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Assessment of antioxidant, acetylcholinesterase, paraoxonase inhibition activities and phenolic content of *Alchemilla lithophila*

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Abstract: In the present study, antioxidant activity and inhibition of acetylcholinesterase (AChE) and paraoxonase (hPON 1) of *Alchemilla lithophila* extracts were evaluated for the first time. Besides, there is no research on the contents of phenolic compounds except for fatty acids. In this context, phenolic compounds of *A. lithophila* were investigated by liquid chromatography/ mass spectrometry (LC-MS/MS). The methanol extract of the *A. lithophila* exhibited significant inhibition on the AChE (IC_{50} value for methanol extract 0.162 ± 0.25 mg /mL, R^2:0.992). Besides, antioxidant activities of the *A. lithophila* extracts were examined using by the methods $ABTS^{\bullet+}$and $DPPH^{\bullet}$ free radical scavenging potentials, FRAP and CUPRAC metal-reducing activities. $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ scavenging activities were found for methanol extract at 70.67% and water extract at 75.38%, respectively. Also, FRAP and CUPRAC metal-reducing were determined for water extract 0.796 and hexane extract 1.570 as absorbance. According to LC-MS/MS analyses, the amounts of ellagic acid, catechin hydrate, gallic acid, fumaric acid, luteolin, quercetin, kaempferol, acetohydroxamic acid, caffeic acid, syringic acid, hydroxybenzoic acid and salicylic acid were determined by LC-MS/MS, respectively. As a consequence, this study will be a useful resource for determining bioactivity and phenolic compound profile for natural medicine research.

Keywords: Acetylcholinesterase, paraoxonase, antioxidant, *Alchemilla lithophila,* LC-MS/MS.

INTRODUCTION

Parallel to the increasing interest in natural products, it is also reflected in drugs of natural origin. The inadequacy of the treatment with the conventional approach or the occurrence of side effects increases the interest in natural drugs. With this approach, it is essential to elucidate the structure and bioactivity of medicinal aromatic plants (Verma and Singh, 2008; Karimi *et al.*, 2015). Medicinal plants used to treat diseases are an important source of pharmaceutically active substances. Phenolic compounds with therapeutic properties in medicinal plants are members of secondary metabolites. Bioactivity studies on phenolic compounds make them more popular (Canter *et al.*, 2005; Ekor, 2014).

Phenolic compounds, which are products of the shikimic acid pathway in plants, contain hydroxyl groups. The number, configuration and position of these hydroxyl groups play a role in the bioactivity of phenolic compounds. Disruption of oxidative balance has a significant place in the pathogenesis of neurodegenerative, vascular and cancer diseases (Kim *et al.*, 2020; Güzel, 2023). Synthetic drugs used in the treatment of neurodegenerative and cancer diseases have many side effects. Studies are carried out to get rid of these side effects by treating them with natural medicines. Therefore, the phytochemical content of medicinal aromatic plants gains importance (Verma and Singh, 2008).

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Alzheimer's disease, a cognitive and behavioral disorder, is a progressive, chronic, and neurodegenerative disease that causes dementia in older people. Histopathological features of Alzheimer's disease were synaptic degeneration, hippocampal neuronal loss, aneuploidy, tau-hyperphosphorylation, extracellular amyloid plaques and intracellular neurofibrillary tangles. However, Alzheimer's disease histopathological parameters currently only take into account plaques and tangles. Acetylcholine deficiency has been suggested as one of the causes of amyloid plaque formation. The acetylcholinesterase is inhibited to increase the amount of acetylcholine at the synapses. Impulse transmission is ensured with the cholinergic effect approach (Avila, 2006; Swerdlow, 2007).

Paraoxonase (hPON 1), which has antioxidant properties, prevents lipid peroxidation that contributes to the formation of atherosclerosis. Increased oxidative stress and LDL (low-density lipoprotein) cause the formation of atherosclerotic lesions and the formation of atheroma plaques occurs in progressive processes. Studies have shown that the hPON 1 and HDL (high-density lipoprotein) are low in cardiovascular diseases (Ferretti *et al.*, 2005; Venkadeswaran *et al.*, 2016).

