The effect of *Sideritis* species on Alzheimer’s disease: *In vitro* evaluation

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**ABSTRACT:** Alzheimer’s Disease (AD), which is quite prevalent in our society, not only makes the lives of patients and their families more challenging but also brings significant economic burdens. *Sideritis* species are widely used in folk medicine due to their various effects such as anti-inflammatory, antimicrobial, diuretic, and antispasmodic properties. Although there are many studies with the hypothesis that ROS is effective in the pathogenesis of neurodegenerative diseases, there are not enough studies on *Sideritis germanicopolitana* Borrn. subsp. *viridis* Hausskn. ex Borrn. (SGV) and *Sideritis libanotica* Labill. subsp. *linearis* (Bentham) Borrn. (SLL) species. In this study, the antioxidant activities of SGV and SLL plant extracts were compared using DPPH, FRAP, and CUPRAC methods to aid in AD treatment research. It was determined that SGV extract has higher DPPH (0.023±0.005 mg/mL), FRAP (1.074± 0.180 mM FeSO4/mg extract) and CUPRAC (2.988± 0.041 mM trolox equivalent/mg extract) activity potential than SLL extract. Additionally, as a result of cytotoxicity studies performed on SH-SY5Y neuroblastoma cells, ethanol extracts of SGV may be more suitable for use in AD at higher concentrations (250 μg/mL).

**KEYWORDS:** *Sideritis germanicopolitana* *viridis*; *Sideritis libanotica linears*; Alzheimer; antioxidant activity; anticholinesterase activity

1. INTRODUCTION

Alzheimer’s disease (AD) is the leading cause of cognitive impairment and dementia in individuals aged 65 and older, presenting a growing global challenge due to increased longevity. Despite extensive research efforts spanning several decades, effective disease-modifying treatments for the cognitive decline associated with AD have remained elusive. Current therapeutic investigations have predominantly focused on the histopathological hallmarks of AD, namely amyloid plaques and neurofibrillary tangles, and their association with familial mutations in the amyloid-β peptide (Aβ) and tau proteins. However, AD is fundamentally characterized by the misfolding and aggregation of soluble proteins, leading to neuronal dysfunction and loss. Alois Alzheimer’s seminal discovery of plaques and tangles in the brain of a dementia patient paved the way for subsequent research into the role of neuritic Aβ plaques, contributing to our understanding of this neurodegenerative protein-conformational disease [1-3].

While there is currently no definitive cure for AD, there are available medications and treatments aimed at managing its symptoms. These drugs primarily target cholinergic or glutamatergic neurotransmission, providing relief by improving cognition and reducing the social and economic burden associated with AD. Acetylcholinesterase inhibitors, including donepezil, rivastigmine, and galantamine, are approved for mild to moderate AD and enhance cholinergic transmission. Additionally, the NMDA receptor antagonist memantine, approved for moderate to severe AD, reduces excitotoxicity resulting from excessive glutamatergic transmission. These existing drug treatments for AD are symptomatic-based, aiming to mitigate cognitive, behavioral, and psychological symptoms of dementia. They are administered orally or transdermally and offer a limited curative effect [4, 5].

Reactive oxygen species (ROS) are metabolic by products that have important physiological functions but can be harmful in excessive amounts. The gradual increase in ROS levels over time leads to oxidative...
stress, which can impair mitochondrial function and cause damage to various tissues, including the central nervous system. Accumulated oxidative stress has been found to contribute significantly to cognitive aging and the development of neurodegenerative diseases, such as Alzheimer’s disease (AD). This suggests that oxidative stress plays a key role in the pathogenesis of AD and age-related cognitive decline. Additionally, there is a strong connection between oxidative stress and neurodegenerative diseases and aging, leading to the development of neuroprotective therapies aimed at reducing ROS levels and protecting neurons from damage. Understanding the mechanisms through which ROS contributes to neurodegeneration and cell death is crucial for the development of targeted treatments [6, 7].

