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Research Article

A preliminary Study on Antioxidant and Antimicrobial Potential of Ethanol Extracts of *Galanthus gracilis* Celak.

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Abstract

Plants are widely used in the treatment of various diseases. The present study evaluates for the first time the potential antioxidant and antimicrobial activities as well as the total flavonoid contents of the ethanol extracts of leaf and flower parts of *G. gracilis* Celak. species distributed in Muğla province. The antioxidant activities were determined by DPPH, ABTS and β-carotene assays. The antimicrobial activities were evaluated by the disc diffusion method and by determining the minimal inhibitory concentration (MIC). According to the outcomes from DPPH and *β*-carotene assay, the ethanol extract of the flower may be suggested to have a higher antioxidant potential than that of the leaf. The total flavonoid content was found in the highest in the ethanol extract of the leaf. Gram (-) *P. aeruginosa* was detected as the most sensitive bacteria to both extracts. *C. albicans* was determined to be more sensitive to both extracts compared to all tested bacteria. These results suggest that *G. gracilis* possess potential antioxidant and antimicrobial activity.

Keywords: *Galanthus gracilis,* antioxidant activity, antimicrobial activity.

Galanthus gracilis'in Etanol Ekstraktlarının Antioksidan ve Antimikrobiyal Potansiyeli Üzerine Bir Ön Çalışma

Özet

Bitkiler çeşitli hastalıkların tedavisinde yaygın olarak kullanılmaktadır. Bu çalışma, Muğla ilinde yayılış gösteren *G. gracilis* Celak. türünün yaprak ve çiçek kısımlarının etanol ekstraktlarının potansiyel antioksidan ve antimikrobiyal aktiviteleri ile toplam flavonoid içeriğini ilk kez değerlendirmektedir. Antioksidan aktiviteleri DPPH, ABTS ve β-karoten testleri ile belirlenmiştir. Antimikrobiyal aktiviteler disk difüzyon yöntemiyle ve minimum inhibitör konsantrasyonu (MIC) belirlenerek değerlendirilmiştir. DPPH ve β-karoten analizinden elde edilen sonuçlara göre, etanolik çiçek ekstraktının yaprağa göre daha yüksek bir antioksidan potansiyele sahip olduğu önerilebilir. Toplam flavonoid içeriği en yüksek etanolik yaprak ekstresinde bulundu. Gram (-) *P. aeruginosa* her iki ekstreye de en duyarlı bakteri olarak tespit edilmiştir. *C. albicans*, test edilen tüm bakterilere kıyasla her iki ekstrakta da daha duyarlı olduğu belirlendi. Bu sonuçlar, *G. gracilis*'in potansiyel antioksidan ve antimikrobiyal aktiviteye sahip olduğunu göstermektedir.

Anahtar Kelimeler: *Galanthus gracilis,* antioksidan aktivite, antimikrobiyal aktivite.

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1. Introduction

Bacteria have caused massive deaths for centuries, and antibiotics have an essential place in the fight against bacteria. [1]. Multidrug resistance and limited lifespan of drugs have led to the research and use of bioactive molecules of plants as an alternative to antibiotics [2]. Plants provide the most significant potential and alternative medicine source for humanity because of their high drug potential thanks to the bioactive molecules they contain [3,4]. Since free radicals can attack structures such as protein, DNA, lipid, it can cause significant diseases such as diabetes and cancer. Antioxidant substances in plants eliminate these effects [5].

