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RESEARCH ARTICLE

Phytochemical Profile, Antioxidant, Anticholinesterase, and Antiproliferative Activities of Endemic *Onosma discedens*: In Vitro and In Silico Approaches

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ABSTRACT

This study presents the first comprehensive analysis of the phytochemical profile and biological activities of root and stalk extracts from endemic *Onosma discedens* Hausskn. ex Bornm. (OD). The extracts of OD were analyzed by liquid chromatography–electrospray ionization–tandem mass spectrometry (LC–ESI–MS/MS), and 16 phenolics, including vanillic acid (2077.1332 μg/g extract in root) and hesperidin (1185.3621 μg/g extract in stalk), were detected. In antioxidant activity tests, DPPH• radical scavenging activity of root extract (IC_{50}: 60.69 μg/mL) was found to be higher than stalk extract (IC_{50}: 95.66 μg/mL), but both extracts showed lower activity than standard antioxidants. In antiproliferative activity assays, the OD extract exhibited low cytotoxicity against MCF7 cell lines, with an IC_{50} value exceeding 500 μg/mL. The stem extract was more effective on the butyrylcholinesterase (BChE) enzyme (IC_{50}: 546.09 ± 0.533 μg/mL) than on the acetylcholinesterase (AChE) enzyme (IC_{50}: 721.156 ± 0.410 μg/mL). When the plant is evaluated as a whole, it has a dual inhibitory effect on both cholinesterase enzymes. Molecular docking analyses confirmed the interactions of vanillic acid and hesperidin with target enzymes. As a result, it was revealed that OD, which has a rich structure in phenolic compounds and significant bioactivity due to its cholinesterase inhibitory effect, can be utilized to treat neurodegenerative illnesses like Alzheimer's.

1 | Introduction

Natural products are considered one of the main sources of pharmaceutical research due to their wide chemical diversity and biological activities. It is known that alkaloids, flavonoids, terpenoids, and other phenolic compounds, which are among the phytochemical compounds, exhibit acetylcholinesterase (AChE) inhibitory activities [1]. These molecules are not only limited to neurodegenerative diseases but also attract attention with their antioxidant, anti-inflammatory, anticancer, and antibacterial properties. Bioactive compounds of plant origin are increasingly gaining attention in pharmaceutical research in order to avoid

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FIGURE 1 | *Onosma discedens* Hausskn. ex Bornm.

the side effects of synthetic drugs and to develop safer treatment alternatives [2].

Alzheimer's disease (AD) is a neurological illness that causes a gradual loss of memory and cognitive abilities and is becoming a growing global public health problem with the aging population. The multifactorial etiology and complex pathophysiology of AD make effective treatment of the disease significantly difficult. Currently, the pharmacological agents used in the treatment of AD are limited, and the effectiveness of existing drugs is aimed at alleviating symptoms rather than stopping the progression of the disease [3]. For this purpose, AChE inhibitors have been recognized as an essential target in the treatment of AD due to their ability to increase cholinergic transmission. The inhibitory effects of natural compounds on this enzyme have great potential in new drug development studies [4].

The Boraginaceae family includes 2700 plant species known for their phytochemical diversity and medicinal potential. These plants exist in the botanical forms of herbs, shrubs, and trees that grow in arid lands [5]. The *Onosma* genus, represented by 230 species in this family, has been used in traditional medicine for many years in the treatment of various disorders. Studies on *Onosma* species have shown that these plants have antioxidant, anticancer, anti-inflammatory, and antibacterial activities. More than 200 phytochemicals have been identified in this genus [6]. Various studies investigating the phenolic composition and pharmacological effects of some *Onosma* species growing in Türkiye have been reported in the literature [7–12]. However, data on some endemic *Onosma* species are quite limited.

Onosma discedens Hausskn. ex Bornm. (OD) is an endemic *Onosma* species distributed in the Erzincan region of Türkiye (Figure 1), and the current scientific information about its phytochemical and biological potential is quite limited [13, 14]. The pharmacological properties of this plant, which is used for various purposes in traditional medicine, have not yet been investigated in detail. The understanding of OD's phenolic compound profile and biological activity is critical for both scientific literature contribution and the discovery of new bioactive compounds for pharmaceutical applications.

In this study, the antioxidant activities and enzyme inhibition potentials of extracts derived from the root and stalk parts of OD were investigated comprehensively. ABTS•$^{+}$ and DPPH• free radical scavenging activities, ferric reducing antioxidant power (FRAP), and total phenolic and flavonoid contents of the extracts were determined. In addition, the inhibitory effects of OD extracts on AChE and butyrylcholinesterase (BChE) enzymes were evaluated, and their antiproliferative potential against breast cancer cell lines was investigated. In addition, phenolic compounds found in OD extracts were analyzed by liquid chromatography–electrospray ionization–tandem mass spectrometry (LC–ESI–MS/MS) method, and the interactions of the main active compounds with target proteins were investigated by molecular docking analyses.

The main purpose of this first study investigating the biological properties of OD is to comprehensively evaluate the bioactive compounds and pharmacological potential of OD and to contribute to the scientific literature, especially in terms of antioxidant and enzyme inhibitory activities. The findings will provide important information that can guide future pharmaceutical and biomedical research by revealing the potential of OD in traditional and modern medicine.

2 | Results and Discussion

2.1 | Phytochemical Investigation of OD by LC–ESI–MS/MS

Identification and quantitative analysis of phenolic compounds of hydromethanolic (70:30, v/v) root and stalk extracts of OD were carried out using a previously developed and validated LC–MS/MS method [15]. With this analytical method, both extracts were evaluated for a total of 45 bioactive phytochemical compounds. Retention times and amounts (μg/g extract) of the identified phenolic compounds are presented in Table 1. In addition, LC–MS/MS total ion chromatograms of the root and stalk extracts of the OD are shown in Figure 2, respectively.

According to the LC–MS/MS analysis results, a total of 16 phenolic compounds were detected in the root and stalk extracts of OD. Among these compounds, protocatechuic acid, chlorogenic acid, hydroxybenzaldehyde, caffeic acid, caffeine, vanillin, *o*-coumaric acid, and hesperidin were determined as common in both extracts. Vanillic acid (2077.13 ± 2.32 μg/g extract) was found in very high amounts in the root parts of OD and was determined as the major compound. In addition to this compound, caffeic acid (72.15 ± 2.10 μg/g extract), protocatechuic acid (21.07 ± 1.65 μg/g extract), and rutin (11.27 ± 0.34 μg/g extract) were detected. The stalk parts of OD had a significant level of hesperidin (1185.36 ± 3.25 μg/g extract). The extract included phenolic compounds such as caffeic acid (46.57 ± 1.36 μg/g extract), isoquercitrin (40.94 ± 1.22 μg/g extract), and protocatechuic acid (25.45 ± 0.72 μg/g extract). On the other hand, epigallocatechin, vanillic acid, rutin, and capsaicin were detected only in the

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TABLE 1 | The levels of the phenolic compounds in methanol:water (70:30, v/v) extracts of root and stalk parts of OD (μg/g extract).