Medicinal and aromatic plants have a long history of traditional use and are increasingly recognized for their potential in modern medicine. These plants serve as valuable resources for the development of new drugs. While scientific research has contributed to our understanding through publications, there is still a wealth of knowledge held by indigenous communities in remote areas [8]. The growing body of scientific research highlights the remarkable capacity of compounds originating from natural sources to provide robust protection against the detrimental impact of ROS, demonstrating their pivotal role in safeguarding cellular health and mitigating oxidative stress-related damage like AD [9]. Genus Sideritis, belonging to the Lamiaceae family, consists of over 150 species widely distributed in temperate and tropical regions of the Northern Hemisphere. It is subdivided into two subgenera: Sideritis and Marrubiastrum. The southeastern part of Europe and the Eastern Mediterranean region are centers of diversity for Sideritis, particularly in the section Empedoclia, with around 50 species [8,10]. Sideritis species have a long history of traditional use as teas, sweeteners, and for therapeutic purposes. In folk medicine, decoctions and infusions of the plant’s aerial parts are applied orally or topically due to their wide range of beneficial properties, including anti-inflammatory, antimicrobial, anti-ulcerative, diuretic, antispasmodic, antioxidant, analgesic, anticonvulsant, antifungal, and degassing effects. These properties make Sideritis valuable in treating various conditions such as inflammation, the common cold, asthma, bronchitis, and gastrointestinal disorders [11, 12]. Furthermore, recent studies have shown that these plants are also effective in the treatment of diseases such as Type 2 Diabetes Mellitus, Parkinson’s disease, and AD [13-15]. In studies of Sideritis species on AD, it has been understood that this effect is due to the high antioxidant activity and neuroprotective potential they have. When the contents of Sideritis species were evaluated, it was seen that they had main phytochemical contents such as phenolic compounds, flavonoids, tannins and monoterpenoids [16]. Experimental evidence supports the idea that some flavonoids, such as luteolin [17], rosmarinic acid [18], and apigenin [19], may potentially protect against AD. In this study, the antioxidant properties of Sideritis germanicopolitana Bornm. subsp. viridis Hausskn. ex Bornm. (SGV) and Sideritis libanotica Labill. subsp. linearis (Bentham) Bornm. (SLL) species and their cytotoxic effects on the SH-SY5Y cell line have been investigated.

2. RESULTS AND DISCUSSION
2.1. In vitro bioactivity assays

2.1.1. 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging assay

The free radical scavenging activities of extracts obtained from the samples were determined using the DPPH method. The DPPH radical method involves spectrophotometrically measuring changes in concentration resulting from the interaction between the radical and an antioxidant. This reaction occurs as the unpaired electron of the radical is neutralized by a hydrogen atom donated by the antioxidant, allowing assessment of the tested compounds’ antioxidant properties through reaction kinetics [20]. The antioxidant activities of the extracts and the standard ascorbic acid were evaluated by comparing their IC50 values. The results of the activity are displayed in Table 1. According to the findings, the SGV plant showed stronger DPPH radical scavenging activity. However, both extracts showed lower radical scavenging activity than ascorbic acid. On the other hand, these results are significantly higher when compared with Sideritis species, which are known to show low antioxidant activity in the literature [21,22].
Table 1. The DPPH radical scavenging activities of SLL and SGV extracts

<table>
<thead>
<tr>
<th>Extracts</th>
<th>DPPH (IC_{50} mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SGV</td>
<td>0.023±0.005</td>
</tr>
<tr>
<td>SLL</td>
<td>0.070±0.004</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>0.004±0.001</td>
</tr>
</tbody>
</table>

2.1.2. Ferric reducing antioxidant power (FRAP) assay

Numerous investigations have demonstrated a correlation between the antioxidant activity of bioactive compounds and their electron donation capacity, which reflects their ability to reduce. The FRAP assay, an electron transfer-based technique, evaluates the reduction of ferric ions (Fe^{3+})–ligand complex to blue ferrous ions (Fe^{2+}) by antioxidants within acidic environments [23]. The reduction is monitored at 593 nm using a spectrophotometer, and results are expressed as micromolar Fe^{2+} equivalents or with a standard antioxidant. In this study, when comparing the obtained FRAP values, it was observed that the SGV plant exhibited a stronger iron (III) ion reduction potential, while both plants showed lower FRAP values than the BHA compound (Table 2). Similar results for Sideritis albiflora and Sideritis leptoclada by FRAP assays have been reported [24].