Alchemilla lithophila, known as ‘Aslan pençesi and Yıldız pençesi’ in Türkiye, is a perennial herb with woody rhizomes belonging to the Rosaceae family. *Alchemilla* L. is a genus comprising more than 1000 species, with 66 of them being found in Turkey (Ayaz *et al.*, 1999). *Alchemilla* is a medicinal plant used for a variety of

ailments in Turkey, including diarrhea, dysentery, menstrual pain, diuretics, constipation, and vascular diseases. It is also used in skin and mouth itching and inflammation by local residents (Baytop, 1999). Linoleic acid and tridecanoic acid of 49% of the fatty acid composition of the *A. lithophila* were found to be major components. Apart from morphological and taxonomic studies on the *A. lithophila* plant, there is no bioactivity and phenolic compound analysis (Ayaz *et al.*, 1999).

Specific laboratory studies are required to investigate the bioactivity of medicinal and aromatic plants. In this sense, the present work aimed to investigate antioxidant activity of *A. lithophila* extracts by using different antioxidant tests including free radical scavenging ($ABTS^{\bullet+}$ and $DPPH^{\bullet}$) and reducing power (FRAP and CUPRAC) and evaluate inhibition of *A. lithophila* extracts on AChE and hPON 1. Additionally, it was displayed the polyphenol content of the plant by LC-MS/MS.

MATERIALS AND METHODS

Plant sample

The plant material was collected in the Hekimhan/Malatya province of Türkiye in June of 2022 (located at B6 39°04'16"N, 37°59'13"E; altitude 2162 m). *A. lithophila* was identified by Turgay Kolaç (Inonu University, Vocational School of Health Services) with a voucher specimen is deposited in the Herbarium of the Faculty of Pharmacy, University of Inonu (herbarium code: TK 1363). The aerial parts of the plant were washed with distilled water and then dried in the shade for two weeks at 27°C.

Sample extraction

First, the dried herb was pulverized with a grinder. Then the sample (5 g) was extracted separately with 50 mL extraction solvents (methanol, water and *n*-hexane) using the maceration method at 27°C. After the dry extract was filtered through paper and evaporated at 40°C with a rotary evaporator, 1 mg/mL solution was prepared and used in LC-MS/MS analyses, antioxidant capacity and enzyme inhibition experiments.

LC-MS/MS instrument and chromatographic conditions

Qualitative and quantitative determination of 25 selected phenolic compounds were executed using the previously described method (Uğur, 2023) with a Nexera model Shimadzu HPLC connected to a tandem MS device (Shimadzu, Kyoto, Japan). MS detection using a Shimadzu LCMS 8040 triple quadrupole mass spectrometer equipped with an ESI source. LC-ESIMS/MS data were collected and processed by LabSolutions software (Shimadzu, Kyoto, Japan). Multiple reaction monitoring (MRM) modes were used to quantify the analytes and Phytochemicals.

Radical scavenging activities

Colorimetric $DPPH^{\bullet}$ free radical removing activities of *A. lithophila* extracts were carried out according to the Blois method (Blois, 1958). $ABTS^{\bullet+}$ cation scavenging ability was performed according to the method described by Re *et al*. (Re *et al.*, 1999). The radical scavenging effect (%) = $[(A_{control}-A_{sample})/A_{control}] \times 100$

Reducing ability Activities

Iron ions (Fe^{3+}) reduction analysis of *A. lithophila* extracts were performed with a modified version of the FRAP method (Oyaizu, 1986; Elmastaş *et al.*, 2006; Güzel, 2023). Cu^{2+}- Cu^{+} reducing ability of *A. lithophila* extracts were detected by CUPRAC assays as reported by Apak *et al*. (2008).

Enzyme inhibition effects

Inhibitory effects of water and methanol extracts of the *A. lithophila* were tested against AChE and hPON 1 enzymes. Ellman's method was used to evaluate the acetylcholinesterase inhibitory activities (Ellman *et al.*, 1961). In addition, the protocol reported by Güzel was used to demonstrate paraoxonase inhibitory activities (Güzel, 2023).

STATISTICAL ANALYSIS

The experimental procedure was carried out in triplicate. Data were recorded and analyzed as mean ± standard deviation. Experimental results were performed by one-way analysis of variance ANOVA. Duncan's multiple range tests were used to determine differences between means. $P<0.05$ was regarded as significant.

