International Journal of Biochemistry Research & Review

25(1): 1-12, 2019; Article no.IJBCRR.47439
ISSN: 2231-086X, NLM ID: 101654445

Evaluation of Galantamine, Phenolics, Flavonoids and Antioxidant Content of Galanthus Species in Turkey

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Authors' contributions

This work was carried out in collaboration between both authors. Author IB designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author YSK managed the analyses of the study and managed the literature searches. Both authors read and approved the final manuscript.

Article Information

DOI: 10.9734/IJBCRR/2019/v25i130068
Editor(s):
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Complete Peer review History: http://www.sdiarticle3.com/review-history/47439

Original Research Article

Received 24 October 2018
Accepted 18 February 2019
Published 06 March 2019

ABSTRACT

Aims: The aim of the present study was to determine the total phenolic and flavonoid content and antioxidant activities in Galanthus species (*Gaalanthus woronowii*, *Galanthus nivalis*, and *Galanthus elwesii*) indigenous to Turkey.
Study Design: The plant materials used in the study, *Galanthus elwesii* samples were collected in Antalya province, *Galanthus nivalis* samples were collected in Istanbul province, and *Galanthus woronowii* samples were collected in Çaykara, Trabzon province in September 2018.
Place and Duration of Study: Plant samples were stored in Herbarium Material Warehouse at Afyon Kocatepe University. The plant leaves and grated bulbs were dried in an incubator at 60°C. The bulb and leaf samples were then pulverized to 80 mesh particle size for analysis.
Methodology: Total phenolic content was determined spectrophotometrically with Folin-Ciocalteu procedure and calculated as gallic acid equivalent (GAE). Total flavonoid content was determined

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with aluminum chloride colorimetric method and calculated as catechin equivalent (CAE). Antioxidant activities were determined with TEAC (Trolox equivalent antioxidant capacity) and DPPH (diphenyl-p-picrylhydrazyl radical) methods. The phenolic acid and galantamine content were determined by reversed phase HPLC.

Results: The highest total flovanoid content was determined as 33 mg CAE/g DW in *Galanthus woronowii* leaves and as 27 mg CAE / g DW in bulbs. DPPH removal activity was 77% in 500 µg/mL *Galanthus woronowii* leaf extract concentration and 93% in the ascorbic acid control group. The highest antioxidant content was observed in the leaves of *Galanthus woronowii* as 23 µmol Trolox/100 g DW and as 21 µmolTrolox/100 g DW in the bulbs. Higher galantamine content was determined in aerial parts (leaves) when compared to the underground parts (bulbs). The galantamine content in the leaves of all three Galanthus species was about 0.082%. The galantamine content in the bulbs of all three species was about 0.045%. Gallic, protocatechic, vanilic, caffeic, syringic, rosmarinic acid and catechin were identified in the leaves and bulbs of the three species with HPLC phenolic acid analysisIt was determined that the major phenolic acid was gallic acid.

Conclusion: The present study findings demonstrated that Galantthus species has antioxidant capacity. Galanthus spp. leaves had higher antioxidant activity when compared to the bulbs. *Galanthus woronowii* exhibited the highest antioxidant activity among the scrutinized species.

Keywords: Galanthus spp; phenolic; flavonoid; antioxidant.

1. INTRODUCTION

The interest in natural antioxidants, especially those found in fruits and vegetables, have been increasing among consumers and scientists during recent years. Epidemiological studies demonstrated that frequent fruit and vegetable intake is associated with a lower age-related coronary heart disease [1] and cancer risks [2,3]. Several natural nutrients contain dietary antioxidants that could scavenge free radicals. Certain studies demonstrated that phenolics such as flavonoids, phenolic acids, and tannins are more powerful antioxidants when compared to vitamins C and E [4]. These phenolic compounds also serve various biological functions, including anti-inflammatory, anti-carcinogenic and anti-atherosclerotic activities, which may be related to their antioxidant activities [5]. Several studies demonstrated a high degree of correlation between the total antioxidant activity of certain fruits and their phenolic content [6].

In addition to fruit and vegetable-based antioxidants, another important source of antioxidants is traditional medicinal plants, which could exhibit stronger antioxidant activities when compared to traditional dietary plants [7,8]. The Amaryllidaceae family is the most important plant family among 20 plant families that contain alkaloid, and it includes 85 genera and about 1100 perennial bulb species. These plants are distributed in the warm climates and tropical regions in the world [9]. It was reported that *Galanthus* (Amaryllidaceae) genus has about 14 species and 1 hybrid (15 taxa) indigenous to Turkey [10]. The images of the three species in genus Galanthus are presented in Fig. 1.