Table 2. The FRAP values of SLL and SGV extracts

<table>
<thead>
<tr>
<th>Extracts</th>
<th>FRAP (mM FeSO_{4}/mg extract)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SGV</td>
<td>1.074±0.180</td>
</tr>
<tr>
<td>SLL</td>
<td>0.620±0.141</td>
</tr>
<tr>
<td>BHA</td>
<td>5.81 ± 0.004</td>
</tr>
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</table>

2.1.3. Cupric ion reducing antioxidant capacity (CUPRAC) assay

The copper (II) ion-reducing antioxidant capacities of SGV and SLL extracts were evaluated, and the results are presented in Table 3. According to the findings of this study, it was determined that the SGV extract has a stronger potential to reduce Cu(II) to Cu(I) compared to the SLL extract. However, both extracts showed lower reducing power than the ascorbic acid. Our results support previous findings [25].

Table 3. The CUPRAC values of SLL and SGV extracts

<table>
<thead>
<tr>
<th>Extracts</th>
<th>CUPRAC (mM trolox equivalent/mg extract)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SGV</td>
<td>2.988±0.041</td>
</tr>
<tr>
<td>SLL</td>
<td>2.746±0.091</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>5.683±0.337</td>
</tr>
</tbody>
</table>

2.1.4. Inhibition potential of anticholinesterase

One of the significant therapy methods for AD is the inhibition of acetylcholinesterase (AChE), which leads to the breakdown of acetylcholine (ACh) in the brain. Galantamine, an alkaloid derived from plants, acts as an AChE inhibitor and is an approved drug for the treatment of AD [26]. Therefore, the development of novel agents or medications for AD treatment from natural sources holds significant importance. The AChE enzyme inhibition percentages of SGV and SLL extracts were investigated using the Ellman method. The results are displayed in Figure 1. Against AChE, the EtOH extracts of SGV and SLL exhibited moderate
inhibitory activity. When evaluating the extracts against each other, it was determined that the SLL extract exhibited a higher enzyme inhibition value compared to the SGV extract. Despite both plants possessing inhibitory properties, the extracts demonstrated lower AChE activity than the reference compound galantamine. These results are similar to those published by other authors for *Sideritis stricta* [27] and *Sideritis brevibracteata* [28].

![Figure 1](image1.png)

**Figure 1.** Anticholinesterase activity of SLL and SGV extracts.

2.2. **Cell viability assessment**

Cells were treated with the indicated concentrations of SLL extract for 24 h. Cell viability was determined by MTT assay.

![Figure 2](image2.png)

**Figure 2.** Effects of SLL extract on the viability of SH-SY5Y neuroblastoma cells. Error bars represent the mean ±S.E.M. Values of a ≤ 0.0001 vs. Control, b ≤ 0.05 vs. 500 µg/mL, c ≤ 0.0001 vs. 500 µg/mL, d ≤ 0.0001 vs. 250 µg/mL, e ≤ 0.0001 vs. 125 µg/mL treated group were considered significant.

Cells were treated with the indicated concentrations of SGV extract for 24 h. Cell viability was determined by MTT assay.
To determine the non-toxic concentrations of the extracts on SH-SY5Y cells, an MTT assay was conducted (Figure 2 and Figure 3). The indicated concentrations of the extracts were added to the wells and incubated at 37°C for 24 h. EtOH extracts of SLL and SGV species were applied to the cells at 31.75, 62.5, 125, 250, and 500 µg/mL concentrations. The viability of SLL on the SH-SY5Y was 64.70±3.3%, 66.16±1.1%, 76.25±2.6%, 99.02±4.2% and 101.55±4.1% respectively. The viability of SGV on the SH-SY5Y was 81.21±3.2%, 92.5±0.8%, 96.35±0.7%, 98.43±1.6% and 98.60±2.2% respectively. SLL, when applied at concentrations higher than 62.5 µg/mL, resulted in more than 60% decrease in the viability of SH-SY5Y, but SGV did not show any toxic effect on cells up to 250 µg/mL concentration. Therefore, SGV can be used to evaluate the protective effect at higher concentrations. Chalatsa et al. and Ververis et al. found the maximum non-toxic concentration of around 400 µg/mL on SH-SY5Y cells of *Sideritis scardica* [16, 29]. SGV extract may be more appropriate for use in neuroprotective studies at higher concentrations.