RESULTS

LC-MS/MS technique, which has high selectivity and sensitivity, was chosen for screening phytochemicals in *A. lithophila* methanol extract. LOD, LOQ, linear range and R^2 were determined for the phenolic compounds used as standards (Table 1, Figure 1). There is no research on the phenolic compounds of the *A. lithophila*. However, in this study, the LOD and LOQ of 25 phenolic compounds were determined by LC-MS/MS. Ellagic acid 25643.84±9.76 mg/kg, catechin hydrate 8572.77±12.35, gallic acid 3751.76±11.14 mg/kg, fumaric acid 2848.01±14.12 mg/kg, luteolin 771.01±17.84 mg/kg, quercetin 744.82±8.62 mg/kg, kaempferol 509.62±11.54 mg/kg, acetohydroxamic acid 140.56±6.25 mg/kg, caffeic acid 126.46±2.14 mg/kg, syringic acid 64.66±4.24 mg/kg, hydroxybenzoic acid 6.41±3.17 mg/kg and salicylic acid 6.04±2.35 mg/kg from phenolic compounds were quantified in the methanol extract of *A. lithophila*. In Table 2, the calculated phenolic compounds were shown along with their respective standard deviation values. Ellagic acid was determined as the dominant component in the phenolic compounds.

Free radical scavenging ($DPPH^{\bullet}$), cation radical scavenging ($ABTS^{\bullet+}$), cupric reducing (CUPRAC) and ferric reducing (FRAP) tests were carried out for the antioxidant activities of extracts (methanol, water, and *n*-hexane) of the *A. lithophila*. DPPH and ABTS results were given as percent radical scavenging activity, while CUPRAC and FRAP results were given as absorbance readings. According to the results of the $DPPH^{\bullet}$ and FRAP, the water extract demonstrated the highest activity. However, methanol extract was found to be high in the $ABTS^{\bullet+}$ test and *n*-hexane extract was found to be high in the CUPRAC test. The results of antioxidant studies compared to standards (BHA, BHT, and Trolox) were presented in Table 3 with standard deviation values.

A. lithophila extracts were used to investigate their effects on the activity of AChE and hPON 1. The methanol extract of *A. Lithophila* exhibited notable inhibition against the AChE. (Table 4, Figure 2). In contrast, the hPON 1 enzyme wasn't inhibited by *A. lithophila* extracts.

