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Unravelling the phenolic compound reserves, antioxidant and enzyme inhibitory activities of an endemic plant species, *Achillea pseudoaleppica*

Mustafa Abdullah Yılmaz[a], Parham Taslimi[b], Ömer Kılıç[c], İlhami Gülçin[d], Abhijit Dey[e] and Ercan Bursal[f]

[a]Faculty of Pharmacy, Department of Pharmaceutical Chemistry, Dicle University, Diyarbakır, Turkey; [b]Faculty of Science, Department of Biotechnology, Bartin University, Bartin, Turkey; [c]Faculty of Pharmacy, Department of Pharmaceutical Sciences, Adıyaman University, Adı yaman, Turkey; [d]Faculty of Science, Department of Chemistry, Ataturk University, Erzurum, Turkey; [e]Department of Life Sciences, Presidency University, Kolkata, India; [f]Faculty of Health, Department of Nursing, Muş Alparslan University, Muş, Turkey

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ABSTRACT

The present ethnobotanical study unravelled the phenolic reservoir (UHPLC-MS/TQ-MS) and pharmacological activity (antioxidant and enzyme inhibitory activities) of an endemic plant, *Achillea pseudoaleppica* Hub.-Mor. (Asteraceae). The effective antioxidant properties of ethanol and water extracts of *A. pseudoaleppica* leaves were determined by using six different *in vitro* bioanalytical methods including three reducing antioxidant methods and three radical scavenging antioxidant methods. In the other step of the study, the enzyme inhibitory effects of water and ethanol extracts of *A. pseudoaleppica* were determined against acetylcholinesterase (AChE), butyrylcholinesterase (BChE), α-amylase, and α-glucosidase enzymes. The ethanol extract was found to have effective inhibition potential for all four respected enzymes. The IC_{50} values of *A. pseudoaleppica* extract against AChE, BChE, α-amylase, and α-glucosidase enzymes were found to be 2.67 mg/mL, 4.55 mg/mL, 16.51 mg/mL, and 12.37 mg/mL, respectively. Also, UHPLC-MS/TQ-MS analyses revealed quinic acid as the most abundant phenolic compound of the water extract (31.12 ± 1.65 μg/mg) and ethanol extract (11.75 ± 0.82 μg/mg). In addition, the molecular docking interaction of the most abundant phenolic compound of *A. pseudoaleppica* (quinic acid) with AChE, BChE, α-amylase, and α-glucosidase target enzymes were evaluated using Chimera and AutoDock Vina softwares. In conclusion, the rich phenolic content and the potent antioxidant and enzyme inhibitory properties of *A. pseudoaleppica* extracts may support the widespread ethnobotanical use of the plant application.

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Achillea pseudoaleppica; antioxidant; enzyme inhibition; phenolic content; radical scavenging

1. Introduction

Achillea pseudoaleppica Hub.-Mor. is an endemic aromatic perennial herbaceous species from the genus *Achillea* L. (yarrow) of the *Asteraceae* family. This plant species naturally grows in the eastern region of Turkey, especially in steppe, slope, rocky, and screes habitats. The genus *Achillea* L. includes more than 130 species distributed in different parts of the world (Aytac et al., 2016). Many *Achillea* taxa have been reported in ethnobotanical literature against headache, allergic rhinitis, wounds, spasmodic diseases, rheumatism, pneumonia, hemorrhoids, inflammation, menstrual disorders, bleedings, flatulence, stomach pain, and dyspepsia (Venditti et al., 2016) as well as diuretic and emmenagogue properties (Turkmenoglu et al., 2015). Besides being potent traditional folk remedies, many *Achillea* taxa have been reported as a promising medicinal plant in phytotherapy and aromatherapy (Mohammadhosseini et al., 2017). Thus, some of *Achillea* taxa have economic and commercial values. Phenolic acids, flavonoids, lignans, terpenic lactones, and alkamides have been found as the main bioactive compounds of *Achillea* species (Althaus et al., 2014; Eruygur et al., 2019). The essential oils of *Achillea* have been used for pharmacological purposes such as in contemporary medicine, cosmetics, aromatherapy, and phytotherapy (Becker et al., 2016). A former *in vivo* study indicated the anxiolytic, memory-enhancing and antidepressant properties of *Achillea biebersteinii* essential oils (Akbaba et al., 2018). The extracts of *Achillea* species have been extensively studied for their antimicrobial, antihypertensive, antihyperlipidemic, antispasmodic, antidiabetic, antioxidant, antifertility, anti-spermatogenic, and immunosuppressive activities (Ertaş et al., 2014; Al-Jaber et al., 2018).

A. pseudoaleppica is a local endemic rare species traditionally used in female diseases, menstrual irregularities (dried fresh flowers and leaves), intestinal inflammations (fresh and dried flowers and tea made from the leaves), hair-loss (above-ground parts), frequent urination at night (flowers), and skin beauty (above-ground parts). According to the literature review, there are only a few studies on its chemical components (Özen et al., 2003). One of the major essential oil components of *A. pseudoaleppica*, camphor was first

CONTACT Ercan Bursal ercanbursal@gmail.com Faculty of Health, Department of Nursing, Muş Alparslan University, Muş, Turkey.
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obtained from *Cinnamomum camphora* growing in East Asia (Akbaba, 2018). *C. camphora* is known for its analgesic, antiseptic, anti-itch, and stimulant activities. The high amount of camphor in *A. pseudoaleppica* also reveals that it may have activities like *C. camphora*. The synthetic form of camphor is currently being produced for medical, health, and industrial applications (Drikvandi et al., 2020). Camphor was reported for its sedative activity. Camphor is the active ingredient of many ointments used especially against muscle pain (Oshima & Ito, 2021). Memory enhancing, anxiolytic, antioxidant, and antidepressant activities of the essential oils of *A. pseudoaleppica* in scopolamine animal model were investigated and their contributions to complementary and/or integrative therapy for neurodegenerative diseases such as Alzheimer's disease were reported (Akbaba, 2018).

Therapeutic efficacy of medicinal plants is often correlated with their reservoir of diverse secondary metabolites. Phenolic acids in plants are considered as one of the most promising groups of secondary metabolites owing to their plethora of biological and pharmacological attributes mostly linked with their antioxidant properties (Heleno et al., 2015). Screening of phenolics from medicinal and food plants have proved their dietary and health benefit properties. Phenolic compounds were found for the bitter taste, pigments and with immense health benefits (Eggleston et al., 2021). Bioactive compounds such as phenolic acids are potent scavengers of reactive oxygen species (ROS) associated with the pathology of many human ailments. In addition, botanical-derived natural antioxidants are largely favored over synthetic antioxidants. However, plant phenols have not been completely investigated due to their complex chemical nature and widespread presence in plant samples. Phenolic acids, with their age-old uses in food and medicine, are also known to modulate membrane permeability, transcriptional regulation, and signal transduction (Cheynier et al., 2013). Despite possessing a huge pharmacological potential of *A. pseudoaleppica* as manifested by many *in vitro* and *in vivo* assessments, no report exists on the production and variety of phenolic compounds in this endemic plant species.

