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Evaluation of the *in vitro* enzyme inhibition and antioxidant activity of *Clinopodium betulifolium* (Boiss. & Balansa) Kuntze

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ABSTRACT

Clinopodium betulifolium (Boiss. & Balansa) Kuntze is a perennial herb belonging to the Lamiaceae family. There are few studies on *C. betulifolium*, except for its essential oil. In this study, Alzheimer's and cosmetic-related enzyme inhibitory activity and antioxidant activity of *C. betulifolium* species were evaluated. This study extracted C. betulifolium aerial parts by maceration using 70% methanol and water. Antioxidant [DPPH scavenging assay, ABTS cation decolorization, and iron chelating activity] and enzyme inhibition (acetyl-, and butyrylcholine esterase, and tyrosinase) activities of *C. betulifolium* extracts were evaluated using Elisa microplate reader at 2 mg mL^{-1} stock concentration. *C. betulifolium* aqueous extract gave high antioxidant activity (IC_{50}: 34.24 ± 5.01 µg mL^{-1}) in the ABTS method, while its 70% methanol extract (IC_{50}: 100.75 ± 2.62 µg mL^{-1}) was higher than the aqueous extract (IC_{50}: 131.83 ± 4.70 µg mL^{-1}) in the DPPH method. *C. betulifolium* aqueous and 70% methanol extract have moderate anti-tyrosinase activity. Both 70% methanol and aqueous extracts showed similar and high activity against acetylcholinesterase with the IC_{50} values of 73.94 ± 2.78 µg mL^{-1} and 81.71 ± 9.38 µg mL^{-1}, respectively. *C. betulifolium* 70% methanol extract (IC_{50}: 64.08 ± 1.04 µg mL^{-1}) showed higher inhibitory activity than the aqueous extract (IC_{50}: 146.6 ± 8.27 µg mL^{-1}) against butyrylcholinesterase. These results provide basic information for studies that will yield positive results in the development of pharmaceutical formulations or food supplements to be used to treat Alzheimer's and oxidative stress-related diseases.

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Clinopodium betulifolium (Boiss. & Balansa) Kuntze'nin *in vitro* enzim inhibisyonu ve antioksidan aktivitesinin değerlendirilmesi

ÖZET

Clinopodium betulifolium (Boiss. & Balansa) Kuntze, Lamiaceae familyasına ait çok yıllık bir bitkidir. *C. betulifolium* üzerine uçucu yağı dışında pek fazla çalışma bulunmamaktadır. Bu çalışmada *C. betulifolium* türünün Alzheimer ve kozmetikle ilişkili enzim inhibitör aktivitesi ve antioksidan aktivitesi değerlendirildi. Bu çalışmada *C. betulifolium*'un toprak üstü kısımlarından %70 metanol ve su kullanılarak maserasyon tekniği ile ekstraksiyon işlemi yapıldı. *C. betulifolium* ekstrelerinin antioksidan [DPPH radikal süpürücü aktivite, ABTS katyonik renk giderici ve demir şelatlama aktivitesi] ve enzim inhibisyonu (asetil/bütirilkolinesteraz ve tirozinaz) aktiviteleri, 2 mg mL^{-1} stok konsantrasyonunda Elisa mikroplaka okuyucusu kullanılarak değerlendirildi. *C. betulifolium* su ekstresi ABTS yönteminde yüksek antioksidan aktivite verirken (IC_{50}: 34.24 ± 5.01 µg mL^{-1}), DPPH yönteminde %70 metanol ekstresi (IC_{50}: 100.75 ± 2.62 µg mL^{-1}) sulu ekstresinden (IC_{50}: 131,83 ± 4,70 µg mL^{-1}) daha yüksek aktivitede bulunmuştur. *C. betulifolium* su ve %70 metanol ekstreleri orta derecede

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Anahtar Kelimeler
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Anti-tirozinaz
Antioksidan aktivite

anti-tirozinaz aktivitesine sahiptir. Hem %70 metanol hem de su ekstreleri sırasıyla 73,94 ± 2,78 µg mL^{-1} ve 81,71 ± 9,38 µg mL^{-1} IC_{50} değerleriyle asetilkolinesteraza karşı benzer ve yüksek aktivite gösterdi. *C. betulifolium* %70 metanol ekstresi (IC_{50}: 64,08 ± 1,04 µg mL^{-1}), bütirilkolinesteraza karşı su ekstresinden (IC_{50}: 146,6 ± 8,27 µg mL^{-1}) daha yüksek inhibitör aktivite gösterdi. Bu sonuçlar Alzheimer ve oksidatif strese bağlı hastalıkların tedavisinde kullanılacak farmasötik formülasyonların veya gıda takviyelerinin geliştirilmesinde olumlu sonuçlar verecek çalışmalar için temel bilgiler sağlamaktadır.