No.	Compound	RT (min)	Root	Stalk	No.	Compound	RT (min)	Root	Stalk
1	Shikimic acid	1.289	n.d.	n.d.	24	Hesperidin	11.898	3.75 ± 0.11^{e}	1185.36 ± 3.25^{a}
2	Gallic acid	3.299	n.d.	n.d.	25	Isoquercitrin	11.930	n.d.	40.94 ± 1.22^{b}
3	Protocatechuic acid	5.522	21.07 ± 1.65^{c}	25.45 ± 0.72^{c}	26	Rutin	12.735	11.27 ± 0.34^{d}	n.d.
4	Epigallocatechin	6.823	0.97 ± 0.05^{g}	n.d.	27	Quarcetin-3-xyloside	12.488	n.d.	n.d.
5	Catechin	6.935	n.d.	n.d.	28	Kaempferol-3-glucoside	13.274	n.d.	10.50 ± 0.31^{d}
6	Chlorogenic acid	7.435	4.40 ± 0.12^{e}	3.69 ± 0.10^{f}	29	Fisetin	13.268	n.d.	1.87 ± 0.06^{h}
7	Hydroxybenzaldehyde	7.736	1.65 ± 0.07^{f}	2.72 ± 0.08^{g}	30	Baicalin	13.611	n.d.	n.d.
8	Vanillic acid	7.844	2077.13 ± 2.32^{a}	n.d.	31	Chrysin	14.255	n.d.	n.d.
9	Caffeic acid	7.898	72.15 ± 2.10^{b}	46.57 ± 1.36^{b}	32	Daidzein	14.196	n.d.	n.d.
10	Syringic acid	8.461	n.d.	n.d.	33	*Trans*-cinnamic acid	14.340	n.d.	1.24 ± 0.04^{i}
11	Caffeine	8.443	0.17 ± 0.01^{h}	0.16 ± 0.01^{j}	34	Quercetin	14.872	n.d.	n.d.
12	Vanillin	8.694	3.88 ± 0.11^{e}	4.56 ± 0.13^{f}	35	Naringenin	15.050	n.d.	n.d.
13	*o*-coumaric acid	9.503	3.03 ± 0.09^{e}	7.90 ± 0.24^{e}	36	Silibinin	15.682	n.d.	n.d.
14	Salicylic acid	9.824	n.d.	n.d.	37	Hesperetin	15.840	n.d.	n.d.
15	Taxifolin	9.702	n.d.	n.d.	38	Morin	15.904	n.d.	n.d.
16	Resveratrol	9.835	n.d.	n.d.	39	Kaempferol	16.456	n.d.	n.d.
17	Polydatin	9.834	n.d.	n.d.	40	Baicalein	17.025	n.d.	n.d.
18	*Trans*-ferulic acid	10.198	n.d.	n.d.	41	Luteolin	18.212	n.d.	n.d.
19	Sinapic acid	10.447	n.d.	n.d.	42	Biochanin A	17.893	n.d.	n.d.
20	Scutellarin	11.179	n.d.	n.d.	43	Capsaicin	18.152	0.06 ± 0.005^{i}	n.d.
21	*p*-Coumaric acid	11.513	n.d.	n.d.	44	Dihydrocapsaicin	18.657	n.d.	n.d.
22	Coumarin	11.189	n.d.	n.d.	45	Diosgenin	23.373	n.d.	n.d.
23	Protocatechuic ethyl ester	11.661	n.d.	n.d.					

Note: Different letters (a–j) in the same column indicate statistical difference ($p < 0.05$).
Abbreviation: n.d., not detected.

root extract; isoquercitrin, kaempferol-3-glucoside, fisetin, and *trans*-cinnamic acid were detected only in the stalk extract (Table 1). Statistical analysis revealed significant differences in the distribution and concentrations of individual compounds between the root and stalk extracts ($p < 0.05$). Phenolic acids, such as vanillic and caffeic acids, were present at higher levels in the root, whereas flavonoid glycosides, such as hesperidin and isoquercitrin, were characteristic of the stalk. These variations suggest that the distinct phenolic composition of different plant parts may account for the variation observed in their antioxidant activities.

Vanillic acid (4-hydroxy-3-methoxybenzoic acid) is a phenolic compound structurally derived from benzoic acid. Numerous scientific studies have revealed that vanillic acid has a broad spectrum of pharmacological activities, including anti-venom, anti-inflammatory, anticholinesterase, antimicrobial, cardioprotective, hepatoprotective, free radical scavenging, and antioxidant properties [16]. It has also been reported to exhibit chemopreventive potential in various cancer types such as lung cancer, prostate cancer, hepatocellular carcinoma, melanoma, and endometrial carcinoma [17]. In the literature, the vanillic acid content in species belonging to the genus *Onosma* has been quantitatively analyzed, and the vanillic acid content of *O. aucheriana* (1466.63 ± 52.18 μg/g extract), *O. frutescens* (485.14 ± 18.91 μg/g extract), and *O. sericea* (963.48 ± 102.12 μg/g extract) [18]. Similarly, high concentrations were detected in ethyl acetate (60.20 ± 2.67 μg/g extract), methanol (281.01 ± 8.49 μg/g extract), and water (277.38 ± 3.67 μg/g extract) extracts of *Onosma pulchra* and in extracts of *Onosma inexspectata* (1094 ± 53 μg/g extract) and *O. armenium* (594 ± 37 μg/g extract) [19, 20]. On the other hand, very low concentrations were detected in *Onosma gigantea* (0.106 ng/mL), *Onosma isaurica* (0.131 ng/mL), *O*nosma *sieheana* (0.223 ng/mL), and *Onosma taurica* Willd. var. *brevifolia* (0.140 ng/mL). In addition, the vanillic acid was not found in *Onosma haussknechtii* [12]. As a result of these comprehensive analyses, it was observed that the OD root extract (2077.1332 μg/g extract) had a significantly higher vanillic acid content

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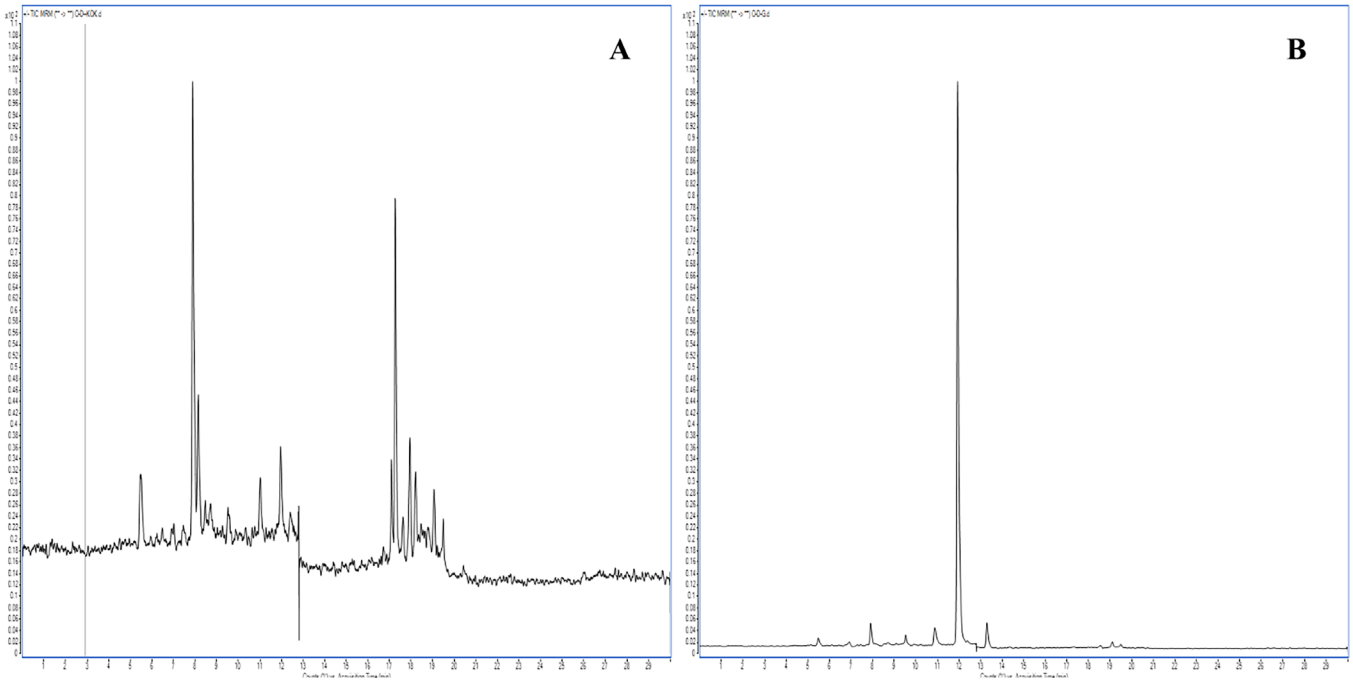


FIGURE 2 | TIC chromatograms of the (A) root and (B) stalk extracts of OD.

compared to all other species belonging to the genus *Onosma*. This finding suggests that this extract has significant potential for pharmacological and therapeutic applications.