Various *Achillea* taxa have been recorded as promising neuroprotective and antidiabetic agents in traditional medicine as well as in pharmacological investigations (Salehi et al., 2020). The two cholinesterase enzymes, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are known to be responsible for hydrolyzing the acetylcholine and butyrylcholine (Turkan et al., 2019). Since acetylcholine is a neurotransmitter in the synaptic cleft, AChE inhibitors can reduce and prevent Alzheimer's disease and Parkinson's disease symptoms. BChE does similar functions as AChE in regulating cholinergic neurotransmission by hydrolyzing acetylcholine (Chohra et al., 2020, Gülçin et al., 2019). α-Glucosidase and α-amylase enzymes have also been recorded as particular concerns in pharmaceutical studies. The inhibitions of α-amylase and α-glucosidase can delay glucose absorption that causes low postprandial plasma blood glucose and prevents postprandial hyperglycemia. Numerous studies have depicted the biological properties and enzyme inhibitory effects of various plant species (Bursal et al., 2019, Gantner et al., 2019, Silinsin & Bursal, 2018). Besides, natural phenolic compounds were reported for their ability to inhibit cholinesterase and α-glucosidase enzymes. *A. pseudoaleppica* has traditionally being used against gastrointestinal inflammations and hair loss and also to enhance skin beauty. Anti-inflammatory properties of plant preparations in many cases are related to its antioxidant capacity (Amaral et al., 2009; Schinella et al., 2002). Moreover, anti-hair fall and skin beauty enhancement properties of herbal formulations also rely on their antioxidant properties (Ribeiro et al., 2015). However, to the best of our knowledge, there is no detailed single study on the enzyme inhibitions concerning the chemical content and biological activity of *A. pseudoaleppica*. Only the determination of essential oils of *A. pseudoaleppica* was reported in a couple of studies (Özen et al., 2003).

The present study aims to unravel the diverse phenolic content of *A. pseudoaleppica* using a quick and reproducible UHPLC-MS/TQ-MS method, to evaluate the antioxidant activity and enzyme inhibitory potential against the AChE, BChE, α-amylase, and α-glucosidase enzymes for initial validation of its neuroprotective and antidiabetic potentials. We aimed to evaluate the inhibition potential of the plant extracts for two major health problems (Alzheimer's disease and DM), separately. Thus, AChE and BChE enzymes were used for their roles in Alzheimer's disease as well as α-amylase and α-glucosidase enzymes were used for their roles in DM.

2. Experimental

2.1. Chemicals and enzymes

The solvents for LC-MS/MS (acetone, formic acid, methanol) were purchased from Merck (Germany). The chemicals such as; ABTS, DMPD, DPPH, neocuproine, and the standard compounds (α-tocopherol, trolox, BHA, BHT) were purchased from Sigma-Aldrich (Germany) to use on the antioxidant methods. Electric eel AChE, equine serum BChE, *Saccharomyces cerevisiae* α-glucosidase, and human pancreatic α-amylase enzymes were used in this study.

2.2. Plant material

A. pseudoaleppica plant samples were collected from Obuz village (Elazığ, Turkey), at 1250–1300 m altitude, calcareous steppe, stony, and screes on 25 May 2019. GPS device used to determine the coordinate (38° 43′53″N; 39° 14′33″E) of the habitat. The plant samples were identified by taxonomist Dr. Omer Kılıç and a voucher specimen was deposited with voucher number 6087 at the Pharmacy Faculty of Adıyaman University.

2.3. Preparation of the plant extracts

The ethanol and water extractions of *Achillea pseudoaleppica* were carried out according to a previous study (Aras et al., 2019). Briefly, the plant leaves were dried at room condition and powdered with a blender. For aqueous extract, the air-

dried plant sample (20 g) was mixed with 200 mL distilled water (1/10:w/v). Also, the air-dried plant sample (20 g) was mixed with 200 mL ethanol (1/10:w/v) to prepare the ethanol extract, as well. The mixtures were homogenized by a magnetic stirrer for 12 h, at room temperature (25 °C). The homogeneous mixtures were filtered with filter papers. The filtrate sample from the water solvent was lyophilized in a lyophilizer (Labconco, Freezone 1 L) at 5 mm Hg at −50 °C for preparing the water extract. The filtrate sample from ethanol solvent was evaporated with a rotary evaporator (Heidolph 94200, Bioblock Scientific) for preparing the ethanol extract. The lyophilized and evaporated samples were stored at −30 °C until being used further.

2.4. Determination of total phenolic and flavonoid contents

The total phenolic and flavonoid contents of the plant sample were determined according to a former study (Bursal et al., 2020). For the total phenolic content determination, the plant extract (0.5 mL) was mixed with Folin-Ciocalteu solution (1.0 mL) and Na_2CO_3 (0.5 mL, 1%). The absorbance of the mixtures were measured at 725 nm after the incubation for 2 h at room temperature. Gallic acid was used as a standard and the total phenolic amounts were given as milligrams of gallic acid equivalents (GAE).

For the total flavonoid content determination, the plant extracts (0.5 mL) was mixed with ethanol (1.5 mL, 95%), aluminum chloride (1.5 mL, 10%), potassium acetate (0.5 mL, 1.0 M), and distilled water (2.3 mL), respectively. The absorbance of the mixtures were measured at 415 nm after the incubation for 30 min at room temperature. Quercetin used as a standard and the flavonoid amounts were given as milligrams of quercetin equivalents (QE) per gram of extract.

2.5. Determination of phenolic compounds by UHPLC-MS/TQ-MS analysis

The following instrumental details were used for mass spectrometer and chromatography conditions (Yilmaz, 2020). A Shimadzu-Nexera model ultrahigh performance liquid chromatography (UHPLC) coupled with a tandem mass spectrometer was used to accomplish a quantitative evaluation of 53 phytochemicals. The reversed-phase UHPLC was equipped with an autosampler (SIL-30AC model), a column oven (CTO-10ASvp model), binary pumps (LC-30AD model), and a degasser (DGU- 20A3R model). The chromatographic conditions were optimized to achieve optimum separation for compounds and to overcome the suppression effects. The chromatographic separation was performed on a reversed-phase Agilent Poroshell 120 EC-C18 model (150 mm × 2.1 mm, 2.7 μm) analytical column. The column temperature was set to 40 °C. The elution gradient was composed of solvent A (water/5 mM ammonium formate/0.1% formic acid) and solvent B (methanol/5 mM ammonium formate/0.1% formic acid). The following gradient elution profile was used: 20-100% B (0-25 min), 100% B (25-35 min), 20% B (35-45 min). Furthermore, the solvent flow rate and injection volume were settled as 0.5 mL/min and 5 μL, respectively.