Galanthus gracilis Celak. is a geophyte species belonging to the genus *Galanthus* (Family: Amaryllidaceae) [6,7,8,9], and the Galanthus genus is a high economic value since it is exported to many countries, including the Netherlands, as an ornamental plant [10]. The *Galanthus* genus is grown as a garden plant in Europe and is considered an early harbinger of spring [11]. Some Galanthus species (*Galanthus trojans*, *Galanthus nivalis*, *Galanthus ikariae*, *Galanthus peshmenii,* etc.) are on the CITES-red list [12]. There are many studies of Amaryllidaceae family alkaloids in the literature, and these alkaloids, especially galantamine and its derivatives, are used for treatment purposes, especially in motor neuron diseases and cancer research due to their phytochemical properties [13,14,15,16,17,18]. There are especially antitumor, antiviral, antiprotozoal, antifungal, antiparasitic, antioxidant, anti-inflammatory, and insect antifeedant, acetylcholinesterase inhibitory activity studies on Amaryllidaceae alkaloids [1,19,20,21,22,23]. Galanthamine alkaloid is used as an effective symptomatic treatment in Alzheimer's disease. [24,25,26,27].

In this study, it was aimed to determine the antioxidant, antibacterial, and antifungal activities of ethanol extracts of leaf and flower parts of *G. gracilis*. This is the first study in the literature to investigate the antioxidant and antimicrobial potential of these extracts from *G. gracilis*, distributed in Muğla province, which is used in traditional treatment by the public.

2. Materials and methods

2.1. Chemicals

Ethanol, Methanol, DPPH, ABTS, Butylated hydroxyanisole (BHA) were purchased Sigma-Aldrich. β-carotene, Quercetin, Gallic acid were purchased from Merck (Darmstadt, Germany). Water was used as ultra distilled pure water. Other chemicals and solvents were of analytical grade.

2.2. Plant material and Extraction

G. gracilis was collected from Yılanlı Mountain in Muğla province in 1200 m altitude in February, 2018 during the flowering period. The plant material was identified by Dr. Yeliz Değerli and stored with voucher specimens (Herbarium No: YD 1016) at Muğla Sıtkı Koçman University, Muğla, Turkey. After air drying at room temperature, the leaves and flowers were sliced into tiny pieces. In a 6-hour ethanol extraction process using a shaking water bath, 100 g of the sliced samples were used. When the ethanol that was used as a solvent was filtered out,

it was evaporated in a rotary evaporator (IKA, RV 10, USA) and lyophilized to evaporate any remaining water (Labconco FreeZone 6, USA) .. The extracts were stored at a temperature of -20 ^{o}C [28].

2.3. Free Radical Scavenging Activity (DPPH)

In the determination of DPPH (2,2-Diphenyl-1-picryl hydrazyl radical), free radical scavenging activity, according to Turan and Mammadov [29] method was used. 4 mL DPPH solution were added 4 concentrations (0.2 -0.8 mg/mL) of the extracts and incubate at room temperature for 30 minutes. Then, the absorbance was measured at 517 nm in a spectrophotometer. Methanol was used as blank solution. Results were given as IC_{50} values.

2.4. ABTS Radical Cation Scavenging Activity

In the determination of ABTS (2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid) radical cation scavenging activity, Re et al. [30] method's was performed. Twelve hours before the assay, 7nM ABTS and 2.45mM potassium persulfate were mixed and kept in the dark at room temperature. Then, it was diluted with ethanol until the absorbance was 0.700 at 734 nm in the spectrophotometer. 0.5 mg/mL extracts mixed with 4.5 mL ABTS solution and incubate at room temperature for 30 minutes. It was then measured at 734 nm. Ethanol was used as blank solution. Results were given as IC_{50} values.

2.5. β-carotene/linoleic acid Method

In the β-carotene-linoleic acid method, the method of Amarowicz et al. [31] was used with some modifications. 2 mg of β-carotene, 1 ml of chloroform, 20 µl of linoleic acid, 200 µl of Tween 20 are mixed, and the chloroform is allowed to evaporate. Then 100 ml dH_2O was added. A mixture containing 24 ml of β-carotene-linoleic acid was added on 1 mg/mL extract dissolved in its solvent. It was measured at 470 nm on a spectrophotometer every half hour during incubation at 50 °C. The results were applied according to the formula of Amarowicz et al. [31].