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INTRODUCTION

For the Lamiaceae family, Turkey is recognized as a significant center of biodiversity. The family is represented by 45 genera, 546 species, and 731 taxa in Turkey (Satil et al., 2011). The Lamiaceae family also includes the genus *Clinopodium* L, a few of which are found in Africa, tropical Asia, and Indo-Malaysia. Still, they are mostly distributed in the New World and temperate Eurasia (Kadereit, 2004).

The initial revision of the genus *Clinopodium* was carried out by Davis and Leblebici (Davis & Leblebici, 1982) for the "Flora of Turkey" and contained just two species. The number of recognized taxa in Turkey has increased to 38 as a result of the taxonomic investigations. *C. troodi* (Post) Govaerts subsp. *grandiflorum* (Hartvig and Å.Strid) Govaerts and *C. troodi* (Post) Govaerts subsp. *vardaranum* (Leblebici) Govaerts are endemic plants in Turkey and East Mediterranean elements (Ayla, 2017).

Several *Clinopodium* species have been studied for their biological and medical applications. This genus has been used for different infections in Ecuadorian folk medicine (De la Torre et al., 2008). Also, In Bulgaria, *C. vulgare* L. has been utilized for the healing of wounds and has shown anti-bacterial properties (Dzhambazov et al., 2002). In traditional medicine, aerial parts of *C. tomentosum* (Kunth) Govaerts have been used to treat respiratory diseases, inflammation, and gastrointestinal problems (Tubon et al., 2020). In Mersin, Turkey, *C. dolichodontum* (P.H.Davis) Bräuchler & Heubl is used for gallstones, analgesic, gastrointestinal pain, relaxant, cold, and flu (Sargin, 2015). *C. vulgare* L. subsp. *vulgare* is used for abdominal pain by wrapping the area in Izmit (Kizilarslan & Özhatay, 2012). *C. acinos* (L.) Kuntze, *C. congestum* (Boiss. & Hausskn. ex Boiss.) Kuntze, *C. graveolens* (M.Bieb.) Kuntze subsp. *graveolens* is used internally for flu and cold in various regions of Turkey. *C. dolichodontum* (P.H.Davis) Brauchler & Heubl shortness of breath and eye diseases, *C. nepeta* (L.) Kuntze subsp. *glandulosum* (Req.) Govaert snake bites, *C. serpyllifolium* (M.Bieb.) Kuntze subsp. *barbatum* (P.H.Davis) Bräuchler internally colic, externally antiseptic wound healing, *C. serpyllifolium* (M.Bieb.) Kuntze subsp. *brachycalyx* (P.H.Davis) Bräuchler diuretic, antiseptic, stomach ache, *C. serpyllifolium* (M.Bieb.) subsp. *fruticosum* (L.) Bräuchler is used for cough and stomach ache in Turkey (Selvi et al., 2022).

Previous phytochemical research on the *Clinopodium* species has revealed the presence of several components, including phenylpropanoids, flavonoids, triterpenoid saponins, and diterpenes, as well as fatty acids and essential oils, that are showing various biological effects (Sarikurkcu et al., 2015; Zeng et al., 2016; Tubon et al., 2020). There are not many studies on *C. betulifolium* (Boiss. & Balansa) Kuntze, known as “kızıl fesleğen” in Turkey, except for taxonomical, and morphological studies, and essential oil research (Kürkçüoglu et al., 2007; Sevim & Atila, 2009).

Antioxidants can protect the body from the damaging effects of free radicals and reactive oxygen species. They prevent the progression of many serious diseases. Researching alternative, and secure sources of antioxidants as well as looking for natural antioxidants, particularly those with a plant origin, have received a lot of attention in recent years (Gulcin, 2020). There are antioxidant activity studies on *C. sericeum*, *C. vulgare, C. nubigenum*, *C. brownei* essential oils (Tepe et al., 2007; Noriega et al., 2018 Benites et al., 2021; P. Noriega et al., 2023). It has been determined that the antioxidant activity of *C. nepeta* and *C. vulgare* extracts is strong (Beddiar et al., 2021; Bektašević et al., 2022).