Galanthus woronowii Losinsk. is one of the fourteen *Galanthus L.* species (fifteen taxa) that are indigenous to Turkey [11,12]. It is prevalent in Caucasus, Trans-Caucasus, southern Russia, Georgia and northeastern Turkey. This species grows in low-to medium altitudes between 20 and 1500 m, however they are usually observed between 200 and 600 m. *G. woronowii* has broad green leaves and is an attractive gardening plant [13,14]. Among the Galanthus species indigenous to Turkey, the bulbs of *G. elwesii Hook* and *G. woronowii* are exported [15,16]. *Galanthus woronowii Losinsk* (Woronowii snowdrop) and *Galanthus nivalis L.* (common snowdrop) are bulbous plant that flowers in spring and grown for its ornamental properties in gardens, which is also used in medicine. *Galanthus nivalis*, common snowdrop, is a perennial bulbous plant indigenous to Europe and southwest Asia. *Galanthus nivalis,* snowdrop or common snowdrop is the most well-known and most common among the 20 species of genus *Galanthus*. Snowdrops are among the first bulbs to bloom in the spring and they can reflect an impressive white feature in natural areas or where they were naturalized. *Galanthus woronowii,* which is indigenous to Turkey, Russia and Georgia, was named to honor Russian botanist and plant collector Georg Woronow (1874-1931).

The herbal pharmaceuticals developed from *Galanthus L.* genus plants contain several active biological compounds: Amarillidaceae alkaloids [17-19], flavonoids, organic and hydroxycinnamic acids [20]. Alkaloids are biologically active substances that reflect the strong pharmacological activities of medicinal plants [21,22]. *G. nivalis* and *G. elwesii* are two of the best known and most commonly grown bulbous plants. Their popularity is due to their beauty and longevity. They have numerous varieties and clones (Davis, 1999). Every year Turkey exports several bulbs collected from the nature. In early 1980s, this trade increased and millions of *G. elwesii* bulbs were exported to the Netherlands. The large volume of Galanthus bulb trade caused great concern, since it was unclear whether such a large bulb collection was sustainable. Thus, Galanthus was included in CITES Annex II in 1990. The harvest of wild *G. elwesii* bulbs is now controlled and monitored, and annual export quotas are determined. Certain wild snowdrop species are endangered, and harvesting bulbs in the wild is illegal in several countries. However, CITES allows the limited trade of the wild bulbs of only three species in Turkey (*G. nivalis, G. elwesii*, and *G. woronowii*) [23].

Galanthus elwesii Hook (Amaryllidaceae) is an easily recognized species with broad yellowish leaves, large flowers and thick marks on the inner parts. It has a relatively wide distribution in the eastern regions of former Yugoslavia, northern Greece, eastern Aegean Islands, southern Ukraine, Bulgaria and Turkey. This species has the widest distribution among other species in Turkey and it is indigenous to northwest, western and southern Anatolia [24,25]. *Galanthus elwesii* (snowdrop) is a small bulbous plant distributed throughout South-Eastern European countries and Eurasia [26], and the plant is cultivated for its elegant flowers. Earlier research on *Galanthus elwesii* led to the isolation of a large variety of Amaryllidaceae alkaloids [27-29]. It was found that many of these compounds exhibited strong acetylcholinesterase inhibitory, cytotoxic and antiviral activities among others [30].

Although the *Galanthus species* have been partially studied, most reported phytochemical studies demonstrated alkaloid structure diversity and no bioactivity studies were conducted. Thus, it is necessary to determine antioxidant capacity, phenolic and flavonoid content of this species.

2. MATERIALS AND METHODS

2.1 Plant Materials

The plant materials used in the study, *Galanthus elwesii*lbradi samples were collected in Antalya province, *Galanthus nivalis* samples were collected in Istanbul province, and *Galanthus woronowii* samples were collected in Çaykara, Trabzon province in September 2018. The plant was collected and identified by Mustafa KARCIOGLU. Plant samples were stored in Herbarium Material Warehouse at Afyon Kocatepe University. The plant leaves and grated bulbs were dried in an incubator at 60°C. The bulbs and leaf samples were then pulverized to 80 mesh particle size for analysis.

2.2 Chemical Materials

Chemicals and solvents used in all experiments were analytical purity. All chemicals used for chromatographic purposes were in HPLC purity. All solvents were filtered with a 0.45 µm filter (Millipore, Bedford, MA, USA). Galantamine hydrobromide, Gallic, protocatechic, vanilic, caffeic, syringic, rosmarinic acids and catechin standard were purchased from Sigma Chemical Co.

Fig. 1A. *Galanthus woronowii*

Fig. 1B. *Galanthus elwesii*

Fig. 1C. *Galanthus nivalis*

2.3 Ultrasonic Assisted Plant Extraction

Ultrasonic assisted extraction was conducted in ultrasonic bath (Bandelin Sonorex with a frequency of 50 kHz). 1 g leave and bulb samples of of the dried plants were weighed and each plant sample and extracted separately with 30 ml 70% methanol for 30 minutes. After the extraction, the mixture was filtered with Whatman brand white band filter paper and extract was stored in a +4°C refrigerator until the analysis. Total phenolic, flavonoid, and phenolic acid content, galantamine analysis and antioxidant capacity were determined using the plant extracts.