The mass spectrometric detection was carried out using a Shimadzu LCMS-8040 model tandem mass spectrometer (MS/MS) equipped with an electrospray ionization (ESI) source operating in both negative and positive ionization modes. LC-ESI-MS/MS data were acquired and processed by LabSolutions software (Shimadzu). The MRM (multiple reaction monitoring) mode was used for the quantification of the phytochemicals. The MRM method was optimized to selectively detect and quantify phytochemical compounds based on the screening of specified precursor phytochemical to fragment ion transitions. The collision energies were optimized to generate optimal phytochemical fragmentation and maximal transmission of the desired product ions. The MS operating conditions were applied as drying gas (N_2) flow (15 L/min), nebulizing gas (N_2) flow (3 L/min), DL temperature (250 °C), heat block temperature (400 °C), and interface temperature (350 °C).

2.6. Antioxidant activity

The antioxidant activity of *A. pseudoaleppica* was analyzed by using six well-known methods to determine the radical scavenging and reducing capacities of its water and ethanol extracts. *In vitro* CUPRAC (cupric ions reducing activity) method, FRAP (ferric ions reducing antioxidant power) method, and Fe^{3+}-TPTZ reducing assays (Aras et al., 2018) were performed to evaluate the reducing potentials of the extracts. Furthermore, radical scavenging activities of the extracts were examined by using DPPH (2,2-diphenyl-1-picryl-hydrazyl) method (Blois, 1958), ABTS (2,2′-azino-bis3-ethylbenzothiazoline-6-sulphonic acid) method (Re et al., 1999), and DMPD (N,N-dimethyl-p-phenylenediamine) method with slight modifications as reported in a previous study (Gülçin et al., 2020). The antioxidant potentials were determined by comparison with the standard antioxidant compounds (BHA, BHT, trolox, and α-tocopherol). The different concentrations (10-30 μg/mL) of the extracts and reference standards were used to examine the effect of the dose-dependent antioxidant potential of the plant extracts.

2.7. Enzyme inhibitory activity

AChE and BChE inhibitory properties of the plant extracts were measured according to a previously described method (Turkan et al., 2019). Acetylthiocholine iodide and butyrylcholine iodide were used as the substrates in reactions. The inhibition of α-glucosidase enzyme was determined by using *p* nitrophenyl-*D*-glucopyranoside (*p*-NPG) substrate as detailed in a previous study (Eruygur et al., 2019). α-Glucosidase and α-amylase enzyme inhibitory properties of the extracts were determined as demonstrated in a previous study (Taslimi & Gulçin, 2017).

2.8. Molecular docking

Chimera and AutoDock Vina computational softwares were used to examine the molecular docking of the major compound of the plant extract (quinic acid) at the active pockets

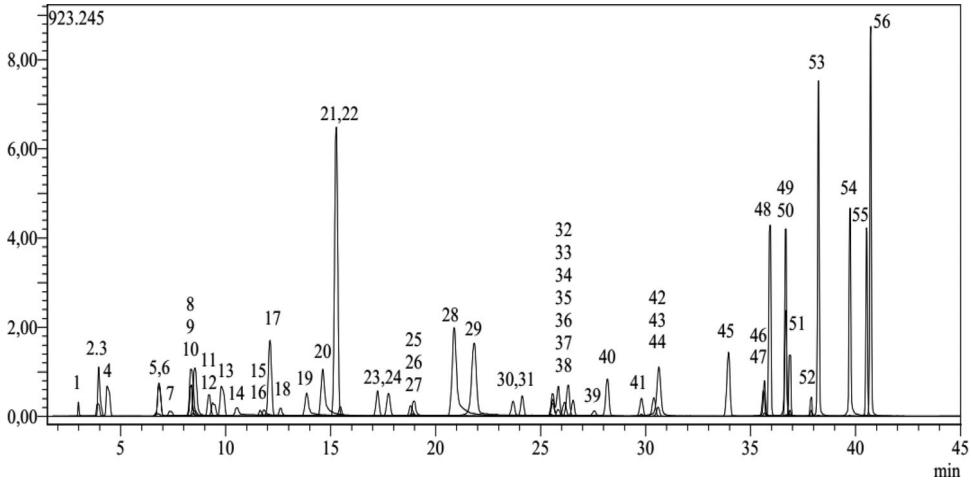

Figure 1. LC-MS/MS chromatograms of standard compounds.

of AChE, BChE, α-glucosidase, and α-amylase target enzymes. The chemical structures of AChE (human, PDB id: 4EY7), BChE (human, PDB id: 4BDS), α-glucosidase (human lysosomal acid- α-glucosidase PDB id: 5NN8), and α-amylase (human pancreatic, PDB id: 2QV4) were downloaded from the website of Protein Data Bank.

All the enzyme structures were optimized on Discovery Studio Visualizer. The optimized enzyme structures were saved as pdb format and loaded to Chimera software. The structure of quinic acid, the major compound of the plant extracts, was downloaded from the website of Pubchem. The structure of quinic acid was optimized on the Avogadro visualization application. The optimized structure of quinic acid was saved as pdb format and loaded as ligand pdb file. Then, the software was launched (Shapovalov & Dunbrack, 2011). The best docking energy scores and the binding interactions of each pose were analyzed on Discovery Studio Visualizer.

3. Results

3.1. Estimation of total phenolic and flavonoid

The present study demonstrated high amounts of total phenolic and total flavonoid contents in *A. pseudoaleppica* extracts. Total phenolic contents of the water and ethanol extracts were determined as 58.53 μg/mg and 65.08 μg/mg GAE (μg of gallic acid equivalent in mg dried extract), respectively. Also, total flavonoid contents of the water and ethanol extracts were determined as 70.32 μg/mg and 79.13 μg/mg QE (μg of quercetin equivalent in mg dried extract), respectively.