Acetyl/butyrylcholinesterase are enzymes that break down acetylcholine, which is associated with Alzheimer's disease and plays an important role in the pathophysiology of the disease (Koçyiğit et al., 2022). The disease, which is especially common in the elderly population, has become a major health problem. Therefore, the discovery of new cholinesterase inhibitors is important in the treatment of the disease

(Erdogan Orhan et al., 2015; Güçlü et al., 2022). Tyrosinase (TYR) is a copper-containing enzyme that plays a role in the biosynthesis of melanin dark pigment, and TYR inhibitors are being investigated as skin-whitening agents in the cosmetic industry (Qian et al., 2020). Among *Clinopodium* species, the enzyme inhibition effects of *C. vulgare* against acetylcholinesterase, *C. nepeta* against cholinesterase and TYR enzyme, and *C. gilliesii* (Benth.) Kuntze against cholinesterase was evaluated (Beddiar et al., 2021; Bektašević et al., 2022; Fernández-Galleguillos et al., 2023).

Clinopodium has potential therapeutic activities including hemostasis, anti-bacteria, anti-inflammation, immunoregulation, lowering blood glucose, antioxidation, and anti-tumor (Yao et al., 2020). Although the traditional uses of the *Clinopodium* genus have been reported, research on the phytochemical investigation and biological activity of this genus is still needed. A comprehensive study regarding the biological effects of *C. betulifolium* has not been published until now. With the aim of searching for the importance of *C. betulifolium* in pharmacognosy, the bioactivity examinations on the aerial parts of *C. betulifolium* were carried out for the first time. The extracts with different polarity solvents of *C. betulifolium* were evaluated through antioxidant [1,1-Diphenyl-2- picrylhydrazyl (DPPH) quenching assay and 2,2'-azinobis-3- ethylbenzothiazoline-6-sulfonic acid (ABTS) cation decolorization, and iron chelating activity] and enzyme inhibition (acetyl-, and butyrylcholine esterase, and tyrosinase) activities.

MATERIALS and METHODS

Plant Material

C. betulifolium was collected from Mersin (C4 Mersin: Taurus Mountains, Güzeldere valley, 50m above the rock; 20.06.2018; Herbarium No: S Dogu & Y Bagci 3076) and identified by Prof. Dr. Yavuz Bagci.

Extraction Methods

The shade-dried and powdered aerial parts of *C. betulifolium* were separately macerated with 70% methanol and water 3 times. Macerates were filtered through pleated filter paper and evaporated to dryness with a Rotary evaporator at 40°C. The extracts were stored in the refrigerator for biological activity experiments.

Total Phenol and Flavonoid Determination

The Folin Ciocalteu (F-C) method was employed to measure total phenol content (TPC) in the extracts (Clarke et al. 2013). The calibration curve of absorbance versus concentration was determined to be mg gallic acid equivalent (GAE) g^{-1} extract for all analyses, which were carried out in triplicate. Aluminium chloride colorimetric method was used to measure the total flavonoid content (TFC) in the extracts. The findings were expressed as mg quercetin equivalent (QE) g^{-1} extract (H. Yang et al., 2011).

Antioxidants Assay

For the ABTS radical scavenging activity of the extracts, 15 mL of 7 mM ABTS and 264 µL of 140 mM potassium persulfate solution are combined to create ABTS•+ radical stock solution. The working solution for ABTS•+ is prepared to provide an absorbance of 0.70 ± 0.02 at 734 nm. 50 µL of sample is combined with 100 µL of ABTS•+ working solution. After mixing for 10 minutes, absorbance is measured at 734 nm (Re et al., 1999). After adding 20 µL of test solution on a 96-well plate, 180 µL of DPPH solution is added. Using an Elisa reader (Multiscan Sky, USA), the plate is measured at 540 nm after 15 min incubation at 25°C in the dark (Clarke et al. 2013). Ascorbic acid was used as a positive control in DPPH and ABTS assay. The iron chelating activity of the extracts was assessed by the interaction of ferrosine-Fe^{2+} complex. Ethylenediaminetetraacetic acid (EDTA) was used as positive control (Chai et al., 2014). All the antioxidant activity analyses were performed in triplicate.