2.4 Determination of Galantamine Content with

Galantamine content of the extract was determined with the analysis method specified in the USP 40-NF 35 monograph. All analyses were conducted with an Agilent 1260 HPLC system equipped with a UV detector. The analytical column was an Agilent Zorbax extended C18 (5 µm, 150 mm X 5 mm) with a mobile phase that included a mixture of solvent A (acetonitrile) and B (water with 4.0 g/L. Potassium dihydrogen phosphate) and employed the isocratic elution (10/90, v/v) at a flow rate of 1.2 mL/min (Table 1). The column temperature was maintained at 30°C and the detection wavelength was set to 288 nm for galantamine. The solvent was filtered through a 0.22 µm filter and degassed. The sample injection volume was 20 µL [31].

Table 1. Analytical conditions of HPLC for galantamine analysis

Parameters	Conditions
Column	Zorbax extended-C18 (C18, 4.6 mm X 150 mm, 5 µm)
Flow rate	1.2 mL/min
Injection volume	20 µL
UV detection	288 nm
Run time	12 min

2.5 Total Phenolic Substance Determination

Phenolic content of the extracts was determined with the Folin-Ciocalteu method modified by Elzaawely and Tawata [32]. 7250 µl deionized water, 500 µL extract, and 250 µL Folin-Ciocalteu reagents were added to 10 mL tubes and mixed and stored in a dark environment for 5 minutes. After adding 2000 µl of Na_2CO_3 solution (7.5%), the volume was completed to 10 mL with deionized water and the mixture was incubated for 30 min. After incubation, the absorbance values of the samples were measured with a Shimadzu brand UV-1800 spectrophotometer at 765 nm wavelength. Gallic acid was used as the standard and the resultss were expressed in mg Gallic acid equivalent (GAE)/1 g dry weight (DW).

2.6 Total Flavonoid Substance Determination

Flavonoid content of the extracts was determined with aluminum chloride colorimetric method [33]. 50 µl of the extract was transferred into 10 ml test tube and 950 µl methanol and 6400 µl deionized water were added, followed by 300 µl of $NaNO_2$ solution (5%). Then, 300 µl of $AlCl_3$ solution (10%) was added to the mixture and remixed. After 5 minutes of incubation, 2000 µl of 1 M NaOH solution was added and the total volume of the mixture was completed to 10 mL. Mixture was incubated for 15 m. and the absorbance value was measured at 510 nm with a Shimadzu brand UV-1800 spectrophotometer. Catechin was used as the standard and the total flavonoid content was expressed as mg catechin equivalent (CAE) / 1 gr. dry weight.

2.7 Antioxidant Activity Analysis

2.7.1 2,2-Diphenyl-1-picrylhydrazyl test (DPPH)

Antioxidant activity of the extracts were determined with the 2,2-diphenyl-1-picrylhydrazyl (DPPH) test as previously described in certain previous modifications [34]. Briefly, 200 µL of each extract (100 µL/mL) was mixed with 3.8 mL DPPH solution and incubated for 1 hour at room temperature in the dark. The absorbance of the mixture was measured at 517 nm. Ascorbic acid was used as the positive control. The ability of the sample to remove the DPPH radical is determined using the following formula:

$$\% \text{ DPPH free radical} = [(A_{Blank} - A_{Sample})/A_{Blank}] \times 100$$

A_{Blank} is the absorbance of the control and A_{Sample} is the absorbance of the test compound.

The sample concentration that provided % 50 inhibition (IC50) was calculated by plotting the inhibition rates against the sample concentrations.

2.7.2 Inhibition of the ABTS*+ Radical Cation (TEAC) test

Antioxidant activities of the samples were determined with Trolox equivalent antioxidant capacity (TEAC) method [35]. This method is based on the inhibition of ABTS radical cation (ABTS * +), produced by the oxidation of 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and potassium persulfate ($K_2S_2O_8$), with the addition of antioxidants to the medium. The antioxidant activity is determined by the reduction of the absorbance of the radical that has blue-green color at 734 nm wavelength for 6 minutes. Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid) was used as the positive control. The sample antioxidant activity figures were expressed as µmol Trolox/100 g.

2.8 Determination of Phenolic Acids by HPLC

The phenolic acids were seperated with an Agilent 1260 series HPLC system equipped with C18 (4.6 mm × 150 mm, 5 µm) column. The flow rate of the mobile phase was 0.5 mL/min. Mobile phase A was water containing 0.02% TFA, and mobil phase B was methanol containing 0.02% TFA. The gradient conditions were as follows: 0-5 min, 25% B; 5-10 min, 25-30% B; 10-16 min, 30-45% B; 16-18 min, 45% B; 18-25 min, 45-80% B; 25-30 min, 80% B; 30-40 min, 80-25% B. The temperature of the column was controlled at 25°C. Injection volume was 10 µL. The detection wavelengths of DAD were set at four positions: 254, 275, 305, and 320 nm [36].

