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Investigation of antioxidant, enzyme inhibitory effects and total secondary metabolite quantification of *Ornithogalum nutans* L. methanol

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Abstract

This study was designed to determine the antioxidant and enzyme inhibitory activities of methanol extracts of different parts (aerial and bulb) of Ornithogalum nutans as well as to investigate the total phenolic and flavonoid amounts. While the total phenolic content of the extracts varied between 1.43±0.01/3.05±0.04 mg GAE/g of extract equivalent, the total flavonoid amount varied between 0.33±0.01/1.79±0.02 mg QE/g. Antioxidant activities were determined by DPPH, ABTS, ß-Carotene/Linoleic acid, CUPRAC, and FRAP methods. The antioxidant activity studies determined that the aerial methanol extract exhibited a higher activity than the bulb. Acetylcholinesterase and tyrosinase enzyme inhibitory activities of the extracts were determined, and the extracts showed lower activity than galantamine (89.41±0.05 %) and kojic acid (89.41±0.05 %) used as standard. The results reveal the potential of O. nutans extracts to be used in pharmacological studies in the pharmaceutical and food industries.

Keywords: *Ornithogalum nutans, antioxidant, acetylcholinesterase, tyrosinase, secondary metabolites*

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Ornithogalum nutans L. Metanol ekstraktlarının antioksidan, enzim inhibitör Etkileri, total sekonder metabolit miktar tayininin araştırılması

Öz

Bu çalışma Ornithogalum nutans türünün farklı kısımlarının (yerüstü ve yeraltı) metanol ekstraktlarının antioksidan ve enzim inhibitör aktivitelerinin belirlenmesinin yanı sıra total fenolik ve flavonoid miktarlarının araştırılması için tasarlanmıştır. Ekstraktların total fenolik miktarı 1.43±0.01/3.05±0.04 mg GAE/g ekstrakt eşdeğeri arasında değişirken, total flavonoid miktarı ise 0.33±0.01/1.79±0.02 mg QE/g arasında değişmektedir. Antioksidan aktiviteleri DPPH, ABTS, ß-Karoten/Linoleik asit, CUPRAC ve FRAP yöntemleri ile belirlemiştir. Yapılan antioksidan aktivite çalışmalarında yerüstü metanol ekstraktının, yeraltına göre daha yüksek bir aktivite sergilediği tespit edilmiştir. Ekstraktların asetilkolinesteraz ve tirozinaz enzim inhibitör aktiviteleri belirlenmiştir ve ekstraktların standart olarak kullanılan galantamin (89.41±0.05 %) ve kojik asite (89.41±0.05 %) göre daha düşük bir aktivite sergilemişlerdir. Sonuçlar, O. nutans ekstraktlarının farmakolojik çalışmalarda ilaç ve gıda endüstrilerinde kullanılabilme potansiyellerini ortaya koymaktadır.

Anahtar kelimeler: *Ornithogalum nutans, antioksidan, asetilkolinesteraz, tirozinaz, sekonder metabolit*

1. Introduction

Turkey, which hosts plant species with different anatomical and morphological characteristics, is a floristic country with high plant biodiversity. The reason why the plant biodiversity is so high is due to the differences in altitude, climate, and habitat diversity, as well as the intersection of three phytogeographic regions. It is known that there are more than 12.000 plant taxa in Turkey, which hosts more than 3000 endemic species [1-3]. With the use of natural medicinal products that are used in traditional medicine and have a rich biodiversity, new therapeutic targets have been provided for many diseases in the scientific world [4]. The fact that plants are used by traditional healers for various medicinal purposes, while various species cause poisoning to farm animals, indicates that more scientific research is needed on the chemical composition of the compounds isolated from these plants, the dosage to be used, the toxicity and possible biological activities [5]. Secondary metabolites are organic compounds produced by microorganisms or plants that are not directly involved in the growth, development, and reproduction of the organism. Secondary metabolites with bioactive properties are used especially in the fields of medicine and pharmacy. Production of secondary metabolites depends on meteorological conditions, geographical location, and growing conditions, and many plants can only grow and mature in certain seasons [6-7]. These metabolites have different biological activities such as anti-inflammatory, antioxidant, antihelminthic, larvacidal, anticancer, antimicrobial, anti-mutagenic, wound healing, and anti-tumor [8]. Oxidative stress, by definition, is the disorganization of the redox balance with an increase in the level of ROS in the cell. Redox homeostasis must be balanced, as excessive oxidation and reduction of cells can have harmful consequences. For this purpose,