Enzyme Inhibition Assay

The inhibition activity of acetyl- and butrylcholinesterase enzymes was conducted out according to the Ellman et al.'s approach with some modifications. 10 µL of 3 mM 5,5'-dithio-bisnitrobenzoic acid (DTNB); 140 µL of 0.1 mM phosphate buffer (pH 6.8); 20 µL of the enzyme (0.22 U mL^{-1} for acetylcholinesterase/0.1 U mL^{-1} for butyrylcholinesterase) produced in phosphate buffer; and 20 µL of test sample/reference standard of varied quantities. 10 µL of the substrate (0.71 mM acetylthiocholine iodide/0.2 mM butyrylthiocholine chloride in phosphate buffer) was added to start the reaction. At 412 nm, absorbance was measured (Epoch, USA). Galantamine served as the positive reference (Ellman et al., 1961; Šinko et al., 2007). According to Yang et al.'s approach, the extracts' TYR inhibitor activities were assessed. To summarize, 20 µL of the sample solution, 20 µL of the enzyme solution (20 µL of mushroom tyrosinase in phosphate buffer), and 100 µL of phosphate buffer (0.1 M, pH 6.8) were added to a 96-well plate. The mixture was then incubated for 30 minutes at 25°C after 20 µL of a 3 mM L-tyrosine solution prepared in phosphate buffer was added as the substrate. At 492 nm, the absorbance was measured using a microplate reader. Kojic acid served as the positive reference (Yang et al., 2012). All the enzyme inhibition analyses were performed in triplicate.

Statistical analysis

The data are represented as the mean ± standard

deviation, with each analysis performed in triplicate. Statistical significance was set at $p< 0.05$.

RESULTS and DISCUSSION

İn this study, *C. betulifolium* total phenol quantification was found to be 40.48 ± 1.51; and 56.37 ± 3.70 mg GAE g^{-1} extract in 70% methanol and aqueous extracts, respectively. TFC content of *C. betulifolium* 70% methanol extract (3.19 ± 4.98 mg QE g^{-1} extract) was found quercetin equivalent. In addition, free radical scavenging and iron chelating activities of *C. betulifolium* extracts were investigated. IC_{50}, or half-maximal inhibitory concentration, is the concentration of an extract required to scavenge free radical by 50%. The lower the IC_{50} value calculated in the antioxidant activity assays, the higher the antioxidant activity. As a result, *C. betulifolium* aqueous extract showed higher antioxidant activity (IC_{50}: 34.24 ± 5.01 µg mL^{-1}) than its 70% methanol extract (IC_{50}: 62.65 ± 0.68 µg mL^{-1}). In the ABTS method, while its 70% methanol extract (IC_{50}: 100.75 ± 2.62 µg mL^{-1}) exhibited higher DPPH radical scavenging activity than its aqueous extract (IC_{50}: 131.83 ± 4.70 µg mL^{-1}). Iron chelation activities of *C. betulifolium* extracts were found close to each other and low (Table 1). Additionally, the findings on the extracts appear to have a strong positive correlation between TPC and DPPH, and ABTS radical scavenging activities (Figure 1).

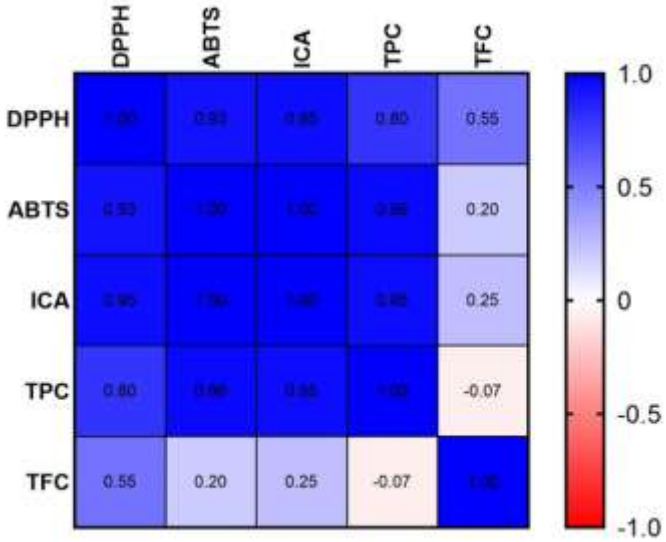

Figure 1. Heatmap of correlations between the analyzed antioxidant parameters*

Şekil 1. Analizlerin antioksidan parametreleri arasındaki korelasyonların ısı haritası

*DPPH (r = 0.80, r=0.55, p<0.0001) and ABTS (r = 0.96, r= 0.20 p<0.0001) radical scavenging, and iron chelating (r = 0.95, r=0.25, p<0.0001) activities.