Hesperidin (hesperetin 7-rutinoside) is a flavonone glycoside commonly found in citrus fruits belonging to the Rutaceae family, primarily lemon, lime, tangerine, and orange. Hesperidin is effective in reducing oxidative stress due to its strong antioxidant properties and has a wide spectrum of pharmacological effects, including anti-inflammatory, antimicrobial, anticarcinogenic, antithrombotic, and antiviral activities. It is particularly notable for its neuroprotective and cardioprotective effects and is an important focus in pharmacological research due to its therapeutic potential [21]. Quantitative analyses on the phenolic profiles of *Onosma* species growing in Türkiye revealed high hesperidin concentrations, especially in species such as *O. aucheriana* (4543.83 ± 32.53 μg/g extract), *O. frutescens* (14312.54 ± 48.69 μg/g extract), and *O. sericea* (91.66 ± 21.64 μg/g extract) [18]. However, significant hesperidin content was detected in species such as the methanolic extract of *O. pulchra* (3038.24 ± 62.33 μg/g extract) and *O. armenium* (248.000 ± 200 μg/g extract) [19, 20] and *O. taurica* var. *brevifolia* (1269 ng/mL), and it was completely absent in *O. gigantea*, *O. isaurica*, *O. sieheana*, and *O. haussknechtii* [12]. Quantitative analysis revealed that OD stalk extract (1185.36 ± 3.25 μg/g extract) contained higher hesperidin concentration than *O. sericea*, *O. armenium*, *O. gigantea*, *O. isaurica*, *O. sieheana*, *O. haussknechtii*, and the ethyl acetate and water extracts of *O. pulchra*. However, the amount of hesperidin in the stalk extract of OD was significantly lower than in species with high hesperidin accumulation capacity, such as *O. aucheriana*, *O. frutescens*, and *O. pulchra* (methanolic extract). These results indicate that the stalk extract of OD can be characterized by a moderate hesperidin content, but some *Onosma* species are much richer sources of this flavonoid. These findings indicate that hesperidin accumulation varies significantly in the *Onosma* genus depending on the species, the climate in which it grows, and the extraction method, and that some species may be a rich source of this valuable flavonoid.

Protocatechuic acid, caffeic acid, and rutin have been reported to have antioxidant, cholinergic enzymes, immunomodulatory, antidiabetic, antiproliferative, and anticancer activities in previous studies [22–24]. LC–MS/MS analyses confirmed the existence of these compounds in OD extracts, which were not detected in other species of the *Onosma* genus even at low concentrations [9, 12, 18–20, 25, 26]. Detailed characterization of root and stalk parts of OD revealed the presence of phenolics such as epigallocatechin, chlorogenic acid, hydroxybenzaldehyde, and vanillin, as well as trace amounts of secondary metabolites such as *o*-coumaric acid, fisetin, *trans*-cinnamic acid, capsaicin, and kaempferol-3-glucoside. These findings indicate the chemical profile of OD extracts differs from other *Onosma* species and constitutes an important reference in evaluating the pharmacological potential of the species.

2.2 | Biological Activities

2.2.1 | Antioxidant Activity

The antioxidant properties of OD extracts were evaluated using DPPH radical scavenging, FRAP reducing power, and by determining their total phenolic content (TPC) and total flavonoid content (TFC). As presented in Table 2, the stalk extract exhibited stronger radical scavenging capacity (60.69 ± 1.82 μg/mL) compared to the root extract (95.66 ± 2.85 μg/mL). However, both extracts exhibited substantially higher IC_{50} values than standard antioxidants such as Trolox (8.76 ± 0.77 μg/mL), butylated hydroxy anisole (BHA) (8.08 ± 0.69 μg/mL), butylated hydroxytoluene (BHT) (10.83 ± 0.73 μg/mL), and ascorbic acid

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TABLE 2 | Antioxidant, total phenolic content (TPC), and total flavonoid content (TFC) results of the stalk and root extracts of OD.

Samples	DPPH IC_{50} (μg/mL)	Total phenolics (mg GAE/g extract)	Total flavonoids (mg QE/g extract)	Reducing power (mg TE/g extract)
Stalk	60.69 ± 1.82^{b}	94.04 ± 0.97^{a}	10.19 ± 0.92^{b}	85.78 ± 0.47^{d}
Root	95.66 ± 2.85^{a}	27.45 ± 0.34^{b}	18.67 ± 1.08^{a}	48.07 ± 0.20^{e}
Trolox	8.76 ± 0.77^{d}	—	—	—
BHA	8.08 ± 0.69^{d}	—	—	338.57 ± 0.31^{b}
BHT	10.83 ± 0.73^{c}	—	—	257.80 ± 1.24^{c}
Ascorbic acid	9.82 ± 0.8^{e}	—	—	394.17 ± 0.98^{a}

Note: Different letters (a–e) in the same column indicate statistical difference ($p < 0.05$).
Abbreviations: BHA, butylated hydroxy anisole; BHT, butylated hydroxytoluene.

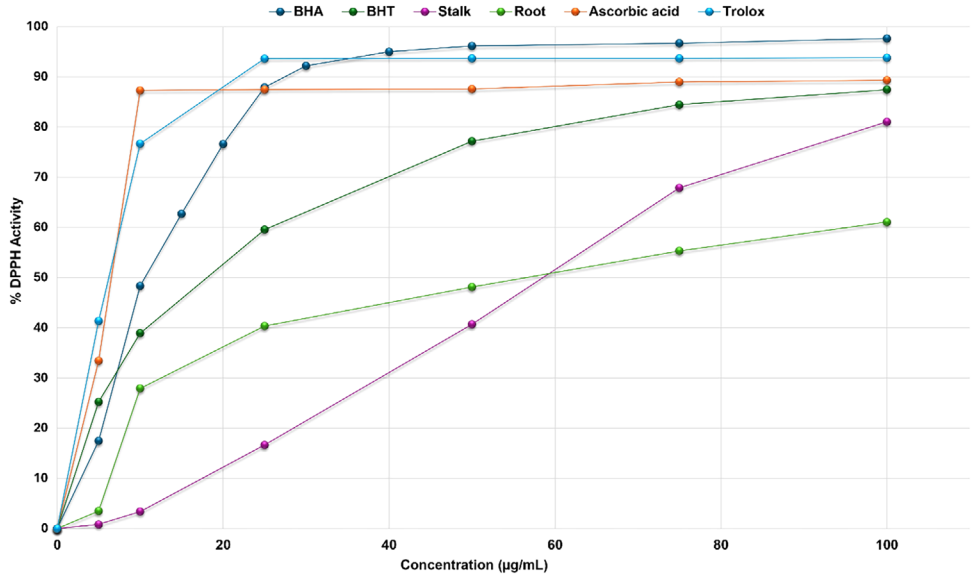


FIGURE 3 | DPPH% values of standards, the stalk, and root extracts of OD. BHA, butylated hydroxy anisole; BHT, butylated hydroxytoluene.

(9.82 ± 0.87 μg/mL), indicating lower radical scavenging potency relative to synthetic and reference antioxidants (Figure 3). Statistical analysis revealed significant differences among all groups ($p < 0.05$); the stalk extract showed significantly higher activity than the root extract, whereas both extracts exhibited significantly lower activity compared to the reference standards.

According to the FRAP test, stalk extract (85.78 ± 0.47 mg Trolox equivalent [TE]/g extract) showed higher capacity compared to root extract (48.07 ± 0.20 mg TE/g extract) (Figure 4). However, both extracts were found to have much lower activity than standard antioxidants such as BHA (338.57 ± 0.31 mg TE/g extract), BHT (257.80 ± 1.24 mg TE/g extract), and ascorbic acid (394.17 ± 0.98 mg TE/g extract). Statistical analysis indicated that all samples were significantly different from each other ($p < 0.05$), with the highest activity observed in ascorbic acid, followed by BHA, BHT, stalk extract, and root extract, respectively.

In terms of the TPC (Figure 4), the stalk extract (94.04 ± 0.97 mg GAE/g extract) was about 3.5-fold higher than the root extract (27.45 ± 0.34 mg GAE/g extract). In contrast, the TFC was higher in the root extract (18.67 ± 1.08 mg QE/g extract) compared to the stalk extract (10.19 ± 0.92 mg QE/g extract). Statistical analysis confirmed significant differences among all groups ($p < 0.05$), indicating that phenolic compounds were higher in the stalk, whereas flavonoids were higher in the root.

The antioxidant capacity of OD was consistent with values previously reported for the genus, with the stalk (60.69 ± 1.82 μg/mL) and root (95.66 ± 2.85 μg/mL) extracts exhibiting moderate radical scavenging activity. These values are comparable to the ethanol extract of *Onosma halophila* ($IC_{50} \approx 45$ μg/mL) [27] and considerably higher than the activities reported for *Onosma lycaonica* and *Onosma papillosa*, with IC_{50} values between 2.69 and 3.41 mg/mL [28]. Similarly, the aerial parts of *O. frutescens* and *O. sericea* exhibited IC_{50} values of 1.1–1.7 mg/mL [6], which indicate lower activity compared with OD. In contrast, the aqueous root extract of *O. aucheriana* exhibited significantly higher scavenging activity ($IC_{50} \approx 9$–10 μg/mL) than both extracts of OD [6]. These comparisons indicate that although OD does not match the very high antioxidant capacity of *O. aucheriana*, it shows stronger radical scavenging potential than several other *Onosma* species.