2.9 Statistical Analysis

The total phenolic and flavonoid contents of each sample were compared with the "Minitab® for Windows Release 1.12" program. An analysis of variance (ANOVA) table was made, general linear modeling was performed and whether the data were statistically significant. When the model was found to be meaningful, pairwise comparisons were made with the Tukey dual comparison test at 95% confidence level and the difference between the samples was examined. In addition, the results of antioxidant activity by DPPH method and total phenolic substance, flavonoid matter and anthocyanin content of all sample types were investigated by regression analysis with Minitab program. HPLC analyzes were performed with three replicates, mean values and standard deviation values were obtained.

3. RESULTS AND DISCUSSION

3.1 Determination of Galantamine Content with HPLC

Determination of the amount of galantamine in the samples was made out by reverse phase HPLC method. For this, the galantamine standard at 5 different concentrations (100, 200, 300, 400 and 500 mg / L.) was injected into the device. A graph of concentration was plotted against the resulting peak area. The obtained linearity graph was given in Fig. 2.

The HPLC chromatogrames of the galantamine standard and *Galanthus spp.* extract are presented in Figs. 3 and 4.

The galantamine content determined in the leaves and bulbs of all three *Galanthus species* are presented in Table 2. The galantamine content was higher in the erial parts (leaves) when compared to the underground parts (bulbs). Galantamine content of the samples are presented in Table 2.

3.2. Total Phenolic Content

The absorbance values for different concentrations of Gallic acid standard solutions were measured at 765 nm with a Shimadzu brand UV-1800 spectrophotometer. Gallic acid standard curve was plotted and presented in Fig. 5.

The total phenolic content in *G. nivalis, G. elwesii* and *G. Woronowii* leaves and bulbs are presented in Table 3.

Table 2. Galanthus spp. galantamine content

Galanthus species	Aerial and underground parts	Galantamine content % ± S.D.
Galanthus nivalis	Leaf	0.075 ± 0.0075
	Bulbs	0.038 ± 0.0090
Galanthus elwesii	Leaf	0.082 ± 0.0085
	Bulbs	0.045 ± 0.0090
Galanthus woronowii	Leaf	0.078 ± 0.0080
	Bulbs	0.040 ± 0.0095