2.6. Determination of Total Flavonoid Contents

The experiment of determination of total flavonoid contents was performed by the aluminum trichloride method [32]. Briefly, 1 ml of 2% aluminum trichloride ($AlCl_3$) in methanol was mixed with the same volume of the extracts (2 mg). Absorption readings at 415 nm were taken after 10 min against a blank sample consisting of a 1 ml extract solution with 1 ml methanol without $AlCl_3$. The concentrations of flavonoid compounds were calculated according to the following equation that was obtained from the standard quercetin. The results were expressed as quercetin equivalent (mg QE/g of extract)

2.7. Antibacterial and Antifungal Activity Test

Antibacterial and antifungal activity of the ethanol extracts of *G. gracilis* was evaluated using the paper disc diffusion technique and by determining the minimal inhibitory concentration (MIC). Lyophilized bacteria and yeast were obtained from the culture collection of the

Department of Basic and Industrial Microbiology, Faculty of Science, Ege University. *Staphylococcus aureus* ATCC 6538/P, *Bacillus cereus* CCM 99, *Escherichia coli* ATCC 35218, and *Pseudomonas aeruginosa* ATCC 27853 were used for antibacterial activity. *C. albicans* ATCC 10239 strains were used for antifungal activity.

2.7.1. Disc Diffusion Assay

Antibacterial activity of raw extracts obtained from *G. gracilis* was tested by the paper disc diffusion technique [33,34,35,36]. The extracts were dissolved in DMSO, and then 20 µL of each extract (5000 µg/mL) of *G. gracilis* were absorbed onto sterile 6-mm diameter filter paper discs (Schleicher and Schüll, Nr 2668, Dassel, Germany). Bacterial strains were pre-cultured on Muller Hinton Broth medium (Merck) and incubated for 24 h at 37 ± 0.1 °C. Candida strains were pre-culted on Sabouraud Dekstroz Broth (Merck) and incubated for 48 h at 25 ± 0.1 °C. Autoclaved Mueller- Hinton Agar (Merck) was added to sterile plates under suitable conditions, and it was allowed to solidify under aseptic conditions. The turbidity of bacteria and fungi was prepared according to McFarland 0.5 scale to obtain a standard inoculum. Then 0.1 mL of the test organisms were inoculated with a sterile drigalski spatula on the surface of the appropriate solid medium in the plates. The sterile disks impregnated with different extracts were then placed on the agar plates and incubated at 37 ± 0.1 °C for 24 h. The sterile disks impregnated with other extracts were then placed on the agar plates and incubated at 25 ± 0.1 °C for 48 h to measure antifungal activity. The inhibition zone (mm) of antibacterial and antifungal activity against test organisms was measured and evaluated. All experiments were done under sterile conditions in duplicated. Erythromycin and Ampicillin (Oxoid) (10 mg/disc) were used as positive controls. DMSO, ethanol were used as negative control.

2.7.2 Determination of MIC values

Minimal inhibitory concentration (MIC) values of bacterial and fungal strains sensitive to *G. gracilis* were determined. For this purpose, a micro-dilution experiment was performed for *Galanthus* samples according to the procedures developed by the National Clinical Laboratory Standards Committee [35,36,37,38]. Dilution series of the extracts were prepared by thawing from 5000 mL DMSO in test tubes and then transferred to the broth in 96-well microtiter plates. Final concentrations in the medium were 200 µg/mL. Before the inoculation of the test organisms, the bacteria strains and yeast strain were adjusted to 0.5 McFarland standards and diluted 1:100 (v/v) in Mueller–Hinton broth and Saboraud dextrose. The 96-well plates were prepared by dispensing extract into each well of broth and the inocula to obtain 1×10^8 CFU/mL. Extract prepared at the concentration of 128 µg/mL was added into the first wells. Then its serial dilutions (128, 64, 32, 16, 8, 4, 2, and 1 µg/mL) were transferred into the consecutive wells. Plates were incubated at 37°C for 18–24 h and at 25°C for 48 h for the yeast. All the tests were performed in broth and repeated twice. The MIC was defined as the lowest concentration that showed clear against a black background (no visible growth). The MIC was defined as the lowest concentration of an extract or a substance to inhibit the growth of microorganisms after 18–24 h and 48 h for the yeast.