3.2. Quantification of bioactive compounds of A. pseudoaleppica

Ultra-high performance liquid chromatography triple quadrupole mass spectrometry (UHPLC-MS/TQ-MS) technique was used for the identification and quantification of phytochemical compounds in both *A. pseudoaleppica* water and ethanol extracts by using fifty-six reference compounds. The supplementary table (Table S1) represents the analytical method and validation parameters of fifty-three standard compounds and three (ferulic acid, rutin, and quercetin) internal standards (Yilmaz, 2020). The linear equation of standards applied to UHPLC-MS/TQ-MS analysis for quantification and the regression values (R^2) of all compounds were in the range 0.99. The polyphenol compounds present in the extracts were identified by comparing spectrum and retention time (Rajan et al., 2020).

The present study evaluated phenolic compounds of *A. pseudoaleppica* extracts by comparing fifty-three standard phytochemicals (fifty phenolic and three nonphenolic compounds). Figure 1 represents the UHPLC-MS/TQ-MS chromatograms of the reference standards. The UHPLC-MS/TQ-MS chromatograms of the water and ethanol extracts of *A. pseudoaleppica* are indicated in Figure 2, respectively. According to the UHPLC-MS/TQ-MS analyses, quinic acid was identified as the major compound in both water extract (31.12 ± 1.65 μg/mg) and ethanol extract (11.75 ± 0.82 μg/mg). The diversity of compounds of ethanol extract was higher than that of the water extract. Twenty-two out of fifty-three (22/53) different compounds were identified in ethanol extract. Similarly, twelve out of fifty-three (12/53) different compounds were identified in the water extract (Table 1).

3.3. Antioxidant potential of A. pseudoaleppica

In the present study, six well-known *in vitro* methods were used to evaluate the antioxidant activity of *A. pseudoaleppica* by analyzing the radical scavenging and reducing potentials. CUPRAC, FRAP, and Fe^{3+} reducing ability methods were used for that purpose. On the other hand, DPPH, ABTS, and DMPD assays were used for the radical scavenging determinations.

FRAP method has been used for reducing potential measurements of the samples by measuring the reduction of Fe^{3+} to Fe^{2+} ions. According to the results, the reducing potentials of the extracts were higher than α-tocopherol but lower than the other three standards. The results revealed that both extracts had potent reducing abilities.

CUPRAC method also has been used for the determination of Cu^{2+} reducing ability. The method is based on measuring Cu^{2+}-Cu^{+} reduction. The results indicated that

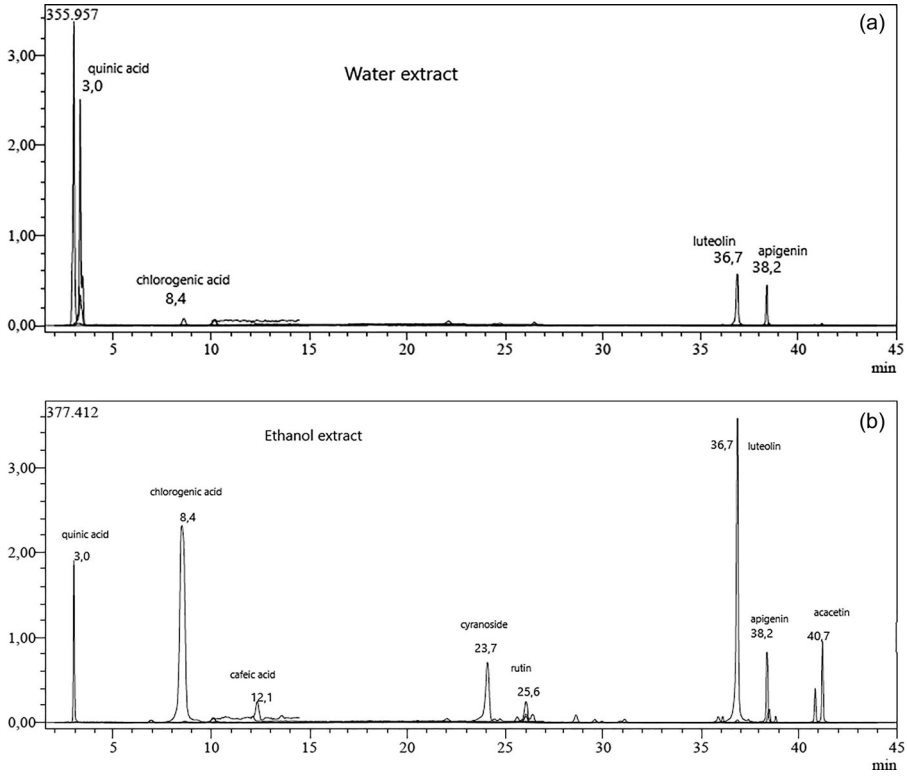

Figure 2. **a**. UHPLC-MS/TQ-MS chromatograms of the water extract of A. pseudoaleppica. **b**. UHPLC-MS/TQ-MS chromatograms of the ethanol extract of A. pseudoaleppica.

Table 1. Major phenolic compounds of *A. pseudoaleppica*.

No.	Retention time	Analyte	Ethanol extract (μg/mg)	**Water extract (μg/mg)**
1.	3.0	Quinic acid	11.753	31.122
2.	6.8	Protocatechuic acid	0.092	0.017
3.	8.5	Protocatechuic aldehyde	0.027	
4.	8.4	Chlorogenic acid	6.88	0.14
5.	12.1	Caffeic acid	0.169	
6.	17.8	p-Coumaric acid	0.065	0.04
7.	21.8	Salicylic acid	0.032	0.035
8.	23.7	Cyranoside	2.614	0.016
9.	25.6	isoquercitrin	0.371	
10.	25.6	Rutin	0.837	
11.	25.8	Hesperidin	0.661	0.007
12.	28.2	Cosmosiin	0.219	
13.	30.4	Astragalin	0.077	
14.	30.6	Nicotiflorin	0.159	
15.	35.7	Quercetin	0.197	
16.	36.7	Luteolin	1.067	0.191
17.	36.7	Hesperetin	0.013	
18.	35.9	Naringenin	0.022	0.006
19.	37.9	Kaempferol	0.012	
20.	38.2	Apigenin	0.14	0.074
21.	40.7	Acacetin	0.048	0.001
22.	40.5	Chrysin	0.003	0.003

Table 2. Absorbance measurements of *A. pseudoaleppica* and standard compounds at 30 μg/mL concentration for evaluation of reducing antioxidant potential.