compounds that provide antioxidant activity in plants are abundant. Although antioxidant activity is undoubtedly found in all plants, it may differ from species to species. Antioxidants are molecules that can eliminate free radical reactions, prevent cell damage, and delay oxidation at a lower concentration than the oxidized substrate. The most common antioxidants investigated in plants are ascorbate, glutathione, phenols, tocopherols, and carotenoids [9]. Geophyte is a plant species that is resistant to adverse climatic and environmental conditions since it has bulb organs such as onions, tubers, and rhizomes in the plant structure [10]. *Ornithogalum* L. (Liliaceae) genus belongs to the Asparagaceae family and is a geophyte plant that generally grows near South Africa and the Mediterranean and includes more than 140 species around the world. The Asparagaceae family, whose general distribution is in South America, Europe, Africa, and Asia, is a bulbous plant community consisting of approximately 41-70 genera and 770-1000 species. The most well-known genus in Asparagaceae is Ornithogalum, a subspecies of Ornithogaleae with 280 species, which is also widely distributed in Anatolia and Asia. There are 42 species of this genus in Turkey, and according to research, 17 of them are defined as endemic to Turkey [11-12]. While *Ornithogalum* L. is used as a medicinal plant in traditional medicine, it can also be used for commercial purposes in the ornamental plant or cut flower sector with its interesting white color [13]. In a study, the toxicity (brine shrimp lethality test) and anthelmintic (*Tubifex tubifex*) activities of the aerial and bulb methanol extracts of *Ornithogalum nutans* L. were investigated. *O. nutans* showed high anthelmintic activity in aerial parts. In addition, they found the lowest toxicity against *Artemia salina* in the aerial extract [14]. In this study, antioxidant (DPPH, ABTS, ß-carotene CUPRAC, and FRAP) and enzyme inhibitory activity (Acetylcholinesterase, and Tyrosinase), as well as total substance amount (Phenolic, and Flavonoid) of *O. nutans* aerial and bulb methanol extracts were determined.

2. Material and methods

2.1. Chemicals

DPPH, ABTS, Trolox, BHA, Sodium carbonate, TPTZ, Sodium acetate, Neocuproine, Folin-Ciocalteu, gallic acid, Ammonium acetate, and quercetin were obtained from Sigma-Aldrich (USA).

2.2. Plant material and extract preparation

Ornithogalum nutans species were collected from Babadağ district of Denizli in 2021 and were diagnosed by Prof. Dr. Olcay DÜŞEN (HERBARIUM NO: 2010000003688). The collected plant samples were dried and cut into small pieces with a blender. Then, 20 g of samples were weighed into Erlenmeyer bottles and 100 mL of methanol was added. Afterwards, the Erlenmeyer bottle was kept in a shaking incubator at 50 ° C for 6 hours, and after this procedure, it was filtered into a balloon jug with the help of blotting paper. Then the solvent was added again and left for another 6 hours. After the filtration process was completed, the filtered samples were taken to the rotary evaporator (Heindolph LABOROTA 4011) to remove the solvent. In order to remove the water in the samples, they were kept in a lyophilizer (Thermo Savant) at -54 °C for 8 hours. After the lyophilization process, the samples were scraped from Petri dishes with the help of a spatula and transferred to glass bottles. The samples were stored at -20 °C until they were used during the experiment [15].

2.3. Determination of antioxidant activity

2.3.1 DPPH free radical scavenging activity

Extract solutions of different concentrations (0.2 mg/mL, 0.4 mg/mL, 0.8 mg/mL, 1.0 mg/mL) were added to 4 mL of DPPH (2,2-Diphenyl-1-Picryl hydrazil) (0,004%) solutions and incubated for 30 minutes at room temperature. After incubation, absorbance measurement was taken at 517 nm. Calculated IC_{50} (half the maximum inhibitory concentration) values of each extract were determined [15].

2.3.2 ABTS free radical scavenging activity

Extract solutions of different concentrations were added to 4.5 mL ABTS (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) solution and incubated for 30 minutes at room temperature. After incubation, the absorbance at 734 nm was measured and the IC_{50} value was calculated [16].

2.3.3 Determination of total antioxidant Activity by β-carotene/linoleic acid method

24 mL of β-carotene/linoleic acid solution was added to 1 mL of the extract solution and the initial absorbance at 470 nm was measured. It was then incubated at 50 °C for two hours. After incubation, the absorbance was measured at 470 nm and the total antioxidant activity (%AA) was calculated using the formula below [17].

$$\% AA = [1- (A_{S0}-A_{K0}) /(A_{S120}-A_{K120})]x\ 100$$

A_{S0} is the absorbance of the sample at minute 0, A_{K0} is the absorbance of the control at minute 0, A_{S120} is the absorbance of the sample at minute 120, and A_{K120} is the absorbance of the control at minute 120.

