhoid, and peptic ulcer in traditional Anatolian folk medicine [14,15]. Pharmacological and phytochemical studies of various different Centaurea species have identified antioxidant, antimicrobial and antipyretic properties [16-19].

The aims of this work were firstly, the gain of more information about total phenolic quantity, the study of the potential natural antioxidant, antimicrobial, antityrosinase, antiacetylcholinesterase, antibutyrylcholinesterase effect of extracts of *P. huber-morathii,* secondly to carry on the relationships between total phenolic content and studied activities.

2. MATERIAL AND METHODS

2.1. Chemicals and Instrumentation

The following chemicals and reagents were used: 2,2-Diphenyl-1-picrylhydrazyl (DPPH), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich); butylated hydroxytoluene (BHT) (Supelco); galanthamine (Sigma); methanol, ethanol, acetic acid, dimethyl sulfoxide, and acetonitrile (Merck); 6-hydroxy–2,5,7,8-tetramethylchroman–2-carboxylic acid (Trolox), 2,4,6-tripyridyl-s-triazine (TPTZ), and Folin–Ciocalteau reagent (Fluka); polytetrafluoroethylene membranes (Sartorius).

Absorbance was calculated using a Spectro UV-Vis Double PC–8 auto cell spectrophotometer (Labomed Inc.). All solutions were prepared with deionized water purified in an Elgacan® C114 Ultra Pure Water System Deioniser (The Elga Group, Buckinghamshire, England).

Evaporation procedures were conducted using the IKA® RV 05 Basic (IKA®, Werke, USA) rotary evaporator system, while extraction was carried out with a heidolp promax 2020 shaker. All dissolution procedures ınvolved the use of a Heidolph Reax top vortex and Elma® Transsonic Digital ultrasonic water bath (Germany). A Hanna Instruments microprocessor pH meter was employed where appropriate.

2.2. Plant Material and Preparation of Samples

P. huber-morathii were collected in 2016 from the Erzincan (Turkey), and identified by one of the authors (Ali Kandemir). The voucher specimens were kept in the herbarium of Erzincan University, Faculty of Science (herbarium number: 10862). Dried and powdered plant was extracted in methanol during a day. The extract was evaporated with a rotary evaporator (IKA-Werke RV05 Basic, Staufen, Germany). The obtained extract was used for antioxidant, antimicrobial, cytotoxicity,

anticholinesterase, and antityrosinase activity studies. The extract for use in HPLC analysis was further dissolved in HPLC grade methanol (10 mg/mL) and filtered through 0.45-µm membranes filter.

2.3. HPLC Conditions

The standards including vanillic acid, *p*-hydroxybenzoic acid, syringaldehyde, *p*-coumaric acid, sinapic acid, benzoic acid and quercetin were used for HPLC analysis. HPLC analysis of phenolic compounds was conducted on a reverse phase column (150 × 4.6 mm i.d, 5 µm) (Waters Spherisorb, Milfort, MA, USA), on a gradient program with the assistance of a two-solvents system [A: 100% methanol; B: 2% acetic acid in water (pH 2.8)], and a constant solvent flow rate set to 1.5 mL min^{-1} on a HPLC system (Shimadzu Corporation, LC 20 AT, Kyoto, Japan) (Table 1). The injection volume was adjusted to 20 µL. Signals were identified at 232, 246, 260, 270, 280, 290, 308, and 328 using DAD detection at a column temperature of 25ºC. HPLC analyses were carried out using validated and modified methods in our previous study [20,21].

2.4. Detection of Antioxidant Capacity

The Folin-Ciocalteu procedure was performed in order to calculate total phenolic quantities in the extract [22]. Gallic acid was used as a positive standard, with the total phenolic content being expressed as mg of gallic acid equivalents per gram of 100 g sample. Briefly, 0.01, 0.02, 0.03, 0.04 and 0.05 mg/mL concentrations of gallic acid were dissolved in methanol. 0.5 mL of each sample was placed into test tubes, and then added 0.5 mL of 0.2 N Folin-Ciocalteu reagent and 1.5 mL of 2% sodium carbonate. The test tubes were incubated for 2 h at 20 °C, after which the absorbance was evaluated spectrophotometrically at 760 nm. All measurements were conducted in triplicate.