	Fe^{3+}-Fe^{2+}reducing		Cu^{2+}-Cu^{+} reducing		Fe^{3+}-TPTZ reducing	
Standards and extracts	λ_{700}	R^2	λ_{450}	R^2	λ_{593}	R^2
BHA	2.234 ± 0.008	0.97	2.181 ± 0.020	0.99	2.334 ± 0.013	0.97
BHT	2.013 ± 0.003	0.92	2.249 ± 0.021	0.95	2.212 ± 0.012	0.96
α-tocopherol	1.013 ± 0.015	0.94	0.879 ± 0.012	0.99	1.560 ± 0.011	0.91
trolox	1.761 ± 0.007	0.98	0.941 ± 0.027	0.92	1.766 ± 0.049	0.93
A. pseudoaleppica water extract	1.205 ± 0.015	0.93	1.034 ± 0.022	0.99	1.282 ± 0.07	0.98
A. pseudoaleppica ethanol extract	1.321 ± 0.016	0.96	1.140 ± 0.009	0.98	1.401 ± 0.001	0.93

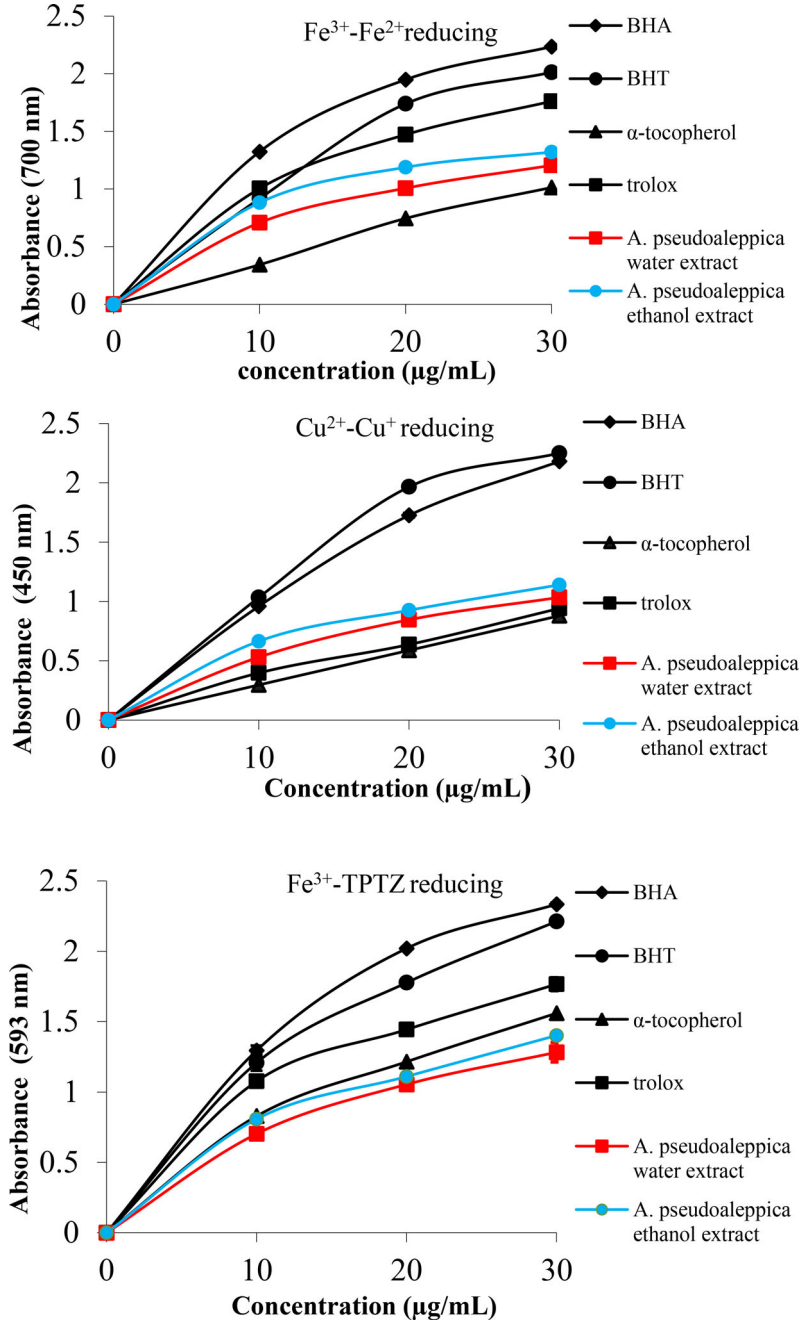

Figure 3. Antioxidant potentials of A. pseudoaleppica extracts and standard compounds by three in vitro reducing methods.

Table 3. Determination of half-maximal concentrations (IC_{50}) of *A. pseudoaleppica* and standards for DPPH, ABTS, and DMPD radical scavenging activities.

Standards and extracts	DPPH· scavenging	R^2	$ABTS^{\bullet+}$ scavenging	R^2	$DMPD^{\cdot+}$ scavenging	R^2
BHA	13.35	0.98	9.57	0.95	32.12	0.94
BHT	14.51	0.94	8.77	0.96	29.53	0.94
α-Tocopherol	21.31	0.98	15.16	0.98	44.65	0.97
Trolox	8.43	0.91	7.51	0.95	35.13	0.94
A. pseudoaleppica water extract	25.57	0.90	14.51	0.93	40.14	0.95
A. pseudoaleppica ethanol extract	23.24	0.95	13.23	0.95	37.70	0.95

the Cu^{2+} reducing powers of the extracts were close to the standard antioxidants. The reduction of *A. pseudoaleppica* extract was measured to be higher than α-tocopherol and trolox but lower than BHA and BHT.

Fe^{3+}-TPTZ reducing assay is the third method to determine the reducing power of the plant extracts. According to this method, the reducing power of the samples and standards were ordered as BHA, BHT, trolox, α-tocopherol, ethanol extract, and water extract. The absorbance data of the extracts and standards for three reducing methods are presented in Table 2. Furthermore, the data of three reducing methods are graphically presented in Figure 3. According to all three methods, the increasing absorbance values indicated high reducing abilities of the extracts of *A. pseudoaleppica*.