2.3.4 CUPRAC (copper ion reducing power) method

After adding 0.5 mL of the extract solution (1 mg/mL) dissolved in its solvent, onto 3 mL of CUPRAC solution (1 mL $CuCl_2$, 1 mL Neocuproine, 1 mL ammonium acetate), it was left to incubate at room temperature for 30 minutes. It was measured at 450 nm after incubation. For the trolox equivalent (mg TE/g extract) results, the formula y=0.078-0.0029 (R^2=0.992) was used [18].

2.3.5 FRAP (Fe^{+3} ion reducing power) assay

The extract solution was added to 2 mL of FRAP reagent solution (3.6 pH, 0.3 M acetate buffer, 10 mM TPTZ, and 20 mM $FeCl_3$) and incubated for 30 minutes at room temperature. After incubation, absorbance was measured at 595 nm. For the trolox equivalent (mg TE/g extract) results, the formula y=0.336-0.038 (R^2=0,9986) was used [19].

2.4. Determination of enzyme inhibitory activity

2.4.1 Acetylcholinesterase

It is based on the formation of a yellow-colored chromophore TNB (5-thio-2-nitrobenzoic acid) from the reaction of thiocholine with acetylcholinesterase hydrolysis of acetylthiocholine with DTNB (5,5-dithio-bis-2-nitrobenzoic acid). The resulting TNB is directly proportional to the AChE activity at 405 nm. 125 µL of DTNB reagent (0.3 mM) and 25 µL of anticholine esterase (0.026 U/mL) enzyme solution were mixed onto 50 µL (1 mg/mL) extract solution and incubated at 25 ºC for 15 minutes. To initiate the reaction, 25 µL of acetylthiocholine iodide (ATCI) substrate (1.5 mM) was added and incubated at 25 ºC for 10 minutes. After incubation, the absorbance value at 405 nm was measured and the % inhibition (AA) was calculated using the following formula [20].

$$\% AA = [(A_k - A_s) /A_k] \times 100$$

A_k is the absorbance value of the control and A_s is the absorbance value of the extract.

2.4.2 Tyrosinase

Tyrosinase inhibitor activity will be carried out using L-DOPA as a substrate. 40 µL of extract solution was taken from 120 µL of phosphate buffer (20 mM pH: 6.8 phosphate buffer) and 20 µL of tyrosinase enzyme (480 U/mL) solution, mixed and incubated at 25 ºC for 15 minutes. After incubation, 20 µL of L-DOPA (2.5 mM) was added and incubated at 25 ºC for another 10 minutes. After incubation, absorbance values were measured at 492 nm and % inhibition (AA) values were calculated using the following formula [21].

$$\% \ AA = [(A_k - A_s) / A_k] \times 100$$

A_k is the absorbance value of the control and A_s is the absorbance value of the extract.

2.5. Quantification analysis of extracts

2.5.1 Determination of total phenolic content

1 mL of FCR (Folin- ciaocelteu Reagent) and 46 mL of distilled water were added to the extract solution and kept at room temperature for 3 minutes. Afterward, 3 mL of Na_2CO_3 solution was added and incubated at room temperature for two hours. After incubation, its absorbance was measured at 760 nm. For the gallic acid equivalent (mg GAE/g extract) results, the formula y=0.0033-0.0002 (R^2=0.9826) was used [22].

2.5.2 Determination of total flavonoid content

After adding 0.2 mL of sodium acetate, 1 mL of aluminum chloride, and 5.6 mL of distilled water to the extract solution, it was incubated at room temperature for 30 minutes. After incubation, its absorbance was measured at 415 nm. For the quercetin equivalent (mg QE/g extract) results, the formula y=0,0737+0,0369 (R^2=0,997) was used [23].

2.6. Statistical analysis

All assays were performed in 3 replicates. The mean ± standard error was analyzed with Microsoft Excel. In studies conducted to determine free radical scavenging activity, the IC_{50} value was calculated using the Minitab 16 statistical program.