Table 1. Extract yield, total phenol and flavonoid contents, and antioxidant activity of *C. betulifolium* extracts.

Çizelge 1. C. betulifolium ekstrelerinin ekstre verimi, toplam fenol ve flavanoit içerikleri ve antioksidan aktivitesi

Plant extract	Extract yield %g g^{-1}	TPC mg GAE g^{-1} extract	TFC mg QE g^{-1} extracts	ABTS (inhibition percentage ± S.D.) 2 mg mL^{-1}	DPPH (inhibition percentage ± S.D.) 2 mg mL^{-1}	Iron-chelating activities (inhibition percentage ± S.D.) 2 mg mL^{-1}
C. betulifolium 70% methanol extract	5.24	40.48 ± 1.51	3.19 ± 4.98	86.15 ± 0.28*** (IC_{50}: 62.65 ± 0.68 µg mL^{-1})	83.84 ± 0.53*** (IC_{50}: 100.75 ± 2.62 µg mL^{-1})	9.93 ± 2.66**
C. betulifolium aqueous extract	14.03	56.37 ± 3.70	-	87.17 ± 0.28*** (IC_{50}: 34.24 ± 5.01 µg mL^{-1})	53.67 ± 3,69*** (IC_{50}: 131.83 ± 4.70 µg mL^{-1})	9.55 ± 3.17**
Ascorbic acid (2 mg mL^{-1})	-	-	-	87.51 ± 0.17***	93.91 ± 0.14***	-
EDTA (2 mg mL^{-1})	-	-	-	-	-	87.06 ± 0.34***

The data are represented as the mean ± standard deviation of three replicates. ** p < 0.01, *** p < 0.001

In the results on enzyme inhibition activity, *C. betulifolium* aqueous (41.18%) and 70% methanol (29.73%) extracts have moderate anti-TYR activities. Both extracts similarly showed high inhibition activities against acetylcholinesterase (IC_{50} values of 70% methanol extract and aqueous extracts: 73.94 ± 2.78, and 81.71 ± 9.38 µg mL^{-1}, respectively). *C. betulifolium* 70% methanol extract (IC_{50}: 64.08 ± 1.04 µg mL^{-1}) showed higher inhibitory activity than the aqueous extract (IC_{50}: 146.6 ± 8.27 µg mL^{-1}) against butyrylcholinesterase (Table 2).

Free radicals and reactive oxygen species cause diseases such as neurodegenerative, atherosclerosis, cancer, and inflammatory disorders by growing damage to important macromolecules such as DNA, protein, and lipid (Na et al., 2011). Studies have proven that plant extracts and natural ingredients reverse the negative effects of these radicals. Antioxidant activity studies are particularly common on phenolic compounds and there are very few studies on other classes of natural products as antioxidant agents (El-Sayed et al., 2008)

In a study on *Clinopodium* genus, the antioxidant effect of *C. nepeta* extracts was evaluated by six methods (DPPH, ABTS$^{\cdot+}$, GOR, CUPRAC, Phenanthroline, FRAP), where the BuOH extract was the most active. Phenolic compounds such as apigenin (21.75 ± 0.41 µg g^{-1}), myricetin (72.58 ± 0.57 µg g^{-1}), and rosmarinic acid (88.51 ± 0.55 µg g^{-1}) were detected in *C. nepeta* (Beddiar et al., 2021). Contraversely, the antioxidant activities of *C. nepeta* BuOH extract (DPPH$^{\cdot}$ IC_{50}: 8.12 ± 0.11 µg mL^{-1}; ABTS$^{\cdot+}$ IC_{50}: 12.82 ± 2.62 µg mL^{-1}) was found to be high than antioxidant activities of *C. betulifolium* MeOH and water extracts (DPPH$^{\cdot}$ IC_{50}: 100.75 ± 2.62 µg mL^{-1}, 131.83 ± 4.70 µg mL^{-1}; ABTS$^{\cdot+}$ IC_{50}: 62.65 ± 0.68 µg mL^{-1}, 34.24 ± 5.01 µg mL^{-1}), respectively.

Table 2. Enzyme inhibition activity of *C. betulifolium* extracts.
Çizelge 2. C. betulifolium ekstrelerinin enzim inhibisyonu.