The ferric reducing antioxidant power (FRAP) assay depends on calculating the iron reducing capacities of a given extract [23]. When exposed to 2,4,6-tripyridyl-S-triazine (TPTZ), the Fe^{2+}-TPTZ complex exhibits a blue color which is read at 593 nm. Briefly, 3.0 mL of fresh FRAP reagent was added to an appropriate volume/concentration of extract. The samples was incubated for 4 min at 37 ^{0}C, after which the absorbance was measured at 593 nm. Trolox was also measured under identical conditions as a standard antioxidant compound for purposes of comparison. The results were stated as µM Trolox equivalent of g sample.

DPPH radical-scavenging activity is connected to the antioxidant's DPPH radical scavenging capacity [24]. In brief, we added 0.75 mL of DPPH reagent (0.1 mM in methanol) to 0.75 mL of extract or standard, and mixed. The samples were incubated in the dark for 30 mins at room temperature. Observed discoloration was measured spectrophometrically at 517 nm. The percentage inhibitions of the discoloration of the extracts were compared with BHT (Butyllated hydroxytoluene) used as standard. The results were expressed as SC_{50} (mg sample per mL).

The CUPRAC levels of extracts were studied spectrophotometrically [25]. Briefly, 1 mL of $CuCl_2$ solution ($1.0x10^{-2}$ M), 1 mL of neocuproine solution ($7.5x10^{-3}$ M) and 1 mL NH_4Ac buffer solution were mixed in a test tube. A range of different extract concentrations were added. The test tubes were then incubated for 30 mins. Absorbance was measured at 450 nm against a reagent blank. CUPRAC values were expressed as μM Trolox equivalent per gram of sample.

2.5. Antimicrobial Activity Assessment

Escherichia coli ATCC 25922, *Yersinia pseudotuberculosis* ATCC 911, *Pseudomonas auroginosa* ATCC 43288, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Listeria monocytogenes* ATCC 43251, *Bacillus cereus* 709 ROMA, *Mycobacterium smegmatis* ATCC607, *Candida albicans* ATCC 60193, and *Saccharomyces cerevisiae* RSKK 251 were supplied by the Hifzissihha Institute of Refik Saydam (Ankara, Turkey). We dissolved the extract in dimethyl sulfoxide (DMSO) for material preparation in a stock solution of 178–256 mg/mL.

The agar-well diffusion method [26], with various modifications previously described elsewhere [27], was used for susceptibility screening. Each bacterium was suspended in Mueller Hinton (MH) (Difco, Detroit, MI) broth, while yeast-like fungi were suspended in yeast extracts broth. The micro-organisms were subsequently diluted to a level of approximately 106 colony-forming units (cfus) per mL. Sabouraud Dextrose Agar (SDA) (Difco, Detriot, MI) was used for yeast-like fungi, and brain heart infusion agar (BHA) was employed for *M. smegmatis* [28]. These were "flood-inoculated" onto the surface of MH and SD agars and then dried. In the following stage, 5-mm diameter wells were produced from the agar with the help of a sterile cork-borer, after 8900- 12800 μg/50 μL of the extract substances was placed into the wells. The plates were incubated for 18 h at 35 ^{o}C. *M. smegmatis* was cultured for 3-5 days on BHA plates at 35 ^{o}C. The zone of inhibition was measured against the test

organism to determine antimicrobial activity. Ampicillin (10 µg), streptomycin (10 µg), and fluconazole (5 µg) were employed as standard drugs, while dimethylsulfoxide served as the control. Finally, minimal inhibition concentration (µg mL^{-1}) of *P. huber-morathii* were calculated.

2.6. Antityrosinase Activity

Tyrosinase inhibitory activity (TIA) (EC 1.14.1.8.1, 30 U, mushroom tyrosinase, Sigma) was measured using different concentrations of kojic acid solutions as standard [29]. Reaction mixture absorbance was read at 490 nm using the spectrophotometric method via a microplate reader (VersaMax Molecular Devices, USA). The percentage of TIA was calculated using the formula % inhibition = [[(A-B)-(C-D)] / (A-B)] x 100

2.7. Acetylcholinesterase (AChE)/Butyrylcholinesterase (BChE) Inhibitory Activity

The modified colorimetric Ellman method was used to investigate acetylcholine esterase inhibitory (AChEI) and butyrylcholin esterase inhibitory (BChE) activities [30]. AChE and BChE were employed as enzymes. Acetylthiocholine iodide and butyrylthiocholine iodide as substrates were used. Also, 5,5'-dithio-bis 2-nitrobenzoic acid (DTNB) was used as the coloring agent. The control and test compounds were dissolved in sodium phosphate buffer (pH 8) range of concentration of 25-200 µg/mL. Next, 130 µL of sodium phosphate buffer, 10 µL of the tested compound and 20 µL of the enzyme were mixed in a 96-well plate and incubated for 15 min at 25 ºC. In the following procedure, 20 µL of DTNB and 20 µL of substrats were added to all wells. Absorbance was measured spectrophotometrically at 412 nm. AChE and BChE inhibition values were calculated using the formula shown below and compared against galantamine used as standard.