3. Results and discussion

Due to the search for new resources in the pharmaceutical industry, modern science is investigating all possible origins. The potential antioxidant activities of natural products have been a source of interest for researchers due to their use as additives and preservatives in food, medicine, and cosmetics [24]. Antioxidant activities of aerial and bulb methanol extracts obtained from *O. nutans* species were determined using DPPH, ABTS, ß-carotene/linoleic acid, CUPRAC, and FRAP methods (Table 1). DPPH and ABTS free radical scavenging activities were determined by calculating IC_{50} values. A low IC_{50} value indicates high radical scavenging activity. Both methods showed higher activity than the standard BHA extracts. The aerial part extract (1.82±0.126/0.32±0.007 mg/mL, IC_{50}, respectively) showed higher scavenging activity in both methods than the bulb extract. In terms of % inhibition values of the extracts, it is lower than standard BHA (94.98±0.70 %). However, it was determined that the aerial extract (50.00±1.24 %) exhibited a higher % inhibition than the bulb extract (36.67±2.30 %). CUPRAC of the extract were calculated as trolox equivalent. The aerial extract (4.59±0.03/0.78±0.04 mg TE/g, respectively) was determined to have a higher reducing power capacity.

Table 1. Antioxidant activity of bulb and aerial methanol extract of *O. nutans*

Solvent/Assay	DPPH (IC_{50}, mg/mL)	ABTS (IC_{50}, mg/mL)	β-carotene/ linoleic acid (%)	FRAP (mg TE/g)	CUPRAC (mg TE/g)
Bulb	2.11±0.004	0.83±0.007	36.67±2.30	0.38±0.01	1.10±0.02
Aerial	1.82±0.126	0.32±0.007	50.00±1.24	0.78±0.04	4.59±0.03
BHA	0.01±0.02	0.019±0.001	94.98±0.70	-	-

In a study, DPPH and ABTS free radical scavenging activities of *O. lanceolatum* ethanolic (80%) extract were investigated. They determined that the aerial extract (12.53±0.03/11.08±0.01 mg/mL, IC_{50}, respectively) exhibited higher antioxidant activity than the bulb extract [25]. Aydın (2020) determined that the total antioxidant capacity of the extract obtained from the root and flower parts of *O. umbelletum* was the highest at 105.50±0.025 µg AAE/mL [26]. Renda et al. (2018) [27] determined in their study that the extract obtained from the aerial parts of *O. orthophyllum* exhibited higher free radical scavenging activity than other extracts. In a study, *O. sintenisii* revealed that the free radical scavenging activities of aerial and bulb methanol extracts were 368 ± 15 µg/mL, IC_{50} and 669 ± 25 µg/mL, IC_{50}, respectively [28]. Zengin et al. (2015) [29] investigated the antioxidant activities of the extracts obtained from different parts of *O. narbonense* with three different solvents in their study. They revealed that the bulb ethyl acetate extract exhibited the highest antioxidant activity for most studies. In a study, the antioxidant activities of *O. sigmoideum* hexane, ethanol, and water extracts were determined by CUPRAC and ABTS experiments. The highest free radical scavenging activity was observed in the water extract (106.01 ± 0.42 µg/mL, IC_{50}). They determined that the highest activity in terms of copper-reducing power capacity was in the ethanol extract (190.03 ± 0.04 µg/mL, $A_{0.50}$) [30]. In a study, the antioxidant activity (ß-carotene, DPPH) of *O. alpigenum* aerial and bulb methanol, ethanol, acetone, and benzene extracts was investigated. Methanol extracts (88.12 ± 0.9 %) exhibited high activity [31]. Taşkın et al., (2021) [32] investigated the antioxidant activity (DPPH, ABTS, FRAP, CUPRAC) of *O. orthophyllum* extracts obtained with different solvents in their study. The aerial methanol extract has high antioxidant activity. Our study is supported by other studies in the literature.

Table 2. Acetylcholinesterase enzyme inhibitory activity of the extracts

Plant/Standart	25 µg/mL	50 µg/mL	100 µg/mL	200 µg/mL
Bulb	11.25±0.10	20.31±0.18	34.73±0.32	48.67±0.70
Aerial	-	-	12.55±0.25	21.45±0.17
Galantamine	73.09±0.05	79.08±0.47	84.76±0.08	89.41±0.05

Acetylcholinesterase and tyrosinase enzyme inhibitory activities of the extracts were determined (Table 2 and Table 3). In enzyme inhibitor studies, the % inhibition value increases depending on the increase in concentration. Aerial and bulb extracts showed lower % inhibition compared to galantamine (89.41±0.05 %) and kojic acid (73.93±0.10%) used in the studies. The bulb extract (48.67±0.70 %) showed higher acetylcholinesterase enzyme inhibition activity than the aerial extract (21.45±0.17 %). At low concentrations of tyrosinase enzyme inhibition activity, both extracts didn't exhibit an inhibitory activity. The aerial extract (25.54±0.40 %) exhibited a higher tyrosinase enzyme inhibitory activity than the bulb (21.47±0.31 %) extract.