DISCUSSION

Free radicals can cause Alzheimer's, atherosclerosis, cancer, diabetes, aging, and other degenerative diseases. Enzymatic and non-enzymatic antioxidants react and neutralize free radicals. There are antioxidant mechanisms that try to maintain the oxidative balance in the body. Dietary antioxidants must be taken to support these mechanisms. Dietary antioxidants are phenolic compounds, a broad member of secondary metabolites (Nimse and Pal, 2015; Al-Mamary and Moussa, 2021). Phytochemicals have bioactivities such as anti-AChE, antioxidant, anti-microbial, anti-diabetic, anti-mutagenic, anti-inflammatory and anti-carcinogenic (Owen *et al.*, 2000; Uğur and Güzel, 2023). Ellagic acid has potential pharmacological effects. Its properties include antioxidant, anti-inflammatory, neuroprotective, hepatoprotective, antidiabetic, antimalarial, anti-atheroma and anti-carcinogenic (Ríos *et al.*, 2018). In a previous study conducted by Ibrahim *et al*., HPLC analyzes of *Alchemilla vulgaris* methanolic root extract showed ellagic acid 12100 mg/kg (Ibrahim *et al.*, 2022). On the contrary, *A. lithophila* phenolic compound showed the highest value of ellagic acid 25643.84±9.76 mg/kg in LC-MS/MS analysis. Catechin hydrate has a potential therapeutic activity in the prevention and treatment of oxidative damage-induced diseases such as atherosclerosis, cancer and neurodegenerative (Kaur *et al*., 2017). While catechin hydrate was found to be 260 mg/kg in the HPLC analysis of the *Menta pulegium* extract (40 mL 62.5% aqueous methanol+10 mL of 6 M HCl), it was measured as 8572.77±12.35 mg/kg in the *A. lithophila* (Proestos *et al.*, 2005). Catechin hydrate, which is rare in plants, was found in *A. lithophila* at a remarkable rate. According to research gallic acid, which has pro-oxidant properties, has strong antioxidant, anticholinergic, anti-inflammatory, antimutagenic and anticancer properties (Cho *et al.*, 2011; Verma *et al.*, 2013). In previous a research carried out by Vlaisavljević *et al*., gallic acid was found in the ethyl acetate extract of *Alchemilla vulgaris* as 2465.79 mg/kg extract in LC-MS/MS analysis, while it was 3751.76±11.14 mg/kg in *A. lithophila* (Vlaisavljević *et al.*, 2019). Fumaric acid is widely used in the treatment of skin diseases such as psoriasis, sarcoidosis, granuloma, necrobiosis lipoidica and malignant melanoma. Also, it has neuroprotective, antioxidant, immunomodulatory and anti-inflammatory effects (Kaur *et al*., 2020). In LC-MS/MS analysis, fumaric acid was found to be 452.78 mg/kg in the ethanol extract of sumac (*Rhus coriaria*), while it was 2848.01±14.12 mg/kg in *A. lithophila* (Tohma *et al.*, 2019). In recent studies, luteolin has been proven to be antioxidant, anti-cancer, anti-prolative, anti-inflammatory, anti-atherosclerotic and anti-allergic (Seelinger *et al.*, 2008; Kwon, 2017). In a previous work carried out by Vlaisavljević *et al*., luteolin was measured as 23.15 mg/kg in the methanol extract of *Alchemilla vulgaris* while it was 771.01±17.84 mg/kg in *A. lithophila* (Vlaisavljević *et al.*, 2019). According to research, quercetin, a member of the flavonoids, has anti-inflammatory, anti-carcinogenic and antiviral effects (Khan *et al.*, 2019). In a previous research conducted by Vlaisavljević *et al*., quercetin was found in the ethyl acetate extract of *Alchemilla vulgaris* as 4541.70 in LC-MS/MS analysis, while in the present study was 744.82±8.62 mg/kg in *A. lithophila* (Vlaisavljević *et al.*, 2019). In recent studies, kaempferol, an aglycone flavonoid, has antioxidant, anti-inflammatory, neuroprotective, antidiabetic, cardioprotective, antimicrobial, antitumor and anticancer bioactivities (M Calderon-Montano *et al.*, 2011). Kaempferol was measured as 364.00 mg/kg by LC/MS/MS in *Alchemilla vulgaris* ethyl acetate extract (Vlaisavljević *et al.*, 2019), while it was measured as 509.62±11.54 mg/kg in *A. lithophila.* In recent studies, acetohydroxamic acid has uses in the biomedical sector such as anti-urease, antioxidant, anti-inflammation (Michaelidou *et al.*, 2007). Acetohydroxamic acid was calculated spectrophotometrically in the methanol extract of *C. niveum* as 344.40 mg/kg mg/kg in LC-MS/MS analysis (Güzel, 2023), while it was 140.56±6.25 mg/kg in *A. lithophila*. In recent studies, caffeic acid, has antioxidant (Gülçin, 2006), anti-inflammatory, antiviral, anti-atherosclerotic and anticancer properties (Huang *et al.*, 2013). In a previous work carried out by Vlaisavljević *et al*., caffeic acid was found in the ethanol extract of *Alchemilla vulgaris* as 69.19 mg/kg in LC-MS/MS analysis (Vlaisavljević *et al.*, 2019), while in present work was 126.46±2.14 mg/kg in *A. lithophila.*

Free radicals cause oxidative stress by increasing oxidation in the cell. Oxidative stress, which causes loss of cellular function, leads to diseases such as Alzheimer's, Parkinson's, cancer, diabetes, cardiovascular and rheumatoid arthritis (Halliwell and Gutteridge, 1990; Valko *et al.*, 2007).