2.8. Statistical Analysis

All assays were performed in 3 replicates. The mean ± standard error and IC_{50} values was analyzed using Microsoft Excel.

3. Results and Discussion

3.1. Antioxidant Activity and Total Flavonoid Content

Because of the bioactive chemicals in plants have such varied and intricate structures, a simple antioxidant test cannot be relied upon to provide accurate findings.Therefore, it is important to do a series of antioxidant tests, all of which should corroborate each other.[39, 40]. The *in vitro* antioxidant activities of the extracts prepared with ethanol solvent of *G. gracilis* were determined by DPPH, ABTS, β-carotene-linoleic acid. (Table 1).

Table 1. Antioxidant activity of the ethanol extracts of *G. gracilis*

Sample	DPPH 0.8 mg/mL%, (IC_{50}, mg/mL)	ABTS 0.8 mg/mL%, (IC_{50}, mg/mL)	*β*-carotene 1mg/mL % (120')	Flavonoid (mg QE/g extract)
Leaf Ethanol Extract	24.98 ± 0.35, (1.69)	73.35 ± 0.31, (0.49)	73.95 ± 0.93	93.38 ± 0.23
Flower Ethanol Extract	40.82 ± 0.45, (0.98)	49.66 ± 0.11, (0.79)	80.82 ± 0.72	33.29 ± 0.62
BHA	67.35 ± 0.10*, (0.03)	90.71 ± 0.29**, (0.04)	88.00 ± 0.92	---

* at 0.05 mg/mL. ** at 0.05 mg/mL.

Free radical scavenging activity assays using DPPH and ABTS were used to determine IC_{50} values. In terms of antioxidant activity, a lower IC_{50} value indicates better results.[41]. The lowest IC_{50} value in the DPPH assay was obtained from the ethanol extract of the flower part (0.98 mg/mL, IC_{50}). But, antioxidant activity was observed far from BHA as the standard antioxidant (0.03 mg/mL, IC_{50}). Similar to DPPH in ABTS assay, although results are far from BHA results (0.04 mg/mL, IC_{50}), the highest antioxidant value was obtained in the ethanol extract of the leaf part (0.49 mg/mL, IC_{50}) (Table 1). The ethanol extract of the flower part (80.82 ± 0.72 %) in 120 minutes showed the closest value to the result of the BHA chemical

antioxidant (88.00 ± 0.92 %) in the β-carotene/linoleic acid method. Benedec et al. [17] studied four Amaryllidaceae family species (*Galanthus nivalis, Narcissus pseudonarcissus, Narcissus poeticus, Leucojum vernum*) and discovered that *G. nivalis* had the greatest DPPH antioxidant activity with an IC_{50} value of 139.88 ± 5.11 g Trolox/mL, IC_{50} value. In a study by Erenler et al. [11] with *G. krasnovii*,the best ABTS activity was found in the extract with obtained from ethyl acetate solvent with IC_{50} value of 14.33 µg/mL.Bulduk et al. [42] found the highest antioxidant activity in the leaf part of *G. woronowii* in DPPH and ABTS antioxidant experiments in their study with *G. nivalis*, *G. elwesii*, *G. woronowii*. These investigations indicate that Galanthus extracts derived from various solvents may exhibit varying levels of antioxidant activity.