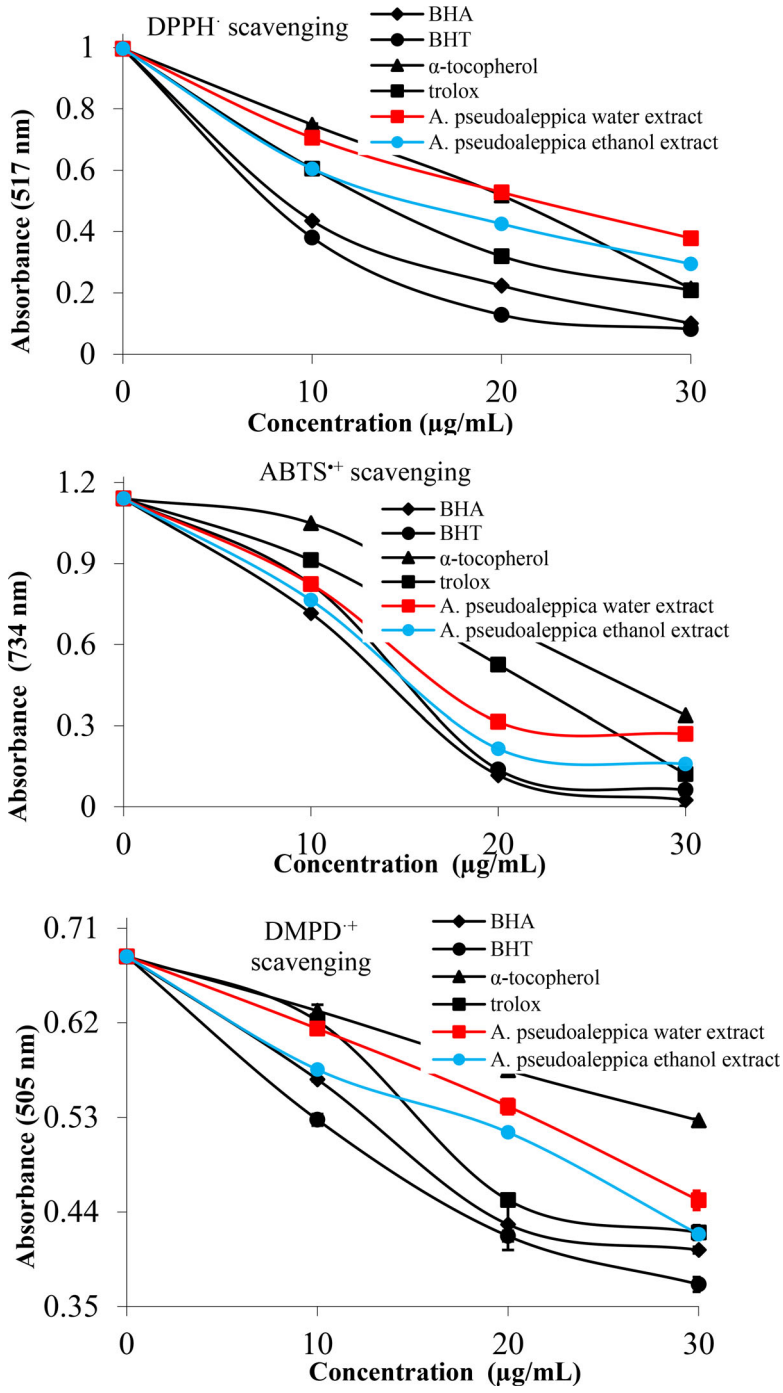

Figure 4. Antioxidant potentials of A. pseudoaleppica extracts and standard compounds by DPPH, ABTS, and DMPD radical scavenging methods.

Table 4. Enzyme inhibition IC_{50} values of *A. pseudoaleppica* extracts.

	Water extract (mg/mL)		Ethanol extract (mg/mL)		Acarbose (mg/mL)		Tacrine (mg/mL)	
Enzymes	IC_{50}	r^2	IC_{50}	r^2	IC_{50}	r^2	IC_{50}	r^2
α-Glucosidase	17.77	0.99	12.37	0.96	22.80	0.99		
α-Amylase	22.35	0.98	16.51	0.99	10.01	0.94		
AChE	9.11	0.99	2.67	0.98			0.12	0.98
BChE	12.62	0.96	4.55	0.99			0.10	0.97

DPPH free radical scavenging potentials of the extracts were determined by measuring and comparing the IC_{50} values of the extracts with standards. The lower IC_{50} value indicates more effective radical scavenging potential. The IC_{50} values of samples are presented in Table 3. Absorbance changes with different concentrations (10-30 µg/mL) of the

extracts and reference standards are graphically presented in Figure 4.

3.4. Enzyme inhibitory activities

Enzyme inhibition studies have gained significant interest in recent times for their roles in metabolic pathways implicated in human diseases. The diverse chemical constituents of medicinal plants are considered great sources of cholinesterase inhibitors. AChE inhibitors have been used for the medical treatments of dementia, Alzheimer's and Parkinson's diseases (Bursal et al., 2020).

In this study, the cholinesterase inhibitory activities of *A. pseudoaleppica* extracts were determined by using AChE and BChE enzymes. The ethanol extract was found to be more effective AChE and BChE inhibitions compared to the water extract. The inhibition levels of the extracts were close to tacrine, a standard reference inhibitor of AChE and BChE enzymes. The IC_{50} values of enzyme inhibition by *A. pseudoaleppica* extracts are summarized in Table 4. The IC_{50} values of the samples against AChE were found to be 2.67 μM for the water extract and 9.11 μM for the ethanol extract. Similarly, IC_{50} values of the water and ethanol extracts for BChE were noted as 4.55 μM and 12.62 μM, respectively. The low IC_{50} values of the extracts indicated their effective potential for AChE and BChE inhibitions as compared to the reference inhibitor.

Also, α-amylase and α-glucosidase enzyme inhibitory activities of *A. pseudoaleppica* extracts were determined. The IC_{50} values of the extracts and the standard compound for α-amylase and α-glucosidase enzymes were calculated. According to the results, the water and ethanol extracts showed lower IC_{50} values implicated in highly effective α-glucosidase enzyme inhibition compare to acarbose. However, less effective α-amylase inhibitions of the extracts were obtained compare to the reference sample (acarbose).

3.5. Molecular docking

The molecular docking studies were carried out to show the binding affinity of the major compound of the plant extract with AChE, BChE, α-amylase, and α-glucosidase target enzymes. The docking scores demonstrated that quinic acid exhibited good binding affinity with all four enzyme targets (Table 5).

AChE-quinic acid free energy of binding score was calculated as −6.2 kcal/mol. Conventional hydrogen bonds of quinic acid with AChE residues (ASP A:71, TYR A:121, and SER A:122) were shown on the 2 D view of the hydrogen bonds donor/acceptor surface on the receptor (Figure 5).

BChE-quinic acid free energy of binding score was calculated as −5.8 kcal/mol. Conventional hydrogen bonds of quinic acid with BChE residues (GLY A:115, TYR A:128, GLU A:197) and carbon hydrogen bonding (HIS A:438) were shown on the 2 D view of the hydrogen bonds donor/acceptor surface on the receptor (Figure 6).

α-Amylase-quinic acid estimated free energy of binding score was calculated as −6.3 kcal/mol. Conventional hydrogen bonds of quinic acid with α-amylase residues (ASN A:301, ALA A:310, THR A:314, and ASP A:317) and carbon hydrogen bonding (GLY A:304) were shown on the 2 D view of the hydrogen bonds donor/acceptor surface on the receptor (Figure 7).