Table 1: Analytical parameters for phenolic compounds by LC-MS/MS

Compounds	Retention time (min)	Precursor ion *(m/z)*	Product ion *(m/z)*	LOD (µg/L)	LOQ (µg/L)	Linear regression	Linear range (µg/L)	r^2	Repeatability (n = 6)	
									Means	RSD %
Acetohydroxamic acid	0.406	76.15	58	6.90	23.01	y = 216.91x + 6165.8	20-750	0.9989	489.34	1.79
Catechin hydrate	2.532	291	139.1	2.05	6.84	y = 1717.9x - 563.99	10-750	0.9988	485.19	1.19
Vanillic acid	2.762	168.95	65	84.78	282.61	y = 48.343x + 662.5	250-1000	0.9993	496.07	5.94
Syringic acid	3.001	199.1	140.1	2.88	9.61	y = 112.03x + 1316.1	10-500	0.9994	483.07	2.91
Resveratrol	3.606	229	135	41.83	139.43	y = 733.34x - 69955	250-1000	0.999	486.42	2.19
Fumaric acid	0.809	115.2	71.1	7.91	26.38	y = 100.91x – 1701.62	40-750	0.9989	499.19	4.02
Gallic acid	1.278	169.1	124.9	3.92	13.06	y = 305.07x - 1859.3	10-100	0.9981	471.32	6.98
Caffeic acid	2.836	179	135	2.87	9.58	y = 1227.2x - 5396.5	10-100	0.9948	474.22	3.69
Phloridzin dihydrate	3.594	435.1	273.1	81.80	272.67	y = 120.23x - 9479.5	250-1000	0.9989	500.19	4.32
Oleuropein	3.567	539.1	377	7.17	23.90	y = 324.26x - 5388.8	40-750	0.9997	496.52	5.26
Protocatechuic acid	3.556	181	108	2.76	9.20	y = 1382.2x - 4393.1	10-500	0.9967	479.92	4.31
Salicylic acid	3.558	137.2	93	22.88	76.25	y = 3838.2x - 149277	75-1000	0.9977	520.39	6.81
Ellagic acid	3.681	301.1	228.9	23.74	79.14	y = 18.841x + 911.46	100-1000	0.9967	502.25	3.45
Myricetin	3.644	317	179.1	4.34	14.45	y = 588.4x - 4990.6	20-500	0.9987	492.54	3.39
2-Hydroxy-1. 4-naphthoquinone	3.664	173.1	145	2.07	6.91	y = 461.45x - 4553.8	10-500	0.9989	540.11	10.83
hydroxybenzoic acid	3.555	137.2	93.1	8.92	29.74	y = 3831.2x - 94423	40-500	0.9996	477.66	4.75
Silymarin	3.996	481.1	453.1	8.00	26.70	y = 199.91x + 950.97	40-750	0.9997	478.05	3.41
Quercetin	3.891	301.1	150.9	7.79	25.98	y = 150.09x - 422.87	20-500	0.9997	487.60	2.99
Naringenin	3.952	271	150.9	68.40	228.10	y = 700.8x - 26469	250-1000	0.9997	481.52	2.73
Butein	3.935	271	134.9	38.50	128.20	y = 62.943x - 2793	100-1000	0.996	492.32	3.25
Luteolin	4.069	285	150.9	6.40	21.40	y = 1389x - 40923	40-1000	0.9988	491.54	2.73
Kaempferol	4.298	285	117	3.90	13.00	y = 62.513x - 821.08	20-1000	0.9982	491.68	3.31
Alizarin	4.594	239	211	15.30	51.10	y = 26.512x - 1721	60-2000	0.9991	512.49	8.30
Curcumin	4.672	367.1	216.9	12.80	42.70	y = 1908.9x - 8252.1	40-1000	0.9994	509.57	4.95
Thymoquinone	3.337	165	137	7.64	25.47	y = 349.23x - 2887.4	20-500	0.9971	482.18	2.71

Table 2: Results of phenolic compounds in *A. lithophila* by LC-MS/MS

Compounds	Means±sd mg/kg	Compounds	Means±sd mg/kg
Acetohydroxamic acid	140.56±6.25	Myricetin	< LOQ
Catechin hydrate	8572.77±12.35	2-Hydroxy-1.4-naphthoquinone	< LOQ
Vanillic acid	ND	4-hydroxybenzoic acid	6.41±3.17
Syringic acid	64.66±4.24	Silymarin	< LOQ
Resveratrol	< LOQ	Quercetin	744.82±8.62
Fumaric acid	2848.01±14.12	Naringenin	< LOQ
Gallic acid	3751.76±11.14	Butein	< LOQ
Caffeic acid	126.46±2.14	Luteolin	771.01±17.84
Phloridzin dihydrate	< LOQ	Kaempferol	509.62±11.54
Oleuropein	< LOQ	Alizarin	< LOQ
Protocatechuic acid	< LOQ	Curcumin	< LOQ
Salicylic acid	6.04±2.35	Thymoquinone	< LOQ
Ellagic acid	25643.84±9.76		

ND: These values are below the limits of the quantification and not determine compound screening of *A. lithophila*.