Plant extract	TYR (percentage ± S.D.[a]) 2 mg mL^{-1} [b]	AChE (percentage ± S.D.[a]) 2 mg mL^{-1} [b]	BChE (percentage ± S.D.[a]) 2 mg mL^{-1} [b]
C. betulifolium 70% methanol extract	29.73 ± 0.33***	72.60 ± 5.87*** (IC_{50}: 73.94 ± 2.78 µg mL^{-1})	89.24 ± 3.44*** (IC_{50}: 64.08 ± 1.04 µg mL^{-1})
C. betulifolium aqueous extract	41.18 ± 2.17***	81.62 ± 5.20*** (IC_{50}: 81.71 ± 9.38 µg mL^{-1})	61.82 ± 2.80*** (IC_{50}: 146.6 ± 8.27 µg mL^{-1})
Kojic acid	80.96 ± 0.51***	-	-
Galantamine hydrobromide	-	99.10 ± 1.18***	84.34 ± 4.85***

[a] Standard deviation, [b] Stock concentration. The data are represented as the mean ± standard deviation of three replicates. *p < 0.05, ** p < 0.01, *** p < 0.001

In another study on *C. vulgare*, the total phenolic content, DPPH, and FRAP values of the extract were found 27.9 ± 0.4 mg gallic acid equivalents per g sample, 0.114 ± 0.0004 mg mL^{-1}, and 1556 ± 3 µM trolox equivalents per g sample, respectively. Bektašević et al. examined the chemical composition of *C. vulgare* hot water and methanol extract using spectroscopic and HPLC/DAD techniques. Among sixteen identified and quantified phenolic compounds the dominant compounds were rosmarinic (26.63 and 34.21 mg g^{-1}) and ellagic acid (23.11 and 29.31 mg g^{-1}) of hot water and methanol extract, respectively (Bektašević et al., 2022). The TPC content of *C. vulgare* hot water (145.5 ± 4.2 mg GAE g^{-1} extract) and methanol (170.0 ± 3.9 mg GAE g^{-1} extract) extracts was higher than the TPC content of our extracts. Controversially, *C. vulgare* methanol extract was shown higher TPC content than water extract, while *C. betulifolium* water extract was found higher methanol content.

C. nepeta extracts showed moderate inhibition on acetylcholinesterase, butyrylcholinesterase, tyrosinase, and α-amylase. *C. nepeta* dichloromethane extract gave the highest activity in both acetyl (IC_{50}: 170.1 ± 1.58 µg mL^{-1}), and butyrylcholinesterase (IC_{50}: 73.06 ± 0.83 µg mL^{-1}) (Beddiar et al., 2021). *C. betulifolium* methanol extract showed higher activity against cholinesterase than *C. nepeta* dichloromethane extract. IC_{50} values of all *C. nepeta* extracts against TYR were higher than 200 µg mL^{-1}. Since no inhibition above 50% occurred in this *C. betulifolium* extract, IC_{50} could not be calculated. Hot water and methanol extract of *C. vulgare* were not inhibited against butyrylcholinesterase. Acetylcholinesterase inhibition of *C. vulgare* extracts was weak (Sarikurkcu et al., 2015). *C. vulgare* subsp. *vulgare* methanol and water extracts had no TYR enzyme inhibitory activity. İn this study, *C. betulifolium*, methanol (29.73 ± 0.33%), and water extracts (41.18 ± 2.17%) had moderate inhibitory activity.

CONCLUSIONS

İn this study, enzyme inhibition and antioxidant activities of *C. betulifolium* were carried out for the first time. *C. betulifolium* extracts showed remarkable radical scavenging activity in both ABTS and DPPH methods. On the contrary, iron chelating capacity of *C. betulifolium* extracts was found to be low. Moreover, *C. betulifolium* extracts showed moderate anti-TYR inhibitory activity. Otherwise, *C. betulifolium* extracts showed significant inhibitory activity against Alzheimer-related enzymes. As a conclusion, our findings revealed that 70% methanol extract of *C. betulifolium* may be promising natural source to conduct advanced studies. The following study will be on the determination the phytochemical contents of *C. betulifolium* and identification of the phytoconstituents, responsible for the mentioned biological activities.

Contribution Rate Statement Summary of Researchers

The authors declare that they have contributed equally

to the article.

Conflict of Interest Statement

The authors of the article declare that there is no conflict of interest between them.

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