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# EFFECTS OF HARVESTING LEAVES FROM DIFFERENT HEIGHTS OF SUMMER SNOWFLAKE (*Leucojum aestivum* L.) ON BULB DEVELOPMENT AND GALANTHAMINE CONTENT

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ABSTRACT. This research was carried out to optimize the optimum leaf length that could be used to obtain maximum galanthamine without damaging the growth of 9-10 g weight summer snowflake (*Leucojum aestivum* L.) during October 2017 and July 2019 for two years. The leaves were harvested at ground level, 5, 10, 15 cm above soil level and control (not harvested). Using a total of 600 bulbs, 30 bulbs were planted in each replication. Equally proportionate sand-soil substrate was used for planting. The length of leaf at harvest (cm), the number of leaves, the percentage of galanthamine in the leaves ranged 15.225-20.775 cm, 2.20-3.60 number, and 0.067-0.094% for the first year and it ranged 19.8-23.4 cm, 4.50-5.50 number, and 0.063-0.096% for the second year in the same order. In the second year, the amount of galanthamine in bulbs ranged from 0.326-0.376%. Harvesting the leaves from soil level negatively affected the bulb quantity and the least bulb was obtained. In control application without leaf harvest, one bulb weight and galanthamine amount reached the highest value. It has been determined that different leaf harvest heights have effects on bulb development and the amount of galanthamine in leaves and bulbs.

## 1. INTRODUCTION

Turkey has an important place in gene centers of world and has a rich population of plants. The number of taxa exceeds 12000 and the number of endemic species exceed 4000 plants. Geophytes constitute a great richness including approximately 850 species [1, 2]. Some of the geophytes come to the forefront with their medicinal properties. Summer snowflake (*Leucojum aestivum* L.) is a perennial plant in the narcissus (Amaryllidaceae) family that stands out with features like beautiful flowers

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and grows in wet and humid areas [3, 4]. Summer snowflake bulbs are used in landscaping in parks, gardens and buildings (around pools and moist areas, along with other ornamental plants [5-7].

Most of the alkaloids of the plants in the Amaryllidaceae family are noted for their antitumor and antiviral properties as well as anticholinesterase activity [8] One of these alkaloids Galanthamine is obtained from plants of the Amaryllidaceae family, like *Galanthus* L.*, Leucojum* L.*, Narcissus* L. and *Lycoris* Herb. since the 1960s, galanthamine has been commercially obtained from natural bulbs of summer snowflake and has been used as a valuable industrial resource [9-11]. It is the most important alkaloid used in the symptomatic treatment of Alzheimer's disease [12]. Its most popularly used alkaloid, lycorine, has an anti-tumor effects [13]. Galanthamine has also been used for many years in the treatment of polio (Poliomyelitis), nerve and muscle disorders [14]. The flowers are also collected and dried for evaluation in brewing and preparation of chamomile tea.

Galanthamine was first isolated from *Galanthus woronowii* Losinsk in 1952. But later the pharmacological properties of the summer snowflake bulb attracted the attention of the pharmaceutical industry. Due to their small size and variability in galanthamine content, *Galanthus* spp. Losinsk species were soon replaced by *L. aestivum* [15]. In Europe, galanthamine is currently obtained from *L. aestivum* and culture forms of the genus *Narcissus* L. [16]. Galanthamine alkaloids, as well as chemicals having different pharmacological effects, such as lectins and chelidonic acid, can also be obtained from the bulbs and leaves of summer snowflake. The plant bulbs are also used to produce ethanol [17, 18]. The amount of alkaloid and especially galanthamine varies greatly depending on plant genotype and geographical locations [19, 20].

The summmer snowflake is found in in the Black Sea natural expansion area of the lakes and in areas close to the Marmara region located within the forest (*Fraxinus angustifolia* Vahl.) ecosystems [21] in the provinces of Beyşehir country of Konya and Erzurum [22].

It is known that the side effects of active substances obtained from plants are less compared to those obtained by chemical synthesis. Therefore, the use of active substances obtained from both summer snowflake and other medicinal plants in the pharmaceutical industry has gained importance in recent years.