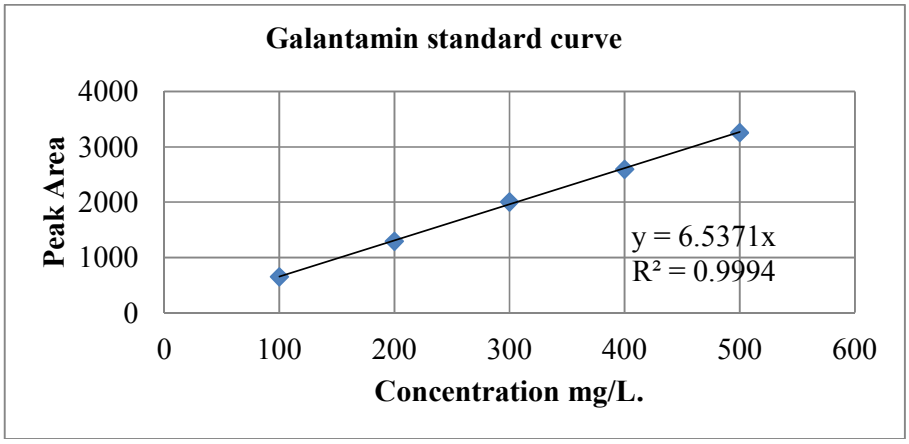

Fig. 2. Galantamine standard curve

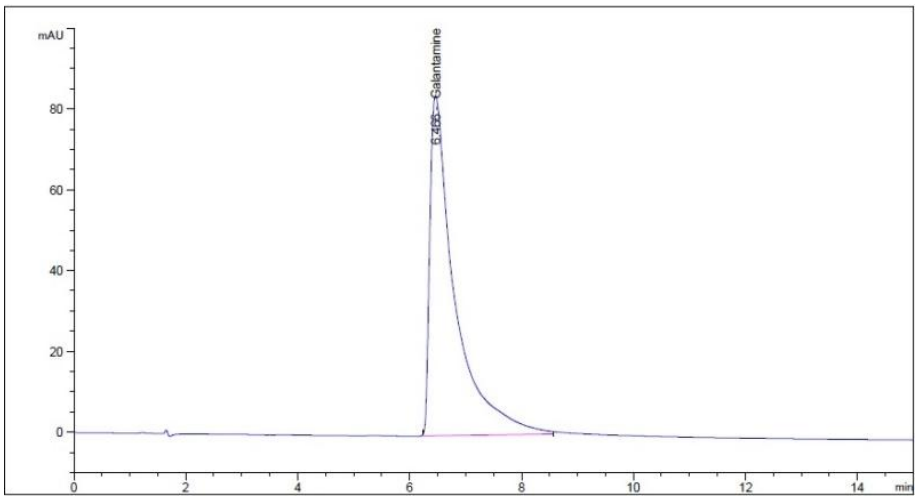

Fig. 3. HPLC chromatograme of galantamine standard solution (400 mg/L.)

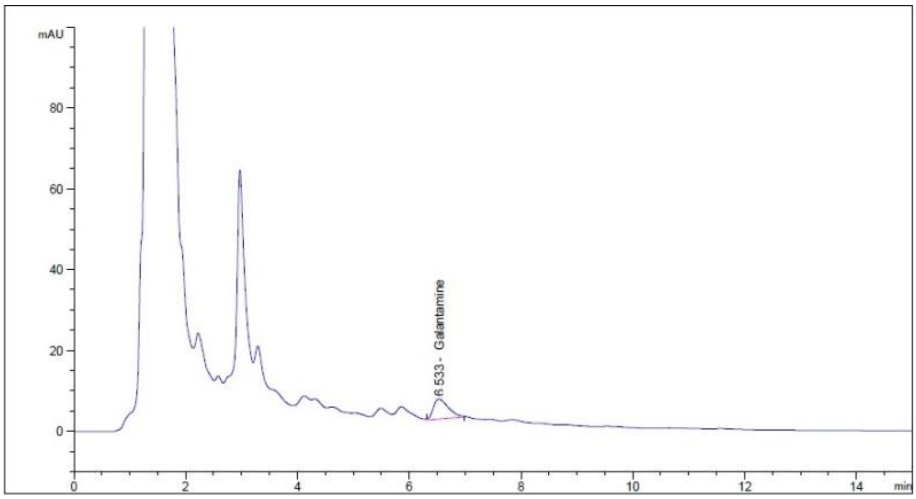

Fig. 4. HPLC chromatograme of *Galanthus woronowii* leaf extract

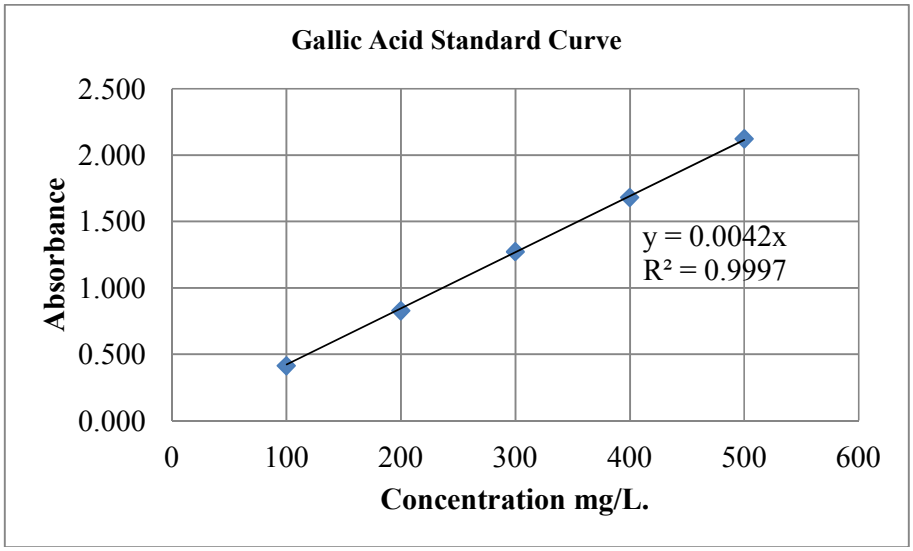

Fig. 5. Gallic acid standard curve

Table 3. Total phenolic content analysis findings

Galanthus species	Aerial and underground parts	Total phenolic content mg GAE / gr. D.W. ± S.D.
Galanthus nivalis	Leaf	36 ± 0.45
	Bulbs	32 ± 0.52
Galanthus elwesii	Leaf	34 ± 0.43
	Bulbs	30 ± 0.50
Galanthus woronowii	Leaf	44 ± 0.42
	Bulbs	36 ± 0.48

A relatively high total phenolic content was determined in the leaves of all three *Galanthus species* when compared to the bulbs. The total phenolic content of the *Galanthus woronowii* species leaves and bulbs were higher when compared to the other species. The highest total phenolic content (22 mg GAE/g DW) was determined in *Galanthus woronowii* bulbs.