3. CONCLUSION

In this study, antioxidant, anticholinesterase, and cytotoxic properties of EtOH extracts obtained from SGV and SLL plants were evaluated. According to the analysis results, it was determined that both plants (SLL has better than SGV) have acetylcholinesterase enzyme inhibition potential. In addition, it was determined that both species (SGV has better than SLL) showed significant antioxidant activity. Furthermore, non-cytotoxic doses of SGV and SLL in SH-SY5Y neuroblastoma cells increased cell proliferation. Although SGV and SLL have a potential anti-Alzheimer’s effect, further *in vitro* and *in vivo* studies are needed in detail to prove the anti-Alzheimer’s effect of SGV and SLL.

4. MATERIALS AND METHODS

4.1. Plant material and preparation of extracts

The aerial parts of SGV were collected in Kastamonu, 2022, in the Black Sea region of Turkey. The aerial parts of SLL were collected in Kahramanmaras, 2022, in the Mediterranean region of Turkey. Both plants were collected during the flowering stage. Plant materials were identified by Dr. Ismail Senkardes and stored in the Herbarium of the Faculty of Pharmacy, Marmara University (MARE Numbers: 22881, 19155).

The aerial parts of SGV and SLL were dried in the shade at a temperature of 25°C. Once dried, the plant materials were ground into a fine powder of appropriate weight using a mechanical grinder (Renas, RBT1250). The powdered dry aerial parts were macerated using a mixture of EtOH-distilled H2O (7:3, v/v) solvent. After the maceration process, the liquid part was filtered through filter paper and extracts were obtained using a lyophilizer (Scanvac, CoolSafe) device. The extracts were kept in the refrigerator at 4°C until the day of the study.
4.2. In vitro bioactivity assays

4.2.1. 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging assay

From the extracts prepared at concentrations of 0.5-5 mg/mL, 10 µL of each was taken and added to 240 µL of 0.1 mM 2,2-diphenyl-1-picryl-hydrazyl (DPPH) solution. The mixture was vortexed for a min and kept in darkness at room temperature for 30 min. The absorbance was measured against the reference at 517 nm using a microplate reader (Epoch, BioTek). The same procedure was carried out for standard solutions of ascorbic acid. The % DPPH radical scavenging activity was calculated using the formula:

\[
\% \text{ DPPH radical inhibition} = \left(\frac{A0 - A1}{A0}\right) \times 100
\]

A0: The absorbance of the control solution
A1: The absorbance of plant extracts and standard solutions

The IC₅₀ value, which is the extract/standard concentration causing a 50% reduction in the DPPH radical concentration, was calculated by placing the % radical scavenging activity against the concentrations studied. The IC₅₀ value was expressed as mg/mL. The investigation was performed three times, and the averages of the data and standard deviation were calculated [30].

4.2.2. Ferric reducing antioxidant power (FRAP) assay

The ferric reducing antioxidant power (FRAP) method was performed by preparing a FRAP reagent consisting of 25 mL of 300 mM acetate buffer (pH 3.6), 2.5 mL of 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) solution, and 2.5 mL of 20 mM FeCl₃ · 6H₂O, which was then incubated at 37°C for 30 min. Subsequently, 10 µL of the extract was added to 190 µL of the FRAP reagent, and the mixture was incubated for 4 min at room temperature. The absorbance of the resulting solution was measured at 593 nm, and the increase in absorbance compared to a reference solution containing only distilled water was recorded. The FRAP value of the extract was determined by referencing a calibration chart prepared using a stock solution of FeSO₄·7H₂O with concentrations of 0.05 mM, 0.1 mM, 0.2 mM, and 0.4 mM. The calibration equation for Fe²⁺ was obtained as:

\[
A = 4.585x + 0.0541 \quad (R² = 0.9926)
\]

The FRAP values of the extracts were expressed as mM Fe²⁺/mg extract and compared to butylated hydroxyanisole (BHA), which was used as a standard [31].