In the present study, total flavonoid amounts of *G. gracilis* ethanol extracts were determined as 33.29 ± 0.62 and 93.38 ± 0.23 mg QE/g of extract for flower and leaf parts, respectively (Table 1). Bati Ay et al. [18], in their study with G. *elwesii*, found the highest flavonoid content in the bulb part with 58.63 mg QE /g at the fruit ripening growing stage. Karimi et al. [14] found the highest amount of flavonoid substances with 2.67 ± 0.02 mg rutin equivalents/g DW (dry weight) values in shoot part in their study with *G. transcaucasicus*. In the study of Benedec et al. [26], the highest flavonoid substance with 12.56 ± 1.43 mg RE/g value was found in *G. nivalis*.. Bulduk et al. [42], in their study with *G. nivalis*, *G. elwesii*, *G. woronowii*, found the highest amount of flavonoid content in the leaf part of the *G.woronowii* with 33 ± 0.28 mg CAE / g. D.W . These studies suggest that different parts of the genus *Galanthus* may differ in their total flavonoid content.

3.2. Antibacterial and Antifungal Activity Assays

Antibacterial and antifungal activity of *G. gracilis* ethanol extracts assessed by disk diffusion and micro-well dilution techniques are reported in Tables 2 and 3, respectively. The disk diffusion test of both extracts of *G. gracilis* ranged from 0.00 ± 0.00 to 0.75 ± 0.03 mm across all bacterial strains. The highest resistant to the leaf and flower extracts tested was demonstrated by the gram-positive bacteria *S. aureus* with an inhibition zone of 0.00 ± 0.00 mm and the gram-negative bacteria *E. coli* with an inhibition zone of, 0.35 ± 0.20 mm, respectively. The most sensitive bacteria to both extracts was gram (-) *P. aeruginosa*. When we look at the inhibition regions that we examined with different extracts for *Candida albicans*, we can say that the inhibition regions of all extract same effective. A 0.80 ± 0.00 mm was found the *C. albicans* disc diffusion experiment for both extracts (Table 2) Benedec et al. [26] evaluated the antimicrobial activity of *G. nivalis* using the disc diffusion method and and reported that the growth of *S. aureus* was strongly inhibited. Türker et al. [43] performed the antibacterial activity of ethanol, methanol, and water extracts of some endemic species including the *G. plicatus subsp. byzantinus* against fish pathogens (*Aeromonas hydrophila*, *Yersinia ruckeri*, *Streptococcus agalactiae*, *Lactococcus garvieae*, *Enterococcus faecalis*) with a disc diffusion assay, and the *G. plicatus subsp. byzantinus* was found to be effective only against A. *hydrophila*, and the water extract was found be the most effective extract with value of 7.50 ± 0.19 mm. Türker and Köylüoğlu [44] reported that among extracts their tested, the antibacterial activity was observed only in ethanol extract in *G. plicatus* subsp. *byzantinus* endemic for Bolu,

Turkey, with 7.25 ± 0.25 mm value against *Staphylococcus epidermidis* (ATCC 12228), 12.50 ± 0.50 mm value against *Streptococcus pyogenes* (ATCC 19615), 8.25 ± 0.62 mm value against *Proteus vulgaris* (ATCC 13315), 7.25 ± 0.25 mm value against *Klebsiella pneumoniae* (ATCC 13883). In another study examined the antimicrobial activity of the extracts of leaf and flower parts of the *G. transcaucasicus*. Among the bacteria, *E. coli* seemed to be the most sensitive, and *S. aureus* were the most resistant strain bacteria to the extracts tested [14].

Table 2. Antibacterial and antifungal activity of the ethanol extracts of *G. gracilis* by the disc diffusion method.

Microorganism (Gram reaction)	Inhibition zone (mm.)						
	Leaf Ethanol Extract	Flower Ethanol Extract	Standards				
			Amp.	Erit.	Nys.	DMSO	Eth.
Antibacterial Activity							
Staphylococcus aureus (ATCC 6538/P) G (+)	0 ± 0.00	0.40 ± 0.23	2,90 ± 0.06	2,80 ± 0.00	-	0,40 ± 0.23	1.20± 0.00
Bacillus cereus (CCM 99) G (+)	0.35 ± 0.20	0.40 ± 0.23	1.40 ± 0.00	3.10 ± 0.00	-	0.75 ± 0.03	1.10 ± 0.00
Escherichia coli (ATCC 35218) G (-)	0.70 ± 0.00	0.35 ± 0.20	2.10 ± 0.06	1.0 ± 0.00	-	0 ± 0.00	1.80 ± 0.00
Pseudomonas aeruginosa (ATCC 27853) G (-)	0.75± 0.03	0.70 ± 0.00	1.35 ± 0.03	3.00± 0.12	-	0 ± 0.00	1.20 ± 0.00
Antifungal Activity							
C. albicans ATCC 10239	0.80 ± 0.00	0.80 ± 0.00	-	-	20 ± 0.00	0.8 ± 0.00	1.20 ± 0.00