A healthy life can be maintained by using nutritional antioxidants to keep oxidative stress in balance in metabolism (Owen *et al.*, 2000). In this sense, the DPPH is a method used to predict free radical-induced oxidative cell damage of biomolecules in metabolism. The activity of antioxidants to neutralize free radicals can be determined by this assay (Necip *et al.*, 2021). At the 0.2 mg/mL concentration, *A. lithophila* of water and methanol extracts showed efficient DPPH scavenging activity as following orders: Trolox (85.35% ±3.12) > BHA (76.22% ±3.62) > Water extracts (75.38% ±1.21) >Methanol extract (52.73% ±0.98) > BHT (43.40% ±3.26) >Hexane extract (10.51% ±0.24). In a previous study, Renda *et al.* (2017) reported that the water extract of *Alchemilla barbatiflora* showed 83.06% $DPPH^{\bullet}$ radical scavenging activity while it showed 75% activity in this study. $ABTS^{\bullet+}$ radical cation scavenging activity is measured spectrophotometrically to calculate the total amount of antioxidant capacity.

Table 3: Radical scavenging and metal-reducing activity of *A. lithophila* extracts

	DPPH$^{\bullet}$ (0.2 mg/mL)	ABTS$^{\bullet+}$ (0.2 mg/mL)	FRAP (0.2 mg/mL)	CUPRAC (0.2 mg/mL)
	%[a]		Absorbance[b]	
Water extract	75.38±1.21	61.83±2.13	0.796± 2.66	1.570±2.26
Methanol extract	52.73±0.98	70.67±1.24	0.450± 1.27	1.303±1.95
Hexane extract	10.51±0.24	10.56±1.02	0,074± 0.86	2.845±2.24
BHA	76.22±3.62	93.65±4.71	1.625±0.38	2.002±0.85
BHT	43.40±3.26	58.21±2.66	1.034±0.23	2.287±1.06
TROLOX	85.35±3.12	90.03±3.07	0.928±0.26	2.025±0.98

BHA: butylated hydroxyanisole; BHT: butylated hydroxytoluene.
[a]The percent (%) of ABTS$^{\bullet+}$ and DPPH$^{\bullet}$ radical scavenging activity.
[b]The values were expressed as absorbance. High absorbance indicates high metal ion reducing activity (Liu *et al.*, 2017).

Table 4: Inhibitory effect of *A. lithophila* extracts on AChE and hPON 1

	Inhibition against AChE		Inhibition against hPON1
	IC_{50}(mg/mL)	r^2	
Methanol extract	0.162± 0.25	0.992	NI
Water extract	NI	-	NI
Tacrine[a]	0.125± 0.18	0.981	-

[a] Tacrine was used as positive control for AChE.
NI: No inhibition.