4.2.3. Cupric ion reducing antioxidant capacity (CUPRAC) assay

To prepare the cupric ion reducing antioxidant capacity (CUPRAC) reagent, 60 µL each of copper(II) solution, neocuproine solution, and 1 M ammonium acetate buffer were mixed together. Then, 60 µL of the extract and 10 µL of ethanol were added to the mixture, and the resulting solution was shaken and kept at room temperature with the lid closed for 60 min. After this incubation period, the absorbance of the solution was measured at 450 nm against a reference solution that did not contain the sample. Trolox was used as a standard, and a stock solution of 1 mM Trolox was prepared to generate a Trolox standard curve. Working solutions with concentrations of 1 mM, 0.8 mM, 0.6 mM, 0.4 mM, 0.2 mM, and 0.1 mM were prepared by diluting the stock solution with ethanol. Trolox solutions with different concentrations were also evaluated with the CUPRAC method. A calibration curve was prepared by plotting concentrations against absorbance, and the equation for the curve was determined to be:

\[
A = 5.281x + 0.05055 \quad (R² = 0.9935)
\]

The CUPRAC values of the extracts were expressed as mM Trolox/mg extract and were compared with ascorbic acid, which was used as a standard [32].

4.2.4. Anticholinesterase activity assay

The inhibition activity of acetylcholinesterase (AChE) was determined using a microplate reader (Epoch, BioTek), following the method described by Ellman et al. [33] with some modifications. Briefly, 50 mM Tris-HCl buffer at pH 8.0 was used to prepare all reagent solutions daily. AChE solution and each sample were mixed in a concentration of 20 µL with 40 µL of Tris-HCl buffer and incubated at 25°C for 10 min. Then, 20 µL of acetylthiocholine iodide (ATCI) substrate (50 mM) was added to the mixture and incubated for 5 min at room temperature. After that, 100 µL of 20 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) containing 1 M NaCl and 0.2 M MgCl₂ · 6H₂O was added to the combination, and the absorbance was read at 412 nm against a reference. The same procedure was applied to the galanthamine used as a standard. Each experiment was conducted in triplicate, and the results were expressed as a percentage of enzyme inhibition relative to the control.
4.3. In vitro cell culture

The human neuroblastoma cell line (SH-SY5Y™, ATCC) was incubated in a Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) penicillin/streptomycin (P/S) at 37°C, 5% CO₂, and 95% relative humidity. During passaging, cells were washed with phosphate-buffered saline (PBS) and incubated with trypsin-EDTA to facilitate their detachment in the cell culture flask. To remove cells from trypsin, centrifugation was performed at 1200 rpm for 5 min. The pellet was resuspended by pipetting in fresh medium. After cell counting, the cells were transferred to T75 cell culture flasks at an appropriate dilution and passaged every 3 days [34].

4.3.1. Cell viability assessment

When the cells reached approximately 80% confluence on the culture surface, they were washed with PBS and then detached using 0.25% trypsin-EDTA solution. Subsequently, the cells were seeded into 96-well plates (Corning) at a density of 1x10⁴ cells per well. Plant extracts were prepared at six different doses. After 24 h of incubation, cells with sufficient density were treated with the respective doses of extracts in triplicate wells, followed by further incubation for 24 h. At the end of these incubation periods, cell viability analysis based on the measurement of total metabolic activity was performed using the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay. The 96-well plates were incubated for 3 h at 37°C, 5% CO₂, and 95% relative humidity. Following the incubation, the medium was aspirated from the cells, and 100 µL of dimethyl sulfoxide (DMSO) was added to dissolve the formed formazan crystals. Subsequently, the plates were incubated on a shaker for 10 min, and then the percentage of viable cells was determined using a multi-plate reader (ELISA reader, BioTek Epoch) at 570 nm [35]. Cell viability was calculated using the formula:

\[
\text{Cell Viability (\%)} = \left( \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}} \right) \times 100\% 
\]

4.4. Statistical analysis

The statistical analyses of the data obtained from the measurements were conducted using GraphPad Prism 9 statistical software. The analysis results were presented as mean ± standard deviation (SD). Intergroup comparisons were performed using one-way and two-way analysis of variance (ANOVA) and Bonferroni post hoc test (\( p < 0.05 \)).

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Evaluation

The Effect of Sideritis Species on Alzheimer's Disease: In Vitro