3.3 Total Flavonoid Content

The absorbances for five concentrations of catechin standard solutions were measured at 510 nm wavelength with a Shumadzu brand UV-1800 spectrophotometer. Catechin standard curve was plotted and showed in Fig. 6.

The total flavonoid content in *G. nivalis, G. elwesii and G. Woronowii* leaves and bulbs are presented in Table 4.

Relatively higher total flavonoid content was determined in all three *Galanthus species* bulbs when compared to the leaves. The total flavonoid content in *Galanthus woronowii* leaves and bulbs were higher when compared to the other species. The highest total flavonoid content was determined in the bulbs of the *Galanthus woronowii* (5.3 mg GAE/g DW) species.

3.4 Antioxidant Activity

3.4.1 1,1-Diphenyl-2-Pixyl-Hydrazyl Test DPPH

Radical scavenging method was used to investigate the antioxidant capacity of the methanol extracts of three *Galanthus species* leaves and onions against free radicals. The DPPH radical is commonly used in the analysis of free radical scavenging activity due to its reaction facility. In this method, primarily the proton transfer reaction with the DPPH free radical due to the antioxidant leads to a decrease in absorbance at 510 nm. This process is based on monitoring the visible area with the spectrophotometer until the absorbance is constant. DPPH scavenging activity was 77% in the 500 µg/mL *Galanthus woronowii* leaf extract concentration and 93% in the ascorbic acid control group (Fig. 7). The percentage inhibition of species extracts was presented in Fig. 7.

3.4.2 Inhibition of the ABTS*+ Radical Cation (TEAC) test findings

The inhibition curve developed with different concentrations of the standard Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid) solutions is presented in Fig. 8.

G. nivalis, G. elwesii and *G. Woronowii* species leaf and bulb antioxidant content are presented in Table 5. The sample antioxidant activities were expressed as µmol Trolox/100 g.

3.5 Chromatographic Analysis of Phenolic Acids

Determination of the amount of phenolic acids in the samples was made out by reverse phase HPLC method. Gallic acid, chlorogenic acid, gentsic acid, vanillic acid, caffeic acid, syringic acid, sinapic acid, p-coumaric acid, ferulic acid, anisic acid, rosmarinic acid, salicylic acid, and cinnamic acid were used as standard material. For this, the phenolic acids standards at 5 different concentrations (20, 40, 60, 80 and 100 mg / L.) was injected into the device. A graph of concentration was plotted against the resulting peak area. Chromatograme of standard phenolic acids was presented in Fig. 9.

The highest phenolic acid content was observed in gallic acid content in the extracts, while the lowest phenolic acid content was observed in the vanilic acid content. Following the gallic acid, significant phenolic acid protocatechic acid content were determined in extracts. Phenolic acid content are presented in Table 6.

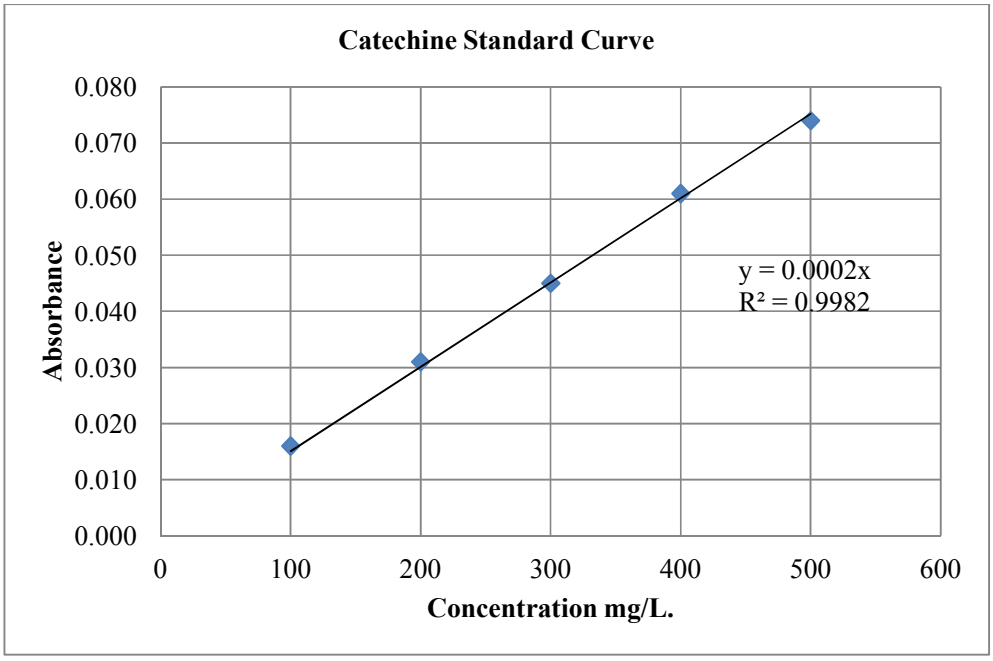

Fig. 6. Catechin standard curve

Table 4. Total flavonoid analysis findings

Galanthus species	Aerial and underground parts	Total flavonoid content mg CAE / gr. D.W.
Galanthus nivalis	Leaf	32 ± 0.25
	Bulbs	24 ± 0.20
Galanthus elwesii	Leaf	22 ± 0.30
	Bulbs	19 ± 0.22
Galanthus woronowii	Leaf	33 ± 0.28
	Bulbs	27 ± 0.18