α-Glucosidase-quinic acid estimated free energy of binding score was calculated as −5.5 kcal/mol. Conventional hydrogen bonds of quinic acid with α-glucosidase residue (ASP A:243) and carbon hydrogen bonding (ASN A:570) were shown on the 2 D view of the hydrogen bonds donor/acceptor surface on the receptor (Figure 8).

4. Discussions

In this present study, quantitative analyses showed the high amounts of flavonoid and phenolic compounds in the extracts of *A. pseudoaleppica* leaves, particularly in the ethanol extract. UHPLC-MS/TQ-MS technique revealed the presence of 23 phenolic compounds in *A. pseudoaleppica* extract of which quinic acid was the major compound. Presence of various phenolic compounds in the plant may be attributed to its antioxidant and enzyme inhibitory properties. Phenols have previously been cited as promising natural antioxidants (Martinez-Gomez et al., 2020) and enzyme inhibitors (Zhai et al., 2018). A number of recent studies also unraveled the phenolic composition, antioxidant, and enzyme inhibition activities of a number of endemic and/or ethnobotanicals (Saravanakumar et al., 2019), superior antioxidant and enzyme inhibitory properties of plant extracts were correlated with their higher phenolic levels (Llorent-Martínez et al., 2017). In addition, quinic acid and its derivatives were reported as a potent antioxidant and enzyme inhibitor (Choi et al., 2021). Therefore, the higher antioxidant and enzyme inhibitory activities of *A. pseudoaleppica* may be implicated to its higher as well as a variety of phenolic compounds of which quinic acid was present in substantially higher amount compared with the other phenolic identified in the plant.

DPPH assay is the most efficient spectrophotometric method for the evaluation of radical scavenging activity. Antioxidant substances can donate hydrogen and scavenge DPPH radicals. According to DPPH results, the extracts showed high free radical scavenging abilities as understood from low IC_{50} values that were close to the standard compounds (BHA, BHT, trolox, and α-tocopherol). ABTS and DMPD assays are the other common methods to determine the radical scavenging potentials (Bursal et al., 2020). These methods are based on the inhibitions of the absorbance of ABTS or DMPD cation radicals caused by antioxidant substances. In the present study, both water and ethanol extracts exhibited effective ABTS and DMPD cation radical scavenging activities with low IC_{50} values that were close to the standard compounds (BHA, BHT, trolox, and α-tocopherol). The results showed that the plant extracts can easily transfer hydrogen atoms to $DMPD^{\bullet+}$ and $ABTS^{\bullet+}$ cation radicals implicated in scavenging the radicals.

α-Amylase and α-glucosidase enzymes have critical metabolic functions in carbohydrate digestion and hydrolysis of polysaccharides (Aras et al., 2019). Diabetes mellitus is a

Table 5. Molecular docking interactions of the major phenolic compound of *A. pseudoaleppica* (Quinic acid) with AChE, BChE, α-amylase, and α-glucosidase.

						Interactions at the active pocket	
No	Enzyme	Source organism	PDB id	Resolution (A°)	Affinity (kcal/mol)	Type of Interactions	Residue Information
1	AChE	Human acetylcholinesterase	4EY7	2.35	−6.2	Hydrogen bonding	ASP A:71 TYR A:121 SER A:122
2	BChE	Human butyrylcholinesterase	4BDS	2.10	−5.8	Hydrogen bonding Carbon Hydrogen bonding	GLY A:115 TYR A:128 GLU A:197 HIS A:438
3	α-Amylase	Human pancreatic α-amylase	2QV4	1.97	−6.3	Hydrogen bonding Carbon Hydrogen bonding	ASN A:301 ALA A:310 THR A:314 ASP A:317 GLY A:304
4	α-Glucosidase	Human lysosomal acid- α-glucosidase	5NN8	2.45	−5.5	Hydrogen bonding Carbon Hydrogen bonding	ASP A:243 ASN A:570

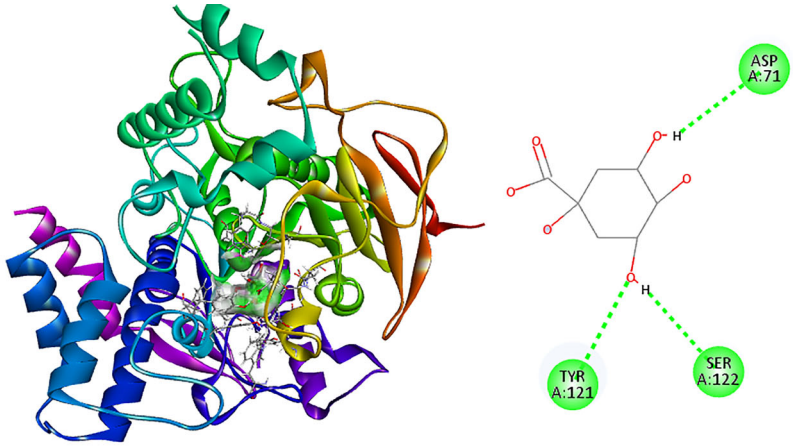

Figure 5. AChE-quinic acid molecular docking interactions.

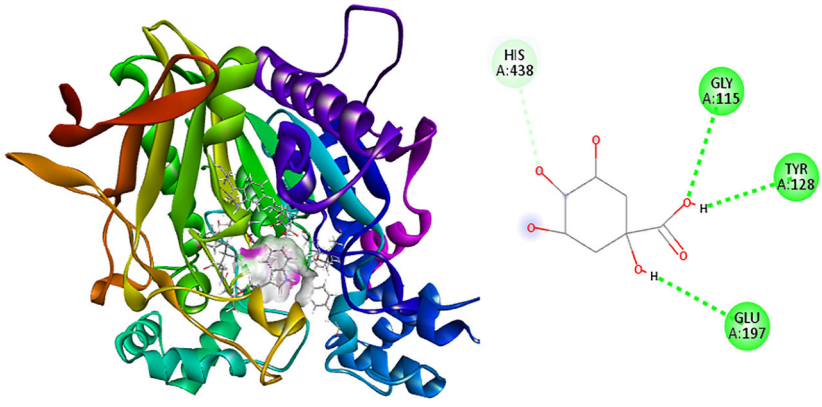

Figure 6. BChE-quinic acid molecular docking interactions.

carbohydrate metabolism disorder caused by high blood glucose levels. Inhibitions of α-amylase and α-glucosidase enzymes have been considered to possess therapeutic values for the treatment of diabetes mellitus (Tohma et al., 2019). Medicinal plants have been reported for having potent α-amylase and α-glucosidase inhibitory activities (Zengin et al., 2019). Earlier, *Achillea* taxa such as *A. schischkinii*, *A. cucullata*, *A. biebersteinii*, *A. millefolium*, and *A. teretifolia* were recorded for their enzyme inhibitory properties (Eruygur et al., 2019). Also, some *Achillea* taxa such as *A. damascene*, *A. fragrantissima*, *A. kellalensis*, *A. millefolium*, *A. santolina*, and *A. sulpherea* have been reported as potent antidiabetic ethnobotanicals (Salehi et al., 2020).