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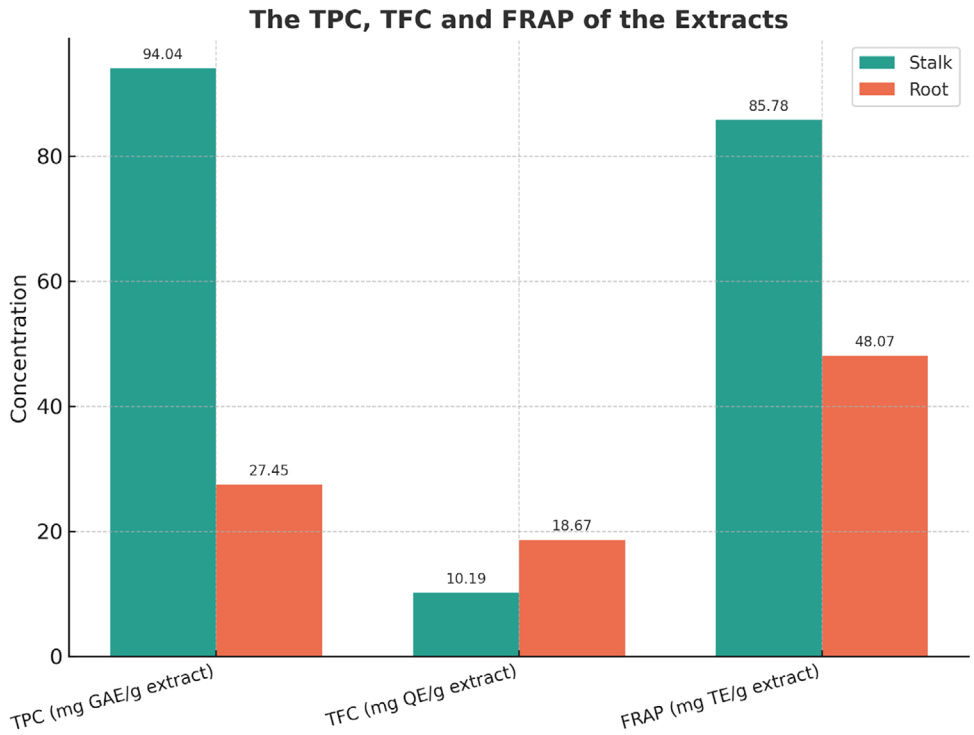


FIGURE 4 | TPC (mg GAE/g extract), TFC (mg QE/g extract), and FRAP (mg TE/g extract) values of OD stalk and root extracts. FRAP, ferric reducing antioxidant power; TFC, total flavonoid content; TPC, total phenolic content.

In the FRAP assay, both stalk and root extracts of OD exhibited lower reducing power compared to standard antioxidants such as BHA, BHT, and ascorbic acid, which are characterized by stronger electron-donating capacity. The reducing capacities of OD were comparable to values reported for several endemic *Onosma* species. *Onosma ambigens* showed FRAP activity of 0.52 mg TE/mL [6], whereas *Onosma mutabilis* had values of about 1.12 mg TE/mL [6], both of which are in a similar range to our findings. In contrast, species, such as *O. lycaonica* and *O. papillosa*, showed stronger reducing capacities, with FRAP EC_{50} values of 0.69 and 0.88 mg/mL, respectively [28], whereas *Onosma riedliana* demonstrated high antioxidant potential attributed to its abundant phenolic constituents [29]. These interspecific differences may be attributed to qualitative and quantitative variations in phenolic and flavonoid composition, as phenolic hydroxyl groups are key contributors to ferric ion reduction. The relatively higher FRAP activity of the stalk compared to the root of OD may reflect its higher phenolic content, consistent with the well-established correlation between phenolic concentration and reducing capacity in *Onosma* species.

In terms of phenolic and flavonoid composition, *O. discedens* showed higher TPC in the stalk extract (94.04 ± 0.97 mg GAE/g) compared to the root extract (27.45 ± 0.34 mg GAE/g), whereas the TFC was higher in the root (18.67 ± 1.08 mg QE/g) than in the stalk (10.19 ± 0.92 mg QE/g). These results are consistent with the variability observed among other *Onosma* species. *O. mutabilis* was reported to contain a TPC of 37.24 mg GAE/g and a TFC of 26.78 mg QE/g [30], values lower in phenolic but higher in flavonoid content compared to the root extract of OD. In contrast, *O. riedliana* demonstrates phenolic content of 97.62 ± 0.20 mg GAE/g, comparable to the stalk extract of OD; its flavonoid levels are also higher [29]. In addition, *Onosma chlorotricum* extracts exhibited TPC values of 56.10–74.12 mg GAE/g and TFC values of 19.3–23.2 mg QE/g [31], which are comparable to those of OD roots but lower than the stalk in terms of phenolic content. Comparative analysis of three Greek *Onosma* spp. revealed one species with high TPC (~69.03 mg GAE/g) and another with elevated TFC (~45.80 mg RE/g), indicating interspecific metabolic variation within the genus [29, 32]. These findings indicate that OD is a species with moderate-to-high phenolic content—in particular in the stalk—and moderate flavonoid levels, supporting the view that organ-specific metabolite distribution contributes to antioxidant potential in *Onosma* species.

2.2.2 | Antiproliferative Activity

The antiproliferative activity of OD extracts was evaluated using the MTT assay on human breast cancer cells (MCF-7). Both root and stalk extracts were tested; however, the root extract showed no activity at the tested concentrations, and no IC_{50} value was calculated. For this reason, it was not included in the graphical presentation. In contrast, the stalk extract exhibited a measurable cytotoxic effect, with an IC_{50} of 500 μg/mL. The results of this assay are presented in Figure 5. This finding indicates that the stalk extract of OD exhibits moderate antiproliferative potential, but not a significant effect.

The root extract showed no activity, indicating that its phenolic constituents do not play a significant role in cytotoxicity against breast cancer cells. The moderate effect observed with the stalk extract may result from the combined or synergistic action

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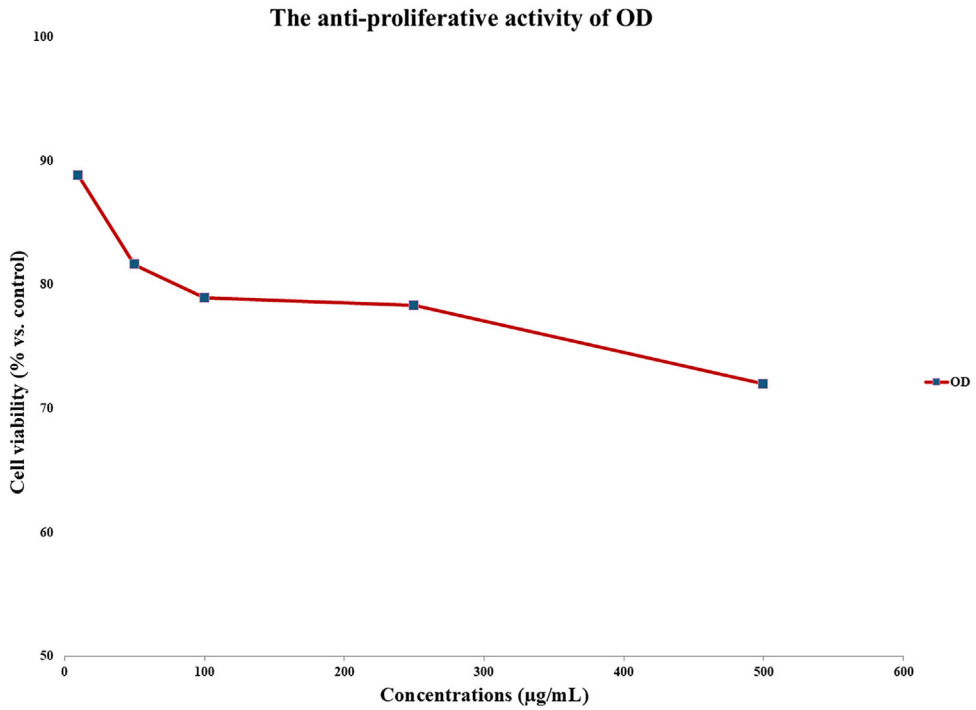


FIGURE 5 | Antiproliferative activity of the stalk extract of OD against MCF-7 cells determined by MTT assay. Data represent cell viability (% of control). Root extract showed no measurable activity and is not presented.

of multiple compounds. LC–ESI–MS/MS analysis of the stalk extract indicated the presence of protocatechuic acid, chlorogenic acid, hydroxybenzaldehyde, caffeic acid, vanillin, *o*-coumaric acid, hesperidin, isoquercitrin, kaempferol-3-glucoside, fisetin, and *trans*-cinnamic acid. Among these, hesperidin, isoquercitrin, and fisetin have been reported to exert anticancer effects such as apoptosis induction and growth inhibition in different cancer cell lines [33–35]. However, the levels of these compounds in the extract may be too low to show a significant antiproliferative effect.