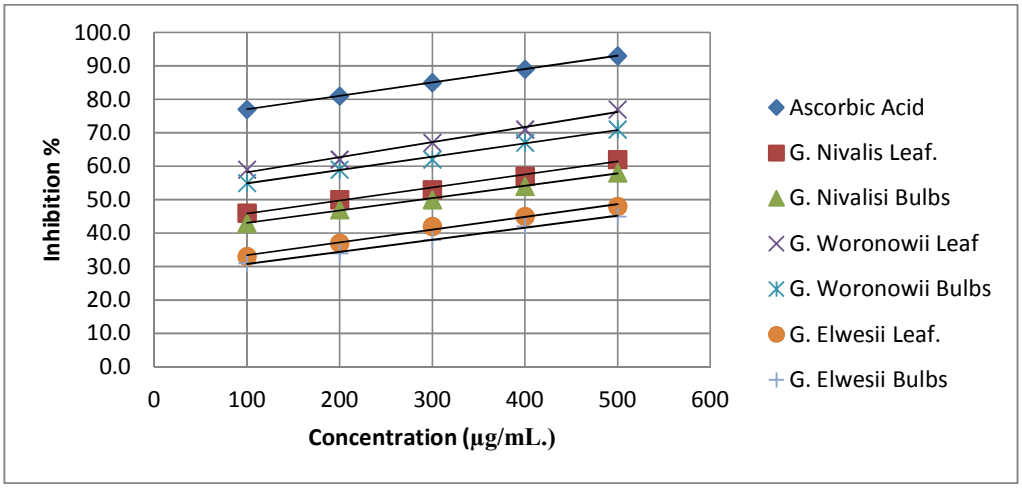

Fig. 7. Free radical scavenging activity of methanolic extracts of *Galanthus* spp. Ascorbic acid was included as a positive control

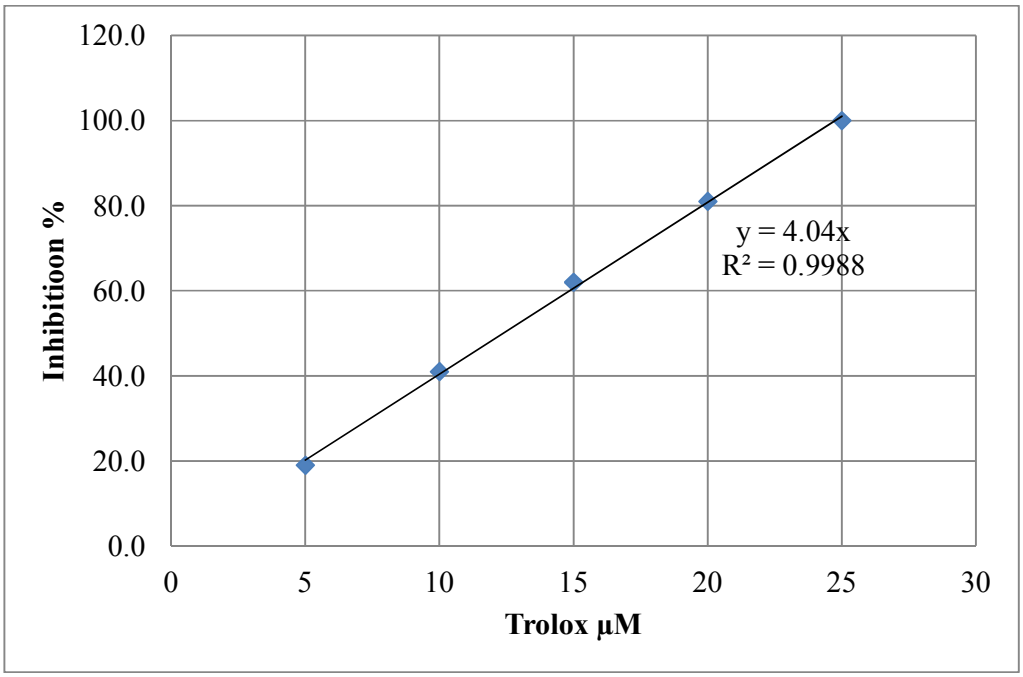

Fig. 8. ABTS*+ radical removal activity by Trolox standards

Table 5. Antioxidant activities

Galanthus species	Aerial and underground parts	Antioxidant activity µmol Troloks/100 g D.W. ± S.D.
Galanthus nivalis	Leaf	20 ± 0.78
	Bulbs	19 ± 0.80
Galanthus elwesii	Leaf	20 ± 0.85
	Bulbs	17 ± 0.78
Galanthus woronowii	Leaf	23 ± 0.64
	Bulbs	21 ± 0.71