Table 3. Tyrosinase enzyme inhibitory activity of the extracts

Plant/Standart	25 µg/mL	50 µg/mL	100 µg/mL	200 µg/mL
Bulb	-	-	12.66±0.20	21.47±0.31
Aerial	-	-	14.80±0.27	25.54±0.40
Kojic acid	45.52±0.14	54.81±0.17	61.73±0.05	73.93±0.10

In a study, the tyrosinase enzyme inhibitory activity of *O. narbonense* extracts was determined. Bulb ethyl acetate extract (33.88 mg KAEs/g extract) showed the highest activity, followed by root (21.46 mg KAEs/g extract) and seed ethyl acetate (15.78 mg KAEs/g extract) extract [29]. Kurt et al., (2018) [30] investigated the acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzyme inhibitory activities of *O. sigmoideum* extracts prepared with three different solvents (hexane, ethanol, water). In the studies, ethanol (154.01 ± 0.93 µg/mL, IC_{50}) and hexane (40.85 ± 0.68 µg/mL, IC_{50}) extract showed the highest activity, respectively. In a study, urease, and acetylcholinesterase enzyme inhibitory activities of *O. orthophyllum* extracts obtained with three different solvents were determined. Petroleum ether (0.038±0.019 mg/mL, IC_{50}) extract showed the highest urease inhibitory activity. The methanol extract (0.019±0.008 mg/mL, IC_{50}) showed the highest AChE inhibitory activity [32].
Total phenolic and flavonoid amounts of the methanol extracts of *O. nutans* species obtained from bulb and aerial were determined (Table 4).

Table 4. Total phenolic and flavonoid amount of bulb and aerial methanol extract of *O. nutans* species

Plant/Assay	Total Phenolic (mg GAE/g)	Total Flavonoid (mg QE/g)
Bulb	1,43±0,01	0,33±0,01
Aerial	3,05±0,04	1,79±0,02

The amounts of phenolic substances were found as gallic acid equivalents. The total flavonoid amount was determined as quercetin equivalent. While the total phenolic content of the extracts ranged between 1.43±0.01/3.05±0.04 mg GAE/g extract

equivalent, the total flavonoid amount varied between 0.33±0.01/1.79±0.02 mg QE/g. In a study, the total phenolic and flavonoid content of *O. lanceolatum* ethanolic (80 %) extract obtained from bulb and aerial was investigated. They found that the aerial extract contained higher phenolics (835±79 mg GAE/100 g) and flavonoids (886±91 mg QE/100 g) [25]. In a study, they determined the total phenolic and flavonoid amounts of the methanol extract (80%) obtained from the shoot of *O. narbonense* as 173.26 ±13.5 µg/mL gallic acid and 22.13±0.9 µg/mL quercetin equivalent, respectively [33]. In their study, they determined that the total phenolic content of the extract obtained from the root and flower parts of *O. umbelletum* was 19.26±0.001 µg GAE/mL, and 18.96±0.001 µg GAE/mL, respectively [26]. Renda et al. (2018) [27] determined the total phenolic content of three different *Ornithogalum* L. species in a study they conducted. They revealed that the aerial extract of *O. orthophyllum* (11.0 mg GAE/g extract) had the highest phenolic content. In a study, the total phenolic content of ethyl acetate, methanol, and water extracts obtained from stems, bulbs, and seeds was determined. It was determined that the highest content was in the bulb ethyl acetate extract (21.05 mg GAEs/g extract) [29]. The aerial extract has a higher amount of substance than the bulb extract. There is a positive correlation between phenolic compounds and antioxidant activity [34-35]. Phenolic compounds can quench radicals because they contain one or more hydroxyl groups in their structure. However, the amount of phenolic does not directly affect antioxidant activity. Flavonoids are secondary metabolites that can show different antioxidant properties depending on the location of the hydroxyl groups in their structures [36-37]. Different properties of the material, such as chemical or physical, whose antioxidant activity is determined, may also have an effect. Within the scope of the study, there is a positive correlation between the total phenolic content of bulb and aerial extracts and their antioxidant activities.

4. Conclusion

The antioxidant and enzyme inhibitory activities of the aerial and bulb methanol extracts of *O. nutans*, as well as the total phenolic and flavonoid amounts were evaluated. There is a correlation between the total phenolic and flavonoid amounts and the higher antioxidant activity of the aerial extract than the bulb extract. *O. nutans* revealed that surface and underground methanol extracts are species that can be evaluated in future pharmacological studies due to their antioxidant and enzyme inhibitory activities. This study will contribute to the literature for the next detailed molecular biological studies. This study will contribute to future literature studies.

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