Like all other bulbous plants, obtaining the bulbs from their natural habitats, with improper use of forest areas, urbanization, agricultural practices, excessive and irregular grazing, etc. in the natural ecosystem are among many reasons that are affecting their growth and development negatively and is resulting in danger of their extinction. The pressure on these plants has increased due to their medicinal properties in recent years. The export of the summer snowflake bulbs growing under wild conditions is prohibited after last permission to remove 3 million summer snowflake bulbs from the wild in the previous years. Only the bulbs obtained after cultivation are allowed to export. This issue is considered by the Ministry of Agriculture and Forestry as it is a wild growing bulbous plant [23]. Turkey's has been engaged in collecting and exporting of bulbs collected from wild to various countries in the world, especially in European countries for a long time. Both bulbs and leaves of the Turkish summer snowflake are in high demand from abroad especially Bulgaria in recent years.

Klosi et al. [4] found the average galanthamine content of summer snowflake bulbs as 0.13% in the first week of April (flowering phase) and 0.14% in late June at the end of flowering period. It is also stated that galanthamine was present in the leaves of the plant as an important source [4, 24]. Georgievaa et al. [20] mentioned that galanthamine or lichorine was found as the main alkaloids in the clusters of shoots obtained under *in vitro* conditions and that the galanthamine content ranged 28 - 2104 μg $g^{-1}$ in bulbs and 454 μg $g^{-1}$ dry weight in shoots.

Petruczynik et al. [25], in their studies examining various extraction procedures using maceration followed by extraction in the ultrasonic bath, found 0.0949 mg $mL^{-1}$ galanthamine and 0.1926 mg $mL^{-1}$ lorin that is significantly higher concentration of alkaloids investigated. Berkov et al. [10] reported 3 fold higher galanthamine concentration (average 74 μg $g^{-1}$ dry weight) from the cultures obtained under light conditions compared to the galanthamine concentration (average 39 μg $g^{-1}$ dry weight) obtained from the clusters under dark conditions. Furthermore, they obtained 5 times higher galanthamine than the concentration obtained from the shoots obtained under *in vitro* conditions.

Many studies on the ecological requirements, anatomy, physiology, galanthamine content, medicinal properties and production methods of summer snowflake have been mentioned in previous studies [13, 18, 19, 21, 26, 27]. However, it has not been discussed how the harvesting of leaves from different heights affects the development of the plant and the amount of galanthamine.

Therefore, the aim of this study was to determine the effect of different leaf heights harvest of summer snowflake (*L. aestivum*) on bulb growth and the potential amount of galanthamine in leaves and bulbs.

## 2. Materials and Methods

### 2.1 Experimental Plan and Applications

This work was carried out between October 2017 and July 2019 (two years) at Uşak University Faculty of Agriculture and Natural Sciences under greenhouse conditions. The summer snowflake bulbs had an average weight of 9-10 g and a circumference of 7-8 cm at the start of the planting and were planted in randomized complete blocks design with 4 replications. The experiment determined the most appropriate level of 4 leaf harvesting heights (the leaves cut at the ground level, 5, 10 and 15 cm above soil) to obtain maximum galathamine. The leaves were not harvested under control treatment in order.

A total of 600 bulbs were used in the study and each replication included 30 bulbs. Sand and soil were mixed in 1:1 percentage to fill the crates for growth and development of summer snowflakes. After planting, the bulbs were watered, irrigated and the other maintenance operations were carried out to the extent needed. Leaf lengths were measured. The leaves harvest operations were performed at the time of the yellowing and drying of the upper parts of the leaves. The leaves left on the soil were waited until they were completely dried by watering when needed.

The bulbs were not removed from the crates during first year and they spent dormant period in the soil, that were watered periodically at drying. Leaf lengths and numbers were measured in the first and second years. They were harvested during two years. At the end of the second year, the bulbs were removed from the soil. The leaves of different length obtained during two years were dried and the galanthamine values were determined for the two years. The number of leaves per plant, fresh leaf weight, leaves height, the amount of galanthamine in the leaves were measured during both years. The number of harvested bulbs, the weight of bulbs and the amount of galanthamine in the bulbs were determined in the second year. The amount of galanthamine in harvested bulbs and harvested leaves was analyzed using HPLC.