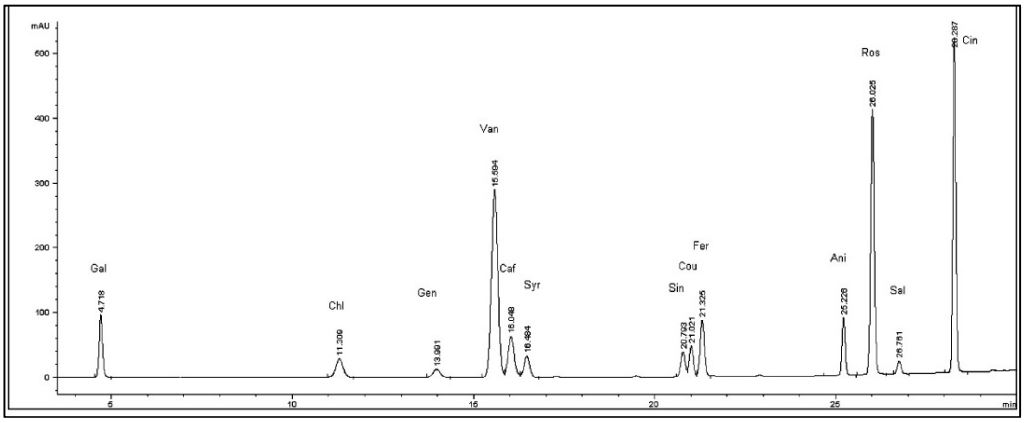

Fig. 9. Typical chromatogram of 13 phenolic acid standards

Gal = Gallic Acid; Chl = Chlorogenic Acid; Gen = Gentsic Acid; Van = Vanillic Acid; Caf = Caffeic Acid; Syr = Syringic Acid; Sin = Sinapic Acid; Cou = P-coumaric Acid; Fer = Ferulic Acid; Ani = Anisic Acid; Ros = Rosmarinic Acid; Sal = Salicylic Acid; Cin = Trans-cinnamic Acid

Table 6. Phenolic acid content of the samples

Galanthus Species	Aerial/Underground Parts	Gallic Acid (ppm) ± 0.88	ProtocatechicAcid (ppm) ± 0.79	Vanilic Acid (ppm) ± 0.37	Coffeic Acid (ppm) ± 0.26	Syringic Acid (ppm) ± 0.48	Coumaric Acid (ppm) ± 0.18	Rosmarinic Acid (ppm) ± 0.67	Catechin (ppm) ± 0.43
Galanthusnivalis	Leaf	17	12	2	5	7	4	12	8
	Bulbs	10	5	1	2	6	3	10	6
Galanthuselwesii	Leaf	15	10	3	4	5	3	14	7
	Bulbs	8	6	1	3	4	3	11	5
Galanthusworonowii	Leaf	18	11	3	3	6	5	14	6
	Bulbs	12	4	2	3	5	2	8	5

4. CONCLUSION

Most previous phytochemical studies on *Gallanthus species* have reported alkaloid structure diversity and no bioactivity studies have been conducted. The present study is the first in this area. Plants contain a significant number of phytochemical components, most of which are known to be biologically active and responsible for various pharmacological activities. Some of these secondary plant metabolites are preferred natural antioxidant sources against synthetic ones due to safety concerns. It was demonstrated that bioactive secondary metabolites reduce the risk and slow the progression of diseases such as cancer, cardiovascular and neurodegenerative diseases, etc. by scavenging free radicals through various biological mechanisms [37]. Phenols have the ability to remove the radicals since they contain hydroxyl groups. These important plant components release the hydrogen atoms from the hydroxyl groups to the radicals to form stable phenoxyl radicals. Therefore, they play an important role in antioxidant activity. Thus, it is very important to determine the plant phenolic compound content to determine the antioxidant capacity of plant extracts [38]. Excess free radical formation leads to cellular damage and several functional disorders in humans such as atherosclerosis, myocardial infarction, cancer and neurodegenerative diseases. However, natural antioxidant compounds are beneficial in repairing cellular free radicals and managing various chronic diseases. Antioxidant tests are highly specific and sensitive to temperatures and incubation periods. Furthermore, the physicochemical properties of the sample are

very important in the analysis of antioxidant properties [39]. Thus, the present study may serve as a guide for future researchers in pharmacology to conduct further studies on these plants by providing different perspectives.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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