When comparing these findings with other *Onosma* species, a significant variability in cytotoxic potential is observed. The ethanol extracts of *Onosma sericeum* roots inhibited MCF-7 cell proliferation to 29%–42% at concentrations of 50–100 µg/mL, indicating stronger cytotoxic activity and highlighting the influence of solvent polarity on bioactivity [10]. Similarly, methanolic, aqueous, and ethyl acetate extracts of *O. mutabilis* showed activity against multiple cancer cell lines, including MCF-7, with IC_{50} values of 29–42 µg/mL, indicating stronger cytotoxic potential than OD [30]. High potency has been reported for nonpolar extracts: Petroleum ether and dichloromethane fractions of *O. frutescens* roots, containing naphthoquinones, were reported with IC_{50} values <8 µg/mL against several cancer cell models, including MCF-7 [36]. Similar strong cytotoxic activity has also been documented for *O. taurica* var. taurica, *Onosma mollis*, *Onosma aksoyii*, *Onosma isaurica*, and *Onosma alborosea* [36, 37]. *Onosma sintenisii* aerial part extracts exhibited cytotoxic effects only at higher concentrations (≈1000 µg/mL, 48 h), inducing apoptosis and growth inhibition in MCF-7 cells, and indicating lower activity comparable to that of the OD stalk extract [38]. In addition, *Onosma armeniacum* root extracts exhibited selective cytotoxic effects against colon (WiDr), lung (A549), and liver (HepG2) cell lines, indicating interspecific variability in the genus [38]. These findings indicate diversity in antiproliferative capacity among *Onosma* species, which is influenced by phytochemical composition—particularly naphthoquinones and phenolic acids—as well as solvent, plant organ, and ecological factors. The absence of activity in the OD root extract can be attributed to limited bioactive content, whereas the moderate activity observed in the stalk extract suggests the presence of cytotoxic constituents that require further phytochemical investigation.

2.2.3 | Enzyme Activities

The inhibitory effects of the OD root and stalk extracts on AChE and BChE are presented in Table 3. Statistical analysis ($p < 0.05$) confirmed significant differences among the samples at each concentration. The root extract exhibited stronger inhibition of AChE (471.65 ± 0.21 µg/mL) compared to the stalk extract (721.16 ± 0.41 µg/mL), whereas the stalk extract exhibited higher inhibition of BChE (546.09 ± 0.53 µg/mL) than the root extract (634.57 ± 0.52 µg/mL). At lower concentrations (125–250 µg/mL), both extracts showed low inhibition (<35%), whereas inhibition increased significantly at higher concentrations (500–1000 µg/mL). However, in all cases, their activities were statistically lower than those of the reference drug. Donepezil exhibited significantly lower IC_{50} values (11.87 ± 0.28 µg/mL for AChE and 22.02 ± 0.26 µg/mL for BChE), highlighting that the inhibitory potential of OD extracts is only moderate compared to the standard inhibitor.

When compared with other *Onosma* species, our findings are consistent with the general trend of moderate cholinesterase inhibition. The extracts from three Greek *Onosma* spp. exhibited

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TABLE 3 | Anticholinesterase activity of the OD extracts and donepezil (standard) against acetylcholinesterase (AChE) and butyrylcholinesterase (BChE).

Samples	Inhibitor activity (%) mean ± STD			IC_{50} (μg/mL) ± STD	
	Conc. (μg/mL)	AChE	BChE	AChE	BChE
OD—Root	125	21.97 ± 0.132[b]	21.36 ± 0.735[b]	471.654 ± 0.211[b]	634.57 ± 0.522[c]
	250	32.2 ± 0.149[b]	32.72 ± 0.634[b]		
	500	56.8 ± 0.225[b]	49.22 ± 0.435[b]		
	1000	76.1 ± 0.336[b]	57.84 ± 0.285[c]		
OD—Stalk	125	8.68 ± 0.497[c]	22.54 ± 0.432[b]	721.156 ± 0.410[c]	546.09 ± 0.533[b]
	250	11.3 ± 0.256[c]	31.24 ± 0.478[b]		
	500	38.9 ± 0.523[c]	48.22 ± 0.765[b]		
	1000	72.1 ± 0.364[c]	78.61 ± 0.457[b]		
Donepezil	125	73.79 ± 0.139[a]	68.59 ± 0.234[a]	11.872 ± 0.28[a]	22.02 ± 0.264[a]
	250	75.68 ± 0.275[a]	71.38 ± 0.364[a]		
	500	91.24 ± 0.332[a]	86.24 ± 0.274[a]		
	1000	98.8 ± 0.378[a]	94.8 ± 0.183[a]		

Note: Values are expressed as mean ± std. ($n = 3$). Different letters (a–c) in the same column indicate statistical difference ($p < 0.05$). Each concentration was evaluated independently, and statistical comparisons were performed only among the samples at the same concentration.

higher AChE than BChE inhibition (≈1.9–2.4 mg GALAE/g), which is consistent with the general genus-specific pattern of AChE selectivity [32]. In agreement with these findings, methanolic extracts of *O. lycaonica* and *O. papillosa* showed stronger AChE than BChE inhibition, indicating that OD exhibits moderate activity [28]. In contrast, ethyl acetate extracts of *O. riedliana* showed high cholinesterase inhibition, associated with flavonoids, including hesperidin, luteolin-7-glucoside, and hyperoside [29]. Similarly, *O. aucheriana* and *O. sericea* demonstrated significant inhibition associated with flavonoid glycosides (e.g., apigenin-7-glucoside, luteolin-7-glucoside) [6, 18]. In addition, *n*-hexane/dichloromethane extracts of *Onosma nigricaule* roots showed combined inhibition of AChE, BChE, and tyrosinase, attributed to isolated naphthoquinones, whereas *Onosma hispida* was reported to produce novel flavanones (hispidone and a dimethoxy derivative) that selectively inhibited AChE ($IC_{50} \approx 11.6$–15.7 mg/mL) and BChE ($IC_{50} \approx 7.9$–28.0 mg/mL) [39, 40].

The activity of OD extracts can be attributed to their phenolic composition. Vanillic acid, identified as a major constituent in the root extract, and hesperidin, present at significant levels in the stalk extract, have both been reported to exhibit cholinesterase inhibitory and neuroprotective activities [41, 42]. In particular, additional phenolics, such as caffeic acid, protocatechuic acid, rutin, and isoquercitrin, may further contribute to the observed inhibition by binding to cholinesterase enzymes and modulating their catalytic activity through hydrogen bonding and steric interactions [43–45].

Overall, these observations suggest that the moderate dual cholinesterase inhibitory effects of OD extracts are consistent with data from other *Onosma* species, representing a common feature of the genus and reflecting the synergistic action of multiple phenolic constituents. Although OD extracts exhibited lower activity than donepezil, the ability to inhibit both AChE (central) and BChE (peripheral) is pharmacologically relevant, as dual inhibition has been proposed as a valuable strategy in AD therapy to improve cholinergic transmission in both systems. In addition, the presence of multiple phenolic compounds could provide additional antioxidant and neuroprotective effects, potentially offering synergistic benefits. However, not comparable to synthetic drugs in potency, such activity, together with their phenolic content, highlights the possible supportive role of OD in complementary approaches to AD management.