Amp.: Ampicillin (10 mg), Erit.: Erythromycin (10 mg), Nys: Nystatin (30 µg/disc), DMSO: Dimethyl sulfoxide, Eth.: Ethanol. Values (mean of two replicates) indicate zone of inhibition in mm and include filter paper disc diameter (6 mm); G: gram reaction; "0": no inhibition

In the micro-well dilution assay (Tables 3), the MICs of the extracts are between 16 ± 0.00 and 64 ± 0.00 µg/mL., and both extracts appear to show significant activity against the bacteria species tested and yeast. MIC value was determined as 64 ± 0.00 µg / mL in all bacteria tested with the leaf ethanol extract. Also a MIC value of 16 ± 0.00 µg / mL was detected in only *C.*

albicans for both extracts. These results make us think that the reason why the extracts we obtained are more effective in fungi than all bacterial species is due to the different cell morphology of the fungi.

In a previous study, the antimicrobial activities of the various groups of the alkaloids of Amaryllidacea were reviewed [1]. In another study, the antibacterial and antitumor activity of different extracts of the *G. plicatus* were investigated, and it has been reported that the antitumor activity of *G. plicatus* gives better results than the antibacterial activity [44].

Table 3. Antibacterial and antifungal activity of the ethanol extracts of *G. gracilis* by micro-well dilution assay (MIC)

	Leaf Ethanol Extract (µg/mL)	Flower Ethanol Extracts (µg/mL)	Standards (µg/mL)		
	Eth.	Eth.	DMSO	Control	Eth.
Antibacterial Activity					
Staphylococcus aureus (ATCC 6538/P) G (+)	64 ± 0.00	32 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
Bacillus cereus (CCM 99) G (+)	64 ± 0.00	16 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
Escherichia coli (ATCC 35218) G (-)	64 ± 0.00	16 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
Pseudomonas aeruginosa (ATCC 27853) G (-)	64 ± 0.00	16 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
Antifungal Activity					
C. albicans (ATCC 10239)	16 ± 0.00	16 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00

G: gram reaction; DMSO: Dimethyl sulfoxide, Eth.: Ethanol., Concentration range (128-1 µg/mL)., '0': No inhibition.

4. Conclusion

The present study is the first study to demonstrate the antioxidant and antimicrobial potential of the ethanol extracts of the leaf and flower parts of *G. gracilis* from Muğla. The antioxidant potential of the ethanol extract of the flower appears to be greater than that of the leaf according

to the results of DPPH and *β*-carotene assay. The total phenolic content was determined to be higher in the ethanol extract of the leaf than in the flower extract. Gram (-) *P. aeruginosa* was detected as the most sensitive bacteria to both extracts. *C. albicans* turned out to be more sensitive than all bacteria tested with an inhibition zone (0.8 ± 0.00 mm) and a MIC value (16 ± 0.00 µg/mL). These results indicate that *G. gracilis* own potential antioxidant and antimicrobial activity

Ethics in Publishing

There are no ethical issues regarding the publication of this study.

Author Contributions

Fiction: A. T, M. T, Y. D; Literature: A. T; Methodology: A. T, M. T; Performing the experiment: A. T, M. T, Y. D; Data analysis: A. T, M. T, Y. D; Manuscript writing: A. T, M. T; Supervision: M. T. All authors approved the final draft.

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