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\textsubscript{50} value for methanol extract 0.162 ± 0.25 mg /mL, R\textsuperscript{2}:0.992). Besides, antioxidant activities of the *A. lithophila* extracts were examined using by the methods ABTS\textsuperscript{•+} and DPPH\textsuperscript{•} free radical scavenging potentials, FRAP and CUPRAC metal-reducing activities. ABTS\textsuperscript{•+} and DPPH\textsuperscript{•} 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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*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⁺ and DPPH) 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' free radical removing activities of *A. lithophila* extracts were carried out according to the Blois method (Blois, 1958). ABTS⁺ cation scavenging ability was performed according to the method described by Re et al. (Re et al., 1999). The radical scavenging effect (%) = ([Acontrol-A sample]/Acontrol] × 100

**Reducing ability Activities**
Iron ions (Fe³⁺) reduction analysis of *A. lithophila* extracts were performed with a modified version of the FRAP method (Oyaizu, 1986; Elmastas et al., 2006; Güzel, 2023). Cu²⁺ - 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 R² 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 3751.76±11.14 mg/kg, fumaric acid 2848.01±14.12 mg/kg, luteolin 771.01±17.84 mg/kg, quercetin 3751.76±11.14 mg/kg, fumaric acid 2848.01±14.12 mg/kg, luteolin 771.01±17.84 mg/kg, quercetin 3751.76±11.14 mg/kg, fumaric acid 2848.01±14.12 mg/kg, luteolin 771.01±17.84 mg/kg, quercetin 3751.76±11.14 mg/kg, fumaric acid 2848.01±14.12 mg/kg, luteolin 771.01±17.84 mg/kg, quercetin 3751.76±11.14 mg/kg, fumaric acid 2848.01±14.12 mg/kg, luteolin 771.01±17.84 mg/kg, quercetin 3751.76±11.14 mg/kg, fumaric acid 2848.01±14.12 mg/kg, luteolin 771.01±17.84 mg/kg, quercetin 3751.76±11.14 mg/kg, fumaric acid 2848.01±14.12 mg/kg, luteolin 771.01±17.84 mg/kg, quercetin 3751.76±11.14 mg/kg, fumaric acid 2848.01±14.12 mg/kg, luteolin 771.01±17.84 mg/kg, quercetin 3751.76±11.14 mg/kg.
Free radical scavenging (DPPH), cation radical scavenging (ABTS+), 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 and FRAP, the water extract demonstrated the highest activity. However, methanol extract was found to be high in the ABTS+ 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, anti-diabetic, anti-malarial, 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, anti-diabetic, 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, acetylated acetic acid has uses in the biomedical sector such as anti-urease, antioxidant, anti-inflammation (Michaelidou et al., 2007). Acetylated acetic acid was calculated spectrophotometrically in the methanol extract of *C. niveum* as 344.40 mg/kg mg/kg in LC-MS/MS analysis (Gülçin, 2006), while it was 140.56±6.25 mg/kg in *A. lithophila*. In recent studies, caffeine, 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., caffeine was found in the methanol 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).
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 method is 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% ±0.24) > 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 radical scavenging activity while it showed 75% activity in this study. ABTS+ radical cation scavenging activity measured spectrophotometrically to calculate the total amount of antioxidant capacity.
Table 3: Radical scavenging and metal-reducing activity of *A. lithophila* extracts

<table>
<thead>
<tr>
<th></th>
<th>DPPH (0.2 mg/mL)</th>
<th>ABTS (0.2 mg/mL)</th>
<th>FRAP (0.2 mg/mL)</th>
<th>CUPRAC (0.2 mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%a</td>
<td>%b</td>
<td>Absorbanceb</td>
<td></td>
</tr>
<tr>
<td>Water extract</td>
<td>75.38±1.21</td>
<td>61.83±2.13</td>
<td>0.796±2.66</td>
<td>1.570±2.26</td>
</tr>
<tr>
<td>Methanol extract</td>
<td>52.73±0.98</td>
<td>70.67±1.24</td>
<td>0.450±1.27</td>
<td>1.303±1.95</td>
</tr>
<tr>
<td>Hexane extract</td>
<td>10.51±0.24</td>
<td>10.56±1.02</td>
<td>0.074±0.86</td>
<td>2.845±2.24</td>
</tr>
<tr>
<td>BHA</td>
<td>76.22±3.62</td>
<td>93.65±4.71</td>
<td>1.625±0.38</td>
<td>2.002±0.85</td>
</tr>
<tr>
<td>BHT</td>
<td>43.40±3.26</td>
<td>58.21±2.66</td>
<td>1.034±0.23</td>
<td>2.287±1.06</td>
</tr>
<tr>
<td>TROLOX</td>
<td>85.35±3.12</td>
<td>90.03±3.07</td>
<td>0.928±0.26</td>
<td>2.025±0.98</td>
</tr>
</tbody>
</table>

BHA: butylated hydroxyanisole; BHT: butylated hydroxytoluene.

aThe percent (%) of ABTS\(^{•+}\) and DPPH\(^{•}\) radical scavenging activity.

bThe 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 hPON1

<table>
<thead>
<tr>
<th></th>
<th>Inhibition against AChE</th>
<th>Inhibition against hPON1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC\textsubscript{50} (mg/mL)</td>
<td>r\textsuperscript{2}</td>
</tr>
<tr>
<td>Methanol extract</td>
<td>0.162±0.25</td>
<td>0.992</td>
</tr>
<tr>
<td>Water extract</td>
<td>NI</td>
<td>-</td>
</tr>
<tr>
<td>Tacrine\textsuperscript{a}</td>
<td>0.125±0.18</td>
<td>0.981</td>
</tr>
</tbody>
</table>

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$^•+$ chromophore, a sturdy framework for antioxidant reactions, is formed from the reaction of ABTS$^•+$ and potassium persulfate (Re et al., 1999; Güzel, 2023). ABTS$^•+$ 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$^•+$ value is reflected by a percentage, where a higher percentage indicates a higher ABTS$^•+$ value (Table 3). In a similar study, Vlaisavljević et al. (Vlaisavljević et al., 2019) found that high phenolic-containing Alchemilla vulgaris extracts inhibited ABTS$^•+$ and DPPH$^•+$ 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. Paraoxonase, 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 phyotherapy 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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REFERENCES
Ibrahim OHM, Abou-Elyousr KAM, Asiry KA, Alhakamy NA and Mousa MAA (2022). Phytochemical characterization, antimicrobial activity and in vitro antiproliferative potential of Alchemilla vulgaris Auct root extract against prostate (PC-3), breast (MCF-7) and colorectal adenocarcinoma (Caco-2) cancer cell lines. Plants, 11(16): 2140.