% Inhibition = 100 - [(A1 / A2) x 100]

A1 = Absorbance of the sample solutions at 412 nm

A2 = Average absorbance of the control solutions at 412 nm.

3. RESULTS

3.1. HPLC Chromatograms

Chromatograms of the phenolic standards and methanolic extract have been presented in Figures 1-2. The quantities of phenolic compounds measured in the samples have been shown in Table 1. As shown the table, *p*-coumaric acid and benzoic acid have been detected in the methanolic extract of the plant.

Table 1. Phenolic composition of the methanolic extract of *P. huber-morathii*

Phenolic compounds	Retention time (min)	Amount (mg/g)
p-hydroxy benzoic acid	4.411	-
Vanillic acid	5.102	-
Syringaldehyde	6.383	-
p-coumaric acid	7.437	2.21
Sinapic acid	7.947	-
Benzoic acid	9.588	11.55
Quercetin	14.720	-

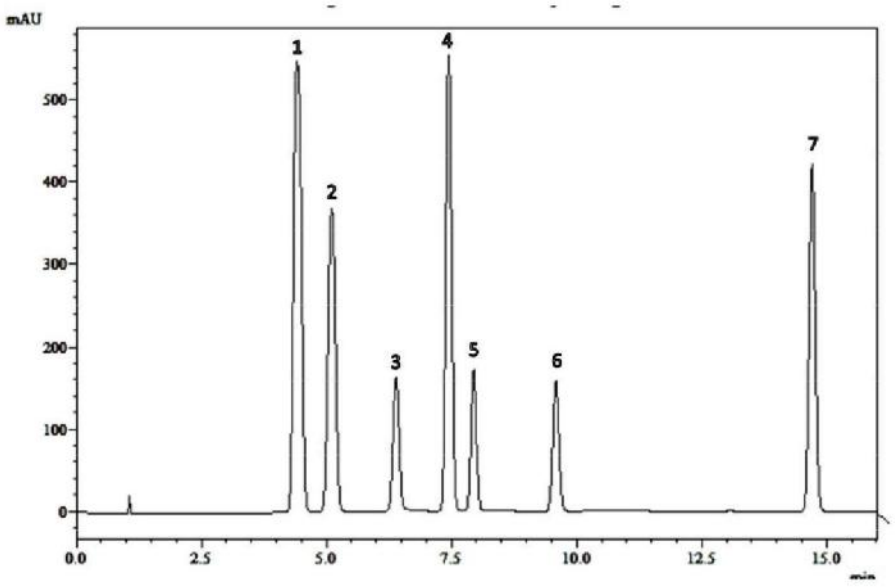

Fig. 1. HPLC chromatogram of phenolic standards

Peak identification: (1) *p*-hydroxy benzoic acid, (2) vanillic acid, (3) syring aldehyde, (4) *p*-coumaric acid, (5) sinapic acid, (6) benzoic acid, (7) quercetin.