2.3 | Molecular Modeling

Molecular docking was performed to investigate the binding affinities of six phytochemicals identified in OD extracts, together with the reference inhibitor donepezil, against AChE (PDB: 4EY7) and BChE (PDB: 4BDS). Donepezil exhibited the strongest binding to both enzymes (−10.11 and −8.7 kcal/mol, respectively), confirming the validity of the docking protocol. Among the natural compounds, caffeic acid exhibited the strongest binding to AChE (−7.6 kcal/mol), whereas rutin (−8.3 kcal/mol) and hesperidin (−8.1 kcal/mol) showed the highest affinities for BChE, as shown in Table 4. Vanillic acid and hesperidin were selected for detailed binding mode analysis because LC–MS profiling identified them as the major phytochemicals in the roots and stalks, respectively.

Docking analysis of vanillic acid with AChE demonstrated the formation of hydrogen bonds with Ser293, Tyr341, and Tyr124 through its carboxyl, carbonyl, phenolic hydroxyl, and ether oxygen groups. In addition, π–π stacking with Tyr286 and π–σ interactions with Tyr341 were observed, which stabilize its orientation within the binding cavity. These interactions are presented in Figure 6, showing both the 3D docking pose (A) and

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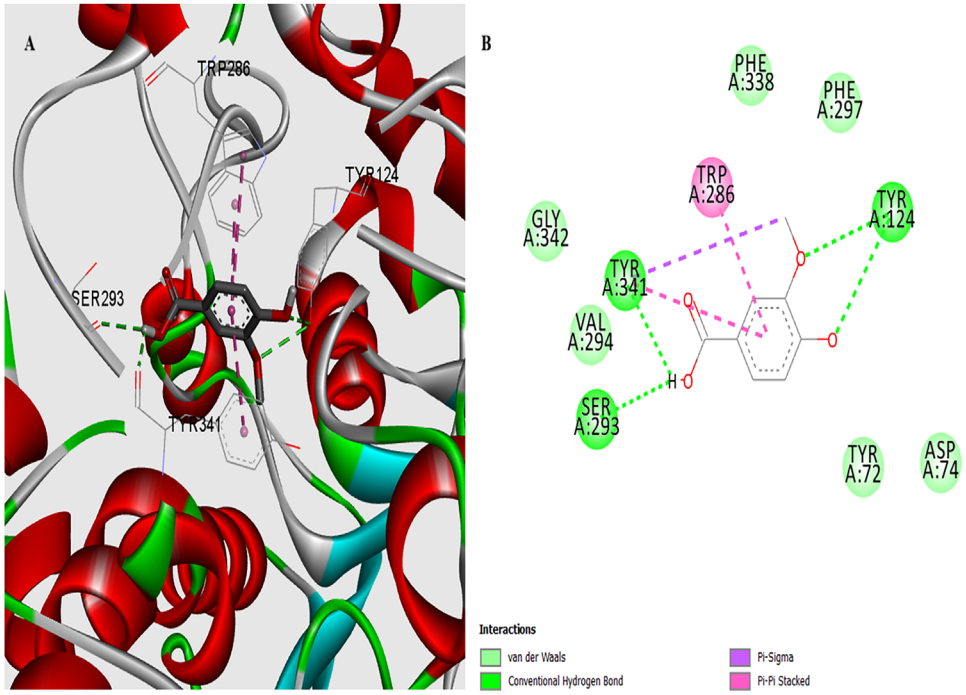


FIGURE 6 | 3D representation of vanillic acid docked within the active site of AChE (PDB ID: 4EY7) and the corresponding 2D interaction diagram illustrating hydrogen bonds, π–π stacking, and other interactions with amino acid residues surrounding the active site.

TABLE 4 | Binding energies of the reference and other compounds to acetylcholinesterase (AchE) (4ey7) and butyrylcholinesterase (BChE) (4bds).

Compounds	Binding energies (kcal/mol)	
	4ey7	4bds
Protocatechuic acid	−6.3	−6.7
Vanillic acid	−6.4	−6.8
Caffeic acid	−7.6	−7.4
Hesperidin	−3.5	−8.1
Isoquercitrin	−6.1	−6.8
Rutin	−1.1	−8.3
Donepezil	−10.11	−8.7

the 2D interaction diagram (B), which highlight the orientation of vanillic acid in the binding site and its interactions with important amino acids. These findings indicate a moderate but consistent interaction profile.

Hesperidin showed strong binding to the BChE active site, forming hydrophobic interactions with Phe329 and Trp82 and an additional hydrogen bond with Ser79. These residues are also known to be critical for the stabilization of donepezil, suggesting that hesperidin shares similar binding features with the standard drug. The predicted interactions are presented in Figure 7, which depicts both the 3D pose (A) and the 2D interaction diagram (B), highlighting the hydrophobic contacts and hydrogen bonds that stabilize the binding.

Comparison with previous reports further validates these findings. Caffeic acid has been reported to act as an effective inhibitor of AChE and BChE, contributing to neuroprotective effects in both in vitro and in vivo models [46]. Similarly, flavonoids such as quercetin and its glycosides, including rutin and isoquercitrin, have been shown to exhibit significant cholinesterase inhibition, with rutin demonstrating stronger affinity for BChE in both enzymatic assays and docking analyses [24]. The computational and biochemical data are consistent with experimental evidence reporting significant inhibitory activity of hesperidin against cholinesterases; although exact values differ, several reports indicate that hesperidin inhibits AChE and BChE at micromolar concentrations, supporting its role in enzyme modulation [41]. In contrast, phenolic acids, such as vanillic and protocatechuic acid, generally exhibit limited-to-moderate inhibition; comparative analyses have shown that caffeic acid produces stronger inhibition of AChE and BChE than chlorogenic acid, whereas vanillic acid exhibited relatively lower activity [47]. In addition, previous reports have reported anticholinesterase activity of vanillic acid in in vivo models of neurodegeneration, showing reduced AChE activity in streptozotocin-induced AD in rats and its derivatives modulating cholinesterase under oxidative stress conditions [48]. However, hydroxybenzoic acids as a class, including protocatechuic acid, have been reported as moderate cholinesterase inhibitors with additional antioxidant properties [24, 47, 49]. Overall, these comparisons support the structure–activity relationship observed in this study: Lower molecular-weight phenolic acids predominantly interact with AChE via PAS residues, whereas higher molecular-weight flavonoid glycosides preferentially bind to BChE as a result of its broader binding cavity.

3 | Conclusions

This study presents a comprehensive study that demonstrates the pharmacological potential of the endemic *O. discedens* species.

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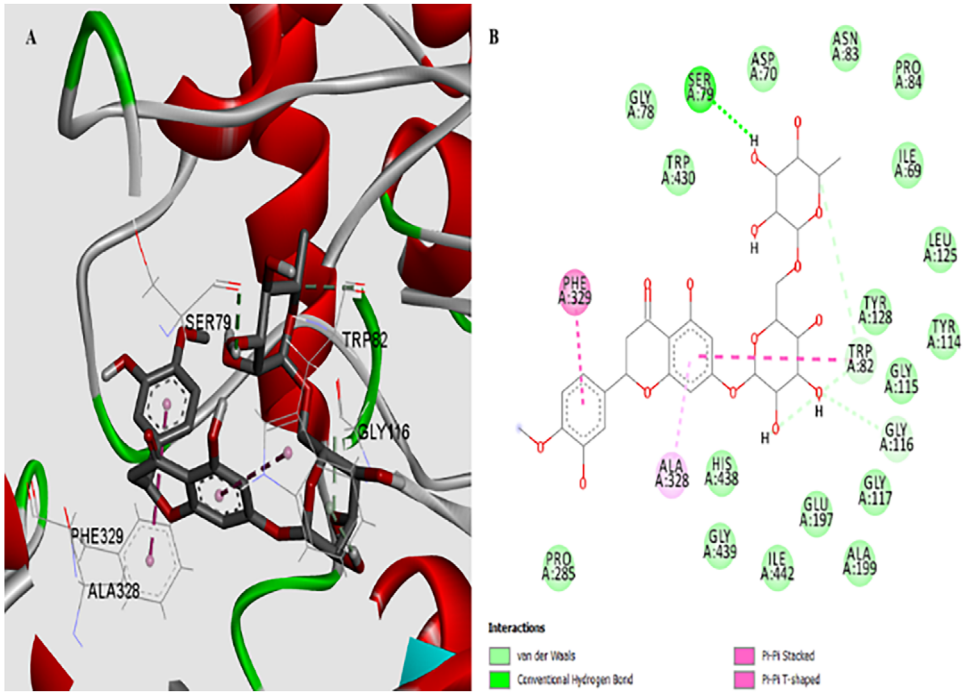


FIGURE 7 | 3D representation of hesperidin docked within the active site of BChE (PDB ID: 4BDS) and the corresponding 2D interaction diagram showing hydrogen bonds and hydrophobic contacts with amino acid residues.