The present study validates the possible anti-diabetic properties of *A. pseudoaleppica* indicated by its inhibitory properties against α-amylase and α-glucosidase. Moreover, the abilities of plant extracts and natural compounds to inhibit AChE and BChE enzymes have been correlated to their anti-Alzheimer's disease activities (Ali et al., 2016; Türkan et al., 2019). Earlier, *A. schischkinii, A. cucullata*, *A.*

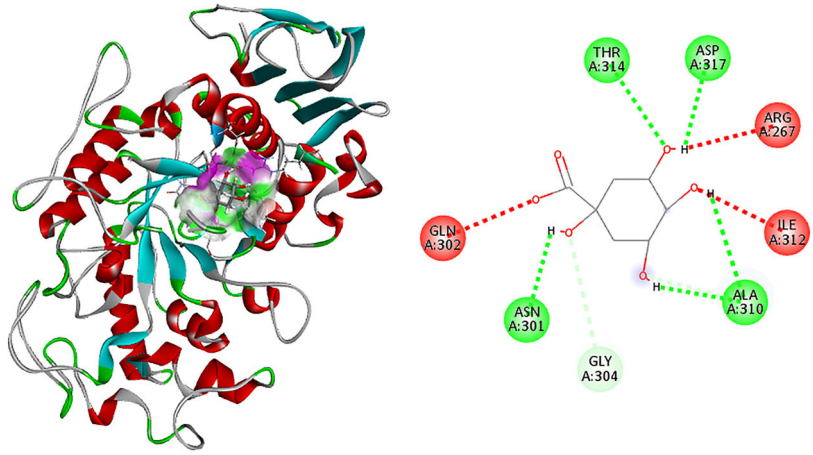

Figure 7. α -Amylase-quinic acid molecular docking interactions.

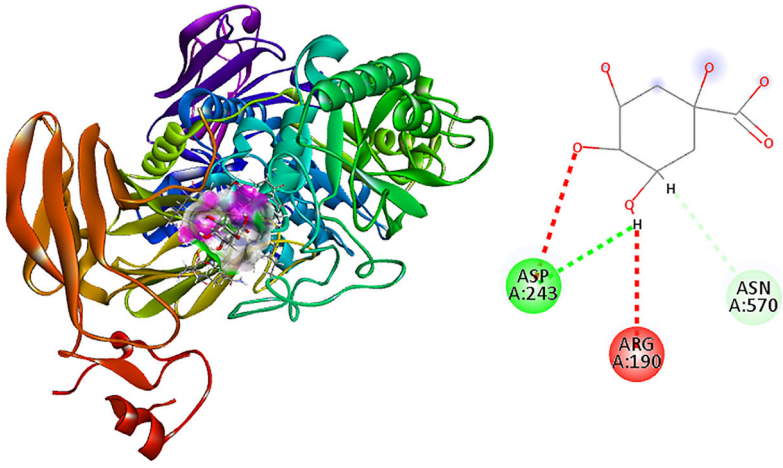

Figure 8. α-Glucosidase-quinic acid molecular docking interactions.

millefolium and *A. fragrantissima* displayed potent anticholinesterase potential (Barut et al., 2017). Furthermore, some *Achillea* spp. have been reported as a neuroprotective ethnobotanical against several neurological ailments (Salehi et al., 2020). Considering the extensive literature on the crucial influence of oxidative stress in many neurodegenerative diseases (*viz.* Parkinson's disease and Alzheimer's disease) and also bearing in mind its promising antioxidative properties rich phenolic content of *A. pseudoaleppica*, it might serve as an excellent ethnobotanical to be used against neurological disorders mediated by oxidative stress. Again, the present study, for the first time reports the anticholinesterase properties of *A. pseudoaleppica* validating the potent ethnopharmacological use of many *Achillea* spp. against an array of neurological disorders including Alzheimer's disease and dementia.

The indigenous flora represents its essence from the traditional ethno-floristic diversity and nurtures a plethora of endemic or specialized plant species that were evolved in varied climatic, topographical, and geographical conditions (Mahomoodally & Aumeeruddy, 2017). Endemic botanicals appreciably contribute toward traditional knowledge and despite their rare and limited availability, the indigenous people use them as potent ethnomedicines. Therefore, the conservation of such endemic plants is of utmost importance for sustainable utilization by the local communities. In addition, endemic ethnobotanicals have always been cited as a prolific source of diverse phytochemicals owing to their superior pharmacological properties. Conservation of this endemic plant species can also offer a novel and steady source of many pharmaceutically active products with therapeutic benefit.

5. Conclusions

The phytochemistry and bioactivity of the endemic medicinal plant, *A. pseudoaleppica*, concerning its phenolic content, antioxidant capacity, and enzyme inhibitory potential were investigated. In summary, quinic acid was identified as the major phenolic compound in *A. pseudoaleppica* extracts. *In vitro* radical scavenging and reducing methods demonstrated effective antioxidant potentials of the water and ethanol extracts of the plant. Besides, possible inhibitory effects of *A. pseudoaleppica* extracts against α-glycosidase, α-amylase, AChE, and BChE enzymes were examined. According to the results, both of the extracts exhibited effective enzyme

inhibition close to the standard inhibitors, whereas the ethanol extract showed more efficacy than the water extract for all four enzymes. Therefore, the present report portrays the medicinal values of *A. pseudoaleppica* as a promising source for phenolic compounds and antioxidant properties as well as a potent inhibitor of α-glucosidase, α-amylase, AChE, and BChE enzymes. Moreover, endemic, economical, medicinal, aromatic, and ethnobotanical plants are needed to be conserved with *in-situ* and *ex-situ* methods for their sustainable utilization by the indigenous communities as well as the prolific source of secondary metabolites for their possible therapeutic applications.

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ORCID

Parham Taslimi http://orcid.org/0000-0002-3171-0633
İlhami Gülçin http://orcid.org/0000-0001-5993-1668
Ercan Bursal http://orcid.org/0000-0001-7289-4507

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