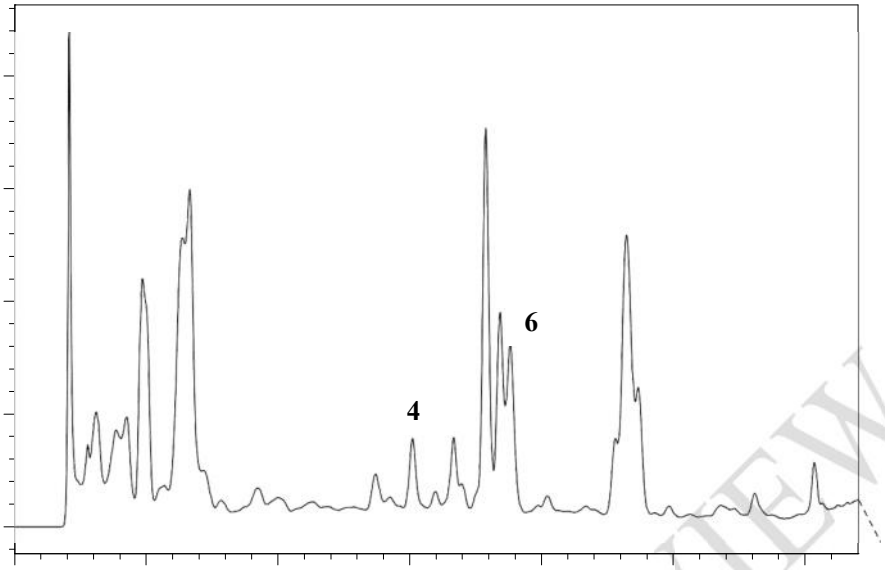

Fig. 2. HPLC chromatogram of *P. huber-morathii* methanol extract
Peak identification: (4) *p*-coumaric acid, (6) benzoic acid.

3.2. Antioxidant Activities of *P. huber-morathii* Extracts

The results of TPC, FRAP, CUPRAC and DPPH scavenging activity studies of the aqueous and methanolic extracts have been defined in Table 2.

Table 2. The antioxidant activities of *P. huber-morathii* extracts

Test Compounds	TPC[†]	FRAP [‡]	CUPRAC[§]	DPPH[¶12]
Aqueous extract	13.9 ± 0.460	841 ± 4.699	1322 ± 8.940	0.3379 ± 0.0049
Methanolic extract	10 ± 0.268	666 ± 3.210	1230 ± 7.915	0.2073 ± 0.0036
BHT				0.0031 ± 0.0002

[†]Total phenolic content expressed in mg of gallic acid equivalent (GAE) per gram of dry plant weight.
[‡] FRAP value expressed as µM trolox equivalents (TE) per gram of dry plant weight.
[§] Trolox equivalent antioxidant capacity (TEAC) value expressed in µM trolox equivalents (TE) per gram of dry plant weight.
[¶12]Concentration of test sample (mg/mL) required to produce 50% inhibition of the DPPH radical.

3.3. Antimicrobial Activities of *P. huber-morathii* Extracts

The antimicrobial activities of *P. huber-morathii* extracts against the bacteria and fungus tested was assessed in terms of the presence of minimal inhibition concentrations (Table 3). The methanolic extract exhibited antimicrobial effect against *E. coli, P. aeruginosa, S. aureus, E. faecalis and M. smegmatis,* but not *Y. pseudotuberculosis.*

Table 3. Antimicrobial activities of *P. huber-morathii* extracts

Tested Compounds	Microorganisms and Minimal Inhibition Concentration (µg/mL)										
	Quantity (µg/mL)	Gram negative			Gram positive				No gram	Yeast Like Fungi	
		Ec	Yp	Pa	Sa	Ef	Lm	Bc	Ms	Ca	Sc
Methanolic Extract	10000	125	-	250	250	350	700	350	62.25	350	350
Aqueous Extract	10000	-	-	-	-	-	-	-	-	-	-
Ampicillin	10	10	18	>128	35	10	10	15	-	-	-
Streptomycin	10								4		
Fluconazole	5									>8	>8

Ec: *Escherichia coli* ATCC 25922, Yp: *Yersinia pseudotuberculosis* ATCC 911, Pa: *Pseudomonas aeruginosa* ATCC 27853, Sa: *Staphylococcus aureus* ATCC 25923, Ef: *Enterococcus faecalis* ATCC 29212, Lm: *Listeria monocytogenes* ATCC 43251, Bc: *Bacillus cereus* 702 Roma, Ms: *Mycobacterium smegmatis* ATCC607, Ca: *Candida albicans* ATCC 60193, Sc: *Saccharomyces cerevisiae* RSKK 251, (-): no activity of test concentrations (10 000 µg/mL).

3.3. Antityrosinase Activity of *P. huber-morathii* Extract

We investigated the antityrosinase activity of *P. huber-morathii* extract. The IC_{50} value of the methanolic extract were found as 575.44 µg/mL, while IC_{50} value of kojic acid as positive standart, were 3.0957 µg/mL.

3.4. Anticholinesterase Activities of *P. huber-morathii* Extracts

We also investigated the AChE, and BChE inhibitory activities of *P. huber-morathii* extracts. AChE and BChE inhibitory activity of the extracts and positive standard galantamine were specified in Table 4.