Currently, approved approaches in the treatment of AD aim to improve cholinergic transmission through cholinesterase inhibition, which improves memory and cognitive functions. The results of our study proved that both root and stem extracts of *O. discedens* are an important source of natural phenolics and flavonoids that exhibit dose-dependent inhibitory activity against both enzymes. In silico analyses revealed that vanillic acid in the root and hesperidin in the stem may be responsible for the inhibitory effects at the microgram level. These findings can be further confirmed by in vitro enzyme tests of compounds isolated from plant medicines.

In particular, the stem extract of *O. discedens* exhibited 3–4 times higher TPC compared to other *Onosma* species in Türkiye. This remarkable result indicates that *O. discedens* may be an important source for antioxidant and therapeutic applications. As AD is a multifactorial neurodegenerative disease, pharmacokinetics, toxicity, compound stability studies, and dose/effect relationships are of critical importance for future studies. Considering these data, *O. discedens* has the potential to be used as a functional food or nutraceutical in the treatment of AD and should be evaluated in more detail in experimental AD models.

This study emphasizes the importance of endemic species in pharmaceutical applications and shows that *O. discedens* is a valuable source of new bioactive compounds. The rich phenolic profile and versatile biological activities of the plant provide a promising basis for future pharmacological studies. However, further studies, such as in vivo studies, standardized extraction protocols, and isolation of active ingredients, are required to fully understand the potential for clinical applications. This study constitutes an important step in terms of supporting the traditional uses of endemic plants with scientific data and the potential to provide a source for new treatment strategies.

4 | Experimental Section

4.1 | Plant Materials

OD specimens were collected in June 2022 during the flowering stage from the rural area of Kemaliye district, Erzincan, Türkiye. The plant material was identified by Prof. Dr. Ali Kandemir (Erzincan Binali Yıldırım University, Department of Biology). A voucher specimen (EBYU-0000032) has been deposited in the Herbarium of Erzincan Binali Yıldırım University. The root and stalk parts were separated and dried at room temperature. The dried samples were powdered before starting the study.

4.2 | Reagents

The chemicals and reagents used in enzyme inhibition tests were obtained from Sigma (Germany) and Aldrich (USA). AChE (CAS no. 9000-81-01) and BChE (CAS no. 9001-08-5) were also acquired from Sigma-Aldrich. Sigma-Aldrich (Saint Louis, Missouri) supplied the antiproliferative compound 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Honeywell (North Carolina, USA) supplied dimethyl sulfoxide (DMSO). All cell culture supplies were bought from Biowest (Saint Louis, Missouri).

4.3 | Preparation of Extracts

The root, stalk, and whole of the OD plant were extracted separately, and the phenolic composition, antioxidant capacity, and enzyme inhibition activities of the root and stalk extracts were analyzed comprehensively. In contrast, the whole plant extract was evaluated only for antiproliferative activity. Each material of 100 g was macerated overnight in 500 mL of methanol:water

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(7:3, v/v) solution. The extraction was carried out in triplicate. The plant residues were filtered, and the solvents were mixed. Extracts were lyophilized overnight after methanol evaporation to yield amorphous solids (9.7, 8.6, and 9.4 g for the whole, stalk, and root of OD, respectively). The extracts were stored in dark flasks at +4°C until used.

4.4 | LC–ESI–MS/MS Conditions

An Agilent 1260 Infinity II LC System and Agilent 6460 Triple Quadrupole Mass Spectrometer (LC–ESI–MS/MS) were used for quantitative analysis of phytochemicals. Chromatographic separation was performed on an Agilent Poroshell 120 EC-C18 analytical column (100 mm × 3.0 mm, 2.7 µm) operating at 40°C. Water (A) containing 5 mM ammonium formate and acetonitrile (B) containing 0.1% formic acid were used as the mobile phase. The gradient elution program was applied as follows: 0–3 min: 3% B, 4–12 min: 50% B, 13–21 min: 90% B, 22–25 min: 3% B. The flow rate was set at 0.5 mL/min, and the total analysis time was 25 min. Mass spectrometry analysis was performed in both negative and positive ion modes. The multiple reaction monitoring (MRM) mode was optimized for accurate detection and quantification of phytochemical molecules. Collision energies (CE) were adjusted to ensure optimum fragmentation and maximum transmission of product ions. Mass spectrometry operating parameters were as follows: drying gas (N2) flow, 15 L/min; nebulizing gas (N2) flow, 11 L/min; a capillary voltage of 4000 V; a gas temperature of 350°C; heat block temperature, 400°C; and interface temperature, 350°C. Data were processed using Agilent MassHunter Software. Limit of detection (LOD), limit of quantification (LOQ), and linearity range were evaluated for method validation [15]. Table 5 shows the precursor ion, product ion, fragmentation voltage (FV), CE, and polarity of the phenolic compounds.

Before analysis, 50 mg of the extracts were diluted in 1 mL of methanol, followed by the addition of 1 mL of *n*-hexane. The mixture was vortexed for 2 min at 4°C then centrifuged at 9000 rpm for 10 min to eliminate the oils. The top phase was eliminated, and the methanol phase was diluted with distilled water in a 1:9 ratio. The samples were ultimately filtered using a 0.45 µm syringe filter, and following that, were injected into the system via an autosampler.

4.5 | Biological Activities of OD Extracts

4.5.1 | Estimation of TPC

The TPC was measured using a spectrophotometric technique with Folin–Ciocalteu reagent [50]. Within the scope of analysis, 100 µL of extract and standard solutions (1 mg/mL) were diluted with 4.5 mL of distilled water, and then 100 µL of Folin–Ciocalteu reagent was added. After the mixtures were incubated for 120 min at room temperature, 300 µL of 2% Na_2CO_3 solution was added. After vortexing, absorbance values were measured at 760 nm wavelength. All analyses were performed in three parallel replicates. The calibration curve was obtained by using gallic acid standard solutions (50–1000 µg/mL), and the obtained linear equation was determined as $y = 0.0009x + 0.0208$. The results are presented as milligrams gallic acid equivalent per gram of extract (mg GAE/g extract).

4.5.2 | Estimation of TFC

The TFC was determined according to the method described by Varol et al. [51]. Extract and standard solutions (1 mg/mL) of 100 µL were mixed with 4.7 mL of methanol. Next, 100 µL of 1 M ammonium acetate (NH_4CH_3COO) solution and 100 µL of 10% aluminum chloride ($AlCl_3$) solution were added. The resulting mixture was vortexed before being incubated at room temperature for 45 min. After the incubation, absorbance values were measured at a wavelength of 415 nm. All analyses were performed in three parallel replicates. From the calibration curve created using various quercetin concentrations (25–1000 µg/mL) ($y = 0.0007x + 0.051$), the results were expressed in mg quercetin equivalent/g extract (mg QE/g extract).

4.5.3 | DPPH• Radical Scavenging Ability

DPPH• radical scavenging activity was determined on the basis of the method previously described in the literature [52]. Briefly, 3 mL solutions containing test material at different concentrations (5–100 µg/mL) were mixed with 1 mL of 0.26 mM DPPH• solution. The mixtures were vortexed and stored in a dark environment and incubated at room temperature for 30 min. At the end of the incubation period, absorbance measurements were performed at 517 nm. DPPH• radical scavenging capacity was calculated using the following formula:

$$\text{Activity}\,(\%) = \left[\left(A_{\text{control}} - A_{\text{sample}}\right)/A_{\text{control}}\right] \times 100, \qquad (1)$$

where A_{control} indicates the absorbance of the control group, whereas A_{sample} represents the absorbance of the test sample. All studies were replicated three times, and findings were provided as mean ± standard deviation in IC_{50} (µg/mL). The obtained data were evaluated by comparing with the standard antioxidants Trolox, BHT, BHA, and ascorbic acid.