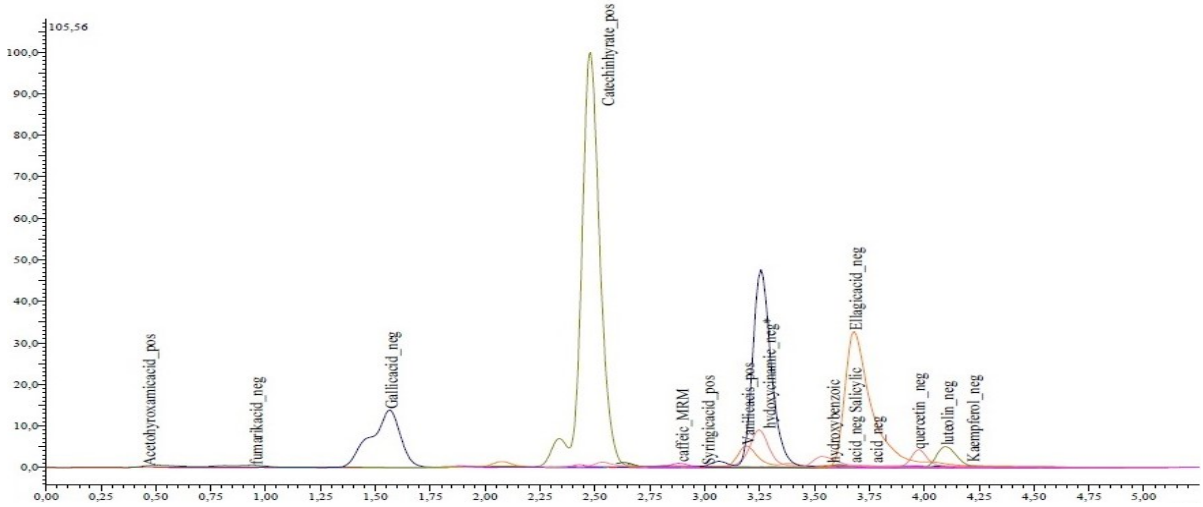

Fig. 1: LC-MS/MS chromatograms of *A. lithophila*

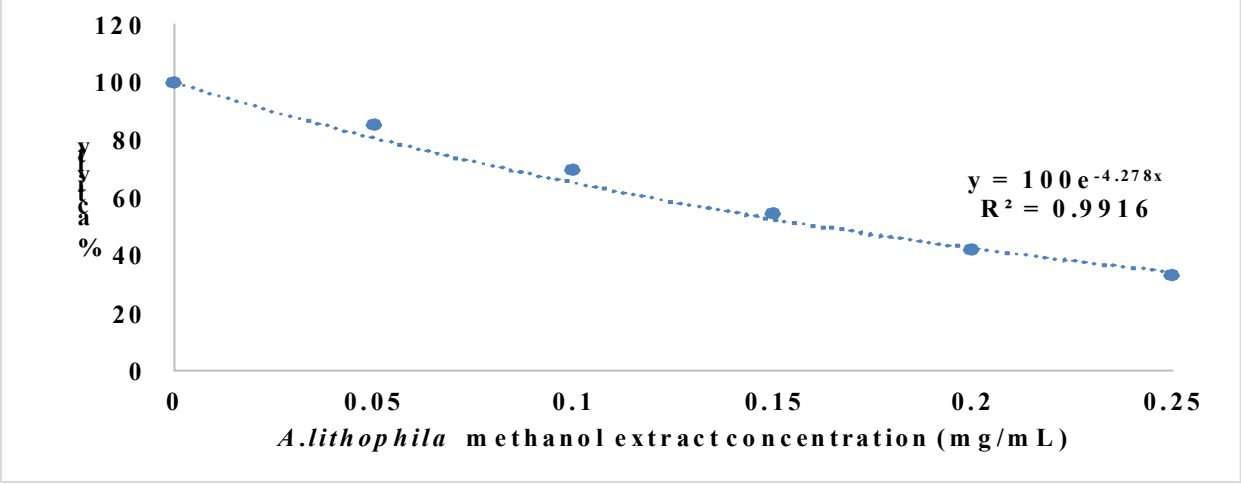

Fig. 2: Inhibition curve on AChE Activity of *A. lithophila* methanol extract

The blue/green $ABTS^{\bullet+}$ chromophore, a sturdy framework for antioxidant reactions, is formed from the reaction of $ABTS^{\bullet+}$ and potassium persulfate (Re *et al.*, 1999; Güzel, 2023). $ABTS^{\bullet+}$ scavenging activity as following orders: BHA (93.65% ±4.71) > Trolox (90.03% ±3.07) > Methanol extracts (70.67% ±1.24) > Water extract (61.83% ±2.13) > BHT (58.21% ±2.66) >Hexane extract (10.56% ±1.02). The $ABTS^{\bullet+}$ value is reflected by a percentage, where a higher percentage indicates a higher $ABTS^{\bullet+}$ value (Table 3). In a similar study, Vlaisavljević *et al*. (2019) found that high phenolic-containing *Alchemilla vulgaris* extracts inhibited $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radicals significantly.