Table 4. Acetylcholinesterase (AChE), and butyrylcholinesterase (BChE) inhibitor activities (% inhibition)

	Samples	25 µg/mL	50 µg/mL	100 µg/mL	200 µg/mL
AChE Inhibitory Activity	AE	9.2 ± 0.5	15.5 ± 0.4	24.9 ± 0.2	30.4 ± 0.8
	ME	3.4 ± 0.7	8.3 ± 0.3	27.8 ± 0.5	38.8 ± 0.7
	Galantamine	64.5±1.2	72.2±0.9	78.6±0.8	84.2 ± 0.3

BChE Inhibitory Activity	AE	21.0 ± 0.2	46.8 ± 0.4	55.3 ± 0.8	68.3 ± 1.3
	ME	10.5 ± 0.4	32.1 ± 0.6	54.9 ± 0.9	72.6 ± 1.5
	Galantamine	41.3±0.7	56.6±0.8	68.7±1.2	80.1 ± 0.4

AE: Aqueous extract; **ME:** Methanolic extract

4. DISCUSSION

Phenolic compounds have been increased popularity for health-promoting effects due to their antioxidant properties. The most widespread types of phenolic compounds in natural sources have been knowed as phenolic acids and flavonoids [31].

According to HPLC analyses, among the phenolic acids, benzoic acid, and *p*-coumaric acid have been detected. *p*-Coumaric acid is a phenolic acid belongs to hydroxycinnamic acid family and important for health care because of antioxidant, anti-inflammatory, antimutagenic, antiulcer, antiplatelet, antidiabetic and anticancer activities. Also İthas been reported to reduce oxidative cardiac damage and atherosclerosis [32]. Benzoic acid and derivatizations have been reported to show antibacterial and antifungal activity and significant antioxidant capacity. Benzoic acid have been used as food preservation because of these properties [33,34].

P. huber-morathii have been also knowned as *Centaurea huber-morathii*. It has been reported that the determination of total phenolic contents, free radical scavenging activity, cupric ion reducing power and ferric reducing antioxidant power was carried on some different *Centaurea* species [17,18, 35-39]. Besides, there have been noticeable research into the antimicrobial activities of different *Psephellus* species. In previosly studies, *Centaurea* species have been reported significant antibacterial and antifungal activities [40-43].

As shown the results of antioxidant capacity studies of the aqueous and methanolic extract of the species, the total phenolic contents have been possessed of considerable values. Furthermore, FRAP, CUPRAC and DPPH scavenging activity belong to the species have been observed to be important. The results from HPLC and antioxidant activity studies have been compatible. Antioxidant capacity of *P. huber-morathii* may be based on its phenolic compounds.

The methanolic extract of the species has showed high antimicrobial activity on *E. coli, P. aeruginosa, S. aureus, E. faecalis and M. smegmatis* microorganisms caused a wide range of diseases. As mentioned above, the high antimicrobial activityof the plant can be related with benzoic acid.

Tyrosinase is a substantial enzyme in the production of melanin. Melanin protects cutaneous tissues against ultraviolet (UV) damage by reducing reactive oxygen species. Overproduction or abnormal melanin pigmentation have given rise to cosmetic concerns in humans. So, potent tyrosinase inhibitors have need to be developed [44]. The IC_{50} value of the methanolic extract on tyrosinase was calculated at 575.44 µg/mL. These findings have indicated that *P. huber-morathii* extract may have been a potential natural source to design and develop of novel tyrosinase inhibitors as anti-browning agents.

AChE and BChE inhibitory activity of some *Centaurea* species have been reported at previously studies, while the ChE inhibitory activity of *P. huber-morathii* have been examined for the first time with this study [45].

AChE inhibitors have been used for treatment of Alzheimer's disease. Recent studies have also looked for novel AChE inhibitors from natural sources [46]. So, Cholinesterase inhibitory activity of the species has been performed. The results have showed that the extracts have been possessed lower acetylcholinesterase inhibitory and closer butyrylcholinesterase inhibitory activities, comparated with galantamine. So, further researches have been necessary to determine for treatment Alzheimer's disease of the plant.

5. CONCLUSION

In the peresent study, all biological activities of *P. huber-morathii* have been examined for the first time. Novel plant-derived bioactive molecules have been urgently needed, and these plant extracts may represent a natural source of antioxidants and antimicrobial agents, particularly in foodstuffs and medicinal products. Further studies have been needed to confirm the bioactive compounds related to antioxidant, antimicrobial, and anticholinesterase activities observed in these extracts.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest

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