4.5.4 | Ferric Reducing Antioxidant Power

The FRAP was evaluated according to the method described by Akman et al. [53]. In the analysis, 250 µL of extract and standard solutions (1 mg/mL) were combined with 1 mL of phosphate buffer (0.2 M, pH 6.6) and 1.25 mL of potassium ferricyanide [$K_3Fe(CN)_6$] (1%) solution. The mixes were incubated for 20 min at 50°C in a water bath. After the incubation period, 0.25 mL of ferric chloride ($FeCl_3$, 0.1%) and 1.25 mL of trichloroacetic acid (10%) were introduced, followed by the recording of absorbance values at a wavelength of 700 nm. All studies were conducted in three parallel duplicates, and the results were represented in mg TE activity/g extract, based on the calibration curve established at varying Trolox doses (10–250 µg/mL, $y = 0.0041x + 0.1289$). The findings acquired in the study were assessed against standard antioxidants, specifically BHT, BHA, and ascorbic acid.

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TABLE 5 | The precursor ion, product ion, fragmentation voltage (FV), collision energy (CE), and polarity of the phenolic compounds.

Compound	Precursor ion	Product ion	Fragment (V)	Collusion energy (V)	Polarity
Rutin	611	464.9	95	9	Positive
Hesperidin	611	302.9	100	14	Positive
Rutin	611	302.8	95	9	Positive
Isoquercitrin	464.9	302.8	95	8	Positive
Scutellarin	462.8	286.8	125	16	Positive
Polydatin	390.9	228.9	85	8	Positive
Epigallocatechin	307	139	99	8	Positive
Taxifolin	304.8	258.9	125	12	Positive
Resveratrol	229	135	105	14	Positive
Resveratrol	229	107	105	19	Positive
Caffeine	195	137.9	111	18	Positive
Vanillin	153	125	79	6	Positive
Coumarin	147.1	103.2	125	22	Positive
Quercetin-3-xyloside	432.7	299.5	165	24	Negative
Chlorogenic acid	353	191	110	12	Negative
Catechin	288.9	245.1	140	12	Negative
Sinapic acid	223.1	208	90	10	Negative
Syringic acid	197.1	181.8	80	12	Negative
Trans-ferulic acid	193.1	133.9	95	14	Negative
Protocatechuic ethyl ester	181	107.9	110	20	Negative
Caffeic acid	178.9	135.1	60	14	Negative
Shikimic acid	173	93.1	100	14	Negative
Gallic acid	169	125.1	90	12	Negative
Vanillic acid	167	151.8	80	12	Negative
o-Coumaric acid	163	119.1	70	10	Negative
p-Coumaric acid	163	119	85	10	Negative
Protocatechuic acid	153	109	80	14	Negative
Salicylic acid	137	93.1	85	18	Negative
Hydroxybenzaldehyde	121	92	110	24	Negative
Silibinin	483.1	464.7	110	11	Positive
Silibinin	483.1	162.9	110	10	Positive
Kaempferol-3-glucoside	448.8	286.9	105	10	Positive
Baicalin	446.8	270.9	140	16	Positive
Diosgenin	415	271	120	18	Positive
Dihydrocapsaicin	308.2	137	107	13	Positive
Capsaicin	306.1	137	95	14	Positive
Morin	302.8	153	145	30	Positive
Fisetin	287	212.9	135	32	Positive
Fisetin	287	137	135	32	Positive
Luteolin	285	133.1	132	36	Positive
Biochanin A	284.9	151.9	170	28	Positive

(Continues)

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TABLE 5 | (Continued)

Compound	Precursor ion	Product ion	Fragment (V)	Collusion energy (V)	Polarity
Baicalein	271	123	150	36	Positive
Krisin	254.9	153	160	32	Positive
Daidzein	254.9	128	128	12	Positive
Trans-cinnamic acid	149	131.1	75	6	Positive
Trans-cinnamic acid	149	103	75	10	Positive
Hesperetin	300.9	164	135	20	Negative
Quercetin	300.8	179	130	20	Negative
Quercetin	300.8	151	130	22	Negative
Kaempferol	284.9	116.9	100	44	Negative
Naringenin	270.9	119.1	115	24	Negative

4.6 | Antiproliferative Activity

The antiproliferative activity of OD extracts was evaluated using the MTT assay on human breast cancer cells (MCF-7) [54]. The human breast cancer cell line MCF7 (HTB-22) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Standardized cell preparation procedures were conducted using a sterile laminar cabinet. MCF7 cell lines were cultivated at 37°C in a 5% CO_2 incubator using Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin.

Using the MTT test, the extract's in vitro antiproliferative efficacy against MCF7 cell lines was assessed. To put it briefly, 96-well microplates were planted with 7×10^3 cells per well. The cells were incubated for 24 h after the extracts were added in increasing doses (10, 50, 100, 250, and 500 μg/mL) following an overnight incubation at 37°C. Less than 0.1% of DMSO was present in the dilutions at the end. After adding 50 μL of MTT solution to reach a final concentration of 0.5 mg/mL, the cells were incubated for 3 h at 37°C. After aspirating to the media, formazan crystals were dissolved in 100 μL of DMSO. Once the contents were dissolved, a microplate spectrophotometer (Epoch spectrophotometer, BioTek Instruments Inc., Vermont, USA) was used to measure the optical density of the wells at 570 nm. By comparing the formazan concentrations of the treated and untreated control cells, the vitality of the cells was assessed. The values were the averages of three separate tests, each with three wells.

4.7 | Enzyme Activities

ChE (AChE/BChE) enzyme inhibitory activities of the extracts (root-stalk) of OD were measured on the basis of Ellman's method as reported in a previous study [55–58]. When AChE activity is inhibited, the enzyme hydrolyzes its substrate, acetylthiocholine, to thiocholine, which combines with Ellman's reagent (DTNB) to create yellow 2-nitrobenzoate-5-mercapto-thiocholine and 5-thio-2-nitrobenzoate, which may be detected at 412 nm. In the inhibition of BChE activity, measurements were carried out in the same way using butyrylthiocholine iodide as a substrate. Donepezil was used as a positive control for both enzymes. In the experiments, four different concentrations (125–1000 μg/mL) were determined for the extracts obtained from the root and stalk of the plant. Three independent experiments were carried out in triplicate at four different concentrations. Enzyme activities were tested at four different concentrations for each compound, and percentage activities were determined. The control activity of the enzymes was accepted as 100%, and the IC_{50} values of each extract were calculated independently for ChE enzymes.

4.8 | Molecular Modeling

4.8.1 | Preparation of Ligands and Proteins

The structures of the designed compounds were first drawn in two dimensions using ChemDraw 2D (version 16.0) software and then transferred to ChemDraw 3D (version 16.0) software to obtain the three-dimensional models. The MM2 forcefield module of ChemDraw 3D software was used for the energy minimizations of the ligands.

For docking studies with AChE and BChE enzymes, 4ey7 and 4bds encoded proteins were chosen from the Protein Data Bank (www.rcsb.org), respectively [59, 60]. To prepare the chosen proteins for the docking approach with AutoDock software, water molecules distant from the ligand (5 Å) at the enzyme binding site were removed using Schrödinger Maestro 12.6 software in order to eliminate crystallographic artifacts and improve docking accuracy by minimizing nonspecific solvent interactions, while retaining structural water molecules potentially involved in ligand stabilization. Polar hydrogens were then added to the protein structure, and Kollman charges were allocated using the AutoDock Tools (version 1.5.6) program.

4.8.2 | Docking Studies

The AutoDockTools interface is used to dock with AutoDock Vina. The central coordinates of crystalline donepezil (*x*: −14.5,

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y: −43.48, z: 26.42) and crystalline tacrine (x: 133.140, y: 115.170, z: 40.24) compounds were located in the active region of the proteins and used as the center of the docking search area. Grid maps (30 × 30 × 30 Å^3) were then created according to these coordinates. The RMSD values of AChE and BChE proteins for AutoDock Vina were 0.76 and 0.67 Å, respectively, following re-docking to validate docking settings. Biovia Discovery Studio Visualizer 2019 software was used to perform 2D and 3D protein–ligand interaction analyses on crystal structures.

4.9 | Statistical Analyses

Statistical analyses were performed using SPSS version 20.0 (IBM Corp., Armonk, NY, USA). One-way ANOVA was applied to determine differences among data, and Tukey's test was used for post hoc comparisons at the $p < 0.05$ significance level.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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