In the present study, Fe^{3+}/ferricyanide complex is reduced to the Fe^{+2} form by antioxidants. The reaction produces Prussian blue which can be measured at 700 nm (Meir *et al.*, 1995; Güzel, 2023). Absorbance for FRAP reducing activity was found as 1.625 for BHA, 1.034 for BHT, 0.928 for Trolox, 0.796 for water extract, 0.450 for methanol extract and 0.074 for *n*-hexane extract at the concentration of 0.2 mg/mL. CUPRAC is a highly preferred method for determining total antioxidants. This method is based on the reduction of Cu(II) to Cu(I) (Apak *et al.*, 2008). Absorbance for CUPRAC reducing activity was found as 2.845 for *n*-hexane extract, 2.287 for BHT, 2.025 for Trolox, 2.002 for BHA, 1.570 for water extract and 1.303 for methanol extract at the concentration of 0.2 mg/mL (Table 2). In a previous study, *Alchemilla vulgaris* extracts has a significant metal-reducing capacity. These results show that *Alchemilla* genus have radical scavenging and metal reduction potential. Polyphenols are crucial compounds present in medicinal plants that exhibit antioxidant properties. The hydroxyl and methoxyl groups in polyphenols determine the antioxidant activity (Al-Mamary and Moussa, 2021).

Alzheimer's disease drugs are “symptomatic” agents that aim to improve cognitive and behavioral symptoms without changing the underlying course of the disease. One of these treatment approaches is the cholinergic hypothesis. The cholinergic hypothesis is to increase the transmitter acetylcholine at synapses by inhibiting acetylcholinesterase. It has been observed that there are some improvements in the mental abilities of patients, although they may not be complete (Cummings, 2021). Drugs with cholinergic effects, such as neuroprotective agent donepezil, may have adverse reactions. The quest for medications that do not come with any adverse effects is becoming more prevalent (Braak and Del Tredici, 2011; Uğur and Güzel, 2023). In recent years, there has been a growing interest in researching medicinal plants with cholinergic properties that don't have any adverse effects. In a previous research, Barut *et al*. (Barut *et al.*, 2017) reported that the methanol extract of *Achillea millefolium* exhibited AChE inhibition with an IC_{50} value of 0.105 mg/mL compared to galantamine (IC_{50} value: 0.017 mg/mL). The inhibition results were shown in Table 4 and Figure 1. The IC_{50} value of A. lithophila methanol extract on AChE was found to be 0.162 mg/mL (r^2: 0.992). The IC_{50} value of tacrine as positive control was found to be 0.125mg/mL. Compared to tacrine, this inhibition value resulting from phenolic compounds is pharmacologically significant.

The paraoxonase is an essential enzymatic antioxidant against acute and chronic diseases associated with cellular oxidative damage. Paraoxanase, a significant component of HDL, neutralizes the toxic products resulting from the oxidation of LDL. Considering the effects of phytochemicals on the enzyme, inhibition or activation of the enzyme will affect homeostasis within the body. Oxidation of lipoproteins such as HDL and LDL will promote atheroma plaques and form atherosclerotic lesions. It will also cause serious diseases such as diabetes and hypercholesterolemia. Also, the paraoxonase enzyme protects against organophosphate exposure, bacterial endotoxins and toxicity of lipopolysaccharides (La Du *et al.*, 1999). No inhibition was determined in the treatment of the *A. lithophila* with paraoxonase. In this sense, the use of *A. lithophila* as a drug will not pose a risk for cardiovascular disease.

CONCLUSION

Modern phytotherapy practices suggest standardization of extraction procedures and chemical profiles of medicinal plants with pharmaceutical potential. In this context, quantitative and qualitative determination of the phytochemical contents of the *A. lithophila* was made for the first time. Among these phenolic compounds, ellagic acid, catechin hydrate, gallic acid, fumaric acid, luteolin, quercetin and kaempferol are the main components in *A. lithophila*. Besides, *A. lithophila* extracts were evaluated for the first time for their antioxidant capacity, anti-acetylcholinesterase activity and antiatherosclerosis-related enzyme. The results revealed that they have both radical-scavenging and metal-chelating abilities. Also, the *A. lithophila* methanol extract showed potent inhibition on the AChE. *A. lithophila* may be promising for Alzheimer's disease in the regulation of iron in amyloid plaques and neurofibrils and the increase of acetylcholine in synapses. In addition, *A. lithophila* is vascular-friendly as it doesn’t inhibit paraoxonase, which has antioxidant and anti-inflammatory properties.

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