### 2.2 HPLC Device Galanthamine Analysis Method

#### 2.2.1 Sample Preparation

300 mg of leaves were weighed, dried and ground and added to 30 ml of 0.1 M HCL solution. After extraction in the ultrasonic bath for 15 minutes, it was filtered with filter paper and the extract was taken to the refrigerator until analysis.

2.2.2 Analysis Method

Analyzes were performed with Agilent brand 1260 HPLC device. Galanthamine hydrobromide (C17H21NO3 HBr) was used as standard. Galanthamine hydrobromide equivalent to 100 mg of Galanthamine was weighed and dissolved in deionized water to make up to 100 ml of deionized water. Working standard solutions of five different content percentages (20, 40, 60, 80, 100 ppm) were prepared using a standard stock solution at a content percentages of 100 ppm. Injected into HPLC. Content percentages graph was generated against peak areas [28] (Fig. 1). Chromatographic System: Detector: UV 288 nm; Column: 4.6-mm´ 15.0-cm; 5-mm packing L1 Mobile Phase A: 4.0 g $L^{-1}$ Monobasic Potassium Phosphate solution was prepared. The pH was adjusted to 6.5 with 5 N Sodium Hydroxide. (90%) Mobile Phase B: Acetonitrile (10%); Flow Rate: 1.2 mL $min^{-1}$; Injection Volume: 20 μL.

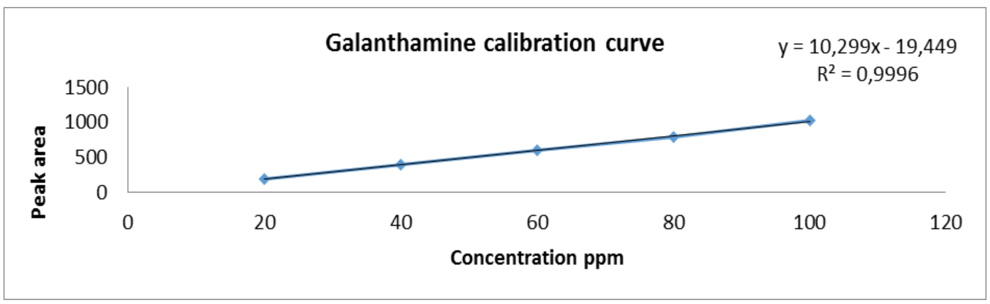


FIGURE 1. Galanthamine calibration curve

2.3 Statistical Analysis

Statistical analysis of the mean values of the measured characters was made with SPSS package program and the differences between the averages were determined by Duncan test [29].

## 3. Results and Discussion

Table 1. Average values and duncan groups of the measured parameters

| Treatments | Number of bulbs | Bulb weight (g)** | One bulb weight (g)* | Galanthamin percentage in bulbs (%)** | Number of leaves | |
|---|---|---|---|---|---|---|
| | | | | | First year ** | Second year |
| **Harvesting at 15 cm height** | 66.500 | 418.000 bc | 6.368 b | 0.342 b | 2.975 b | 4.875 |
| **Harvesting at 10 cm height** | 68.000 | 439.775 ab | 6.495 b | 0.339 b | 3.175 ab | 4.950 |
| **Harvesting at 5 cm height** | 60.250 | 363.900 cd | 6.083 b | 0.326 b | 2.200 c | 5.200 |
| **Harvesting at 0 cm surface level** | 55.500 | 349.125 d | 6.343 b | 0.328 b | 3.175 ab | 4.500 |
| **Control** | 61.750 | 498.600 a | 8.085 a | 0.376 a | 3.600 a | 5.500 |
| **General Average** | 62.400 | 413.880 | 6.675 | 0.342 | 3.090 | 5.025 |

** The differences between the mean values were statistically significant at the 0.01 level.
* The differences between the mean values were statistically significant at the 0.05 level.

Table 1. Continued…

| Treatments | Fresh leaf weight (g) ** | | Galanthamine percentage in leaves (%) | | Leaf length (cm) | |
|---|---|---|---|---|---|---|
| | First year ** | Second year ** | First year ** | Second year ** | First year ** | Second year * |
| **Harvesting at 15 cm height** | 20.598 c | 22.873 c | 0.067 c | 0.063 d | 18.840 c | 21.375 bc |
| **Harvesting at 10 cm height** | 36.240 b | 41.995 b | 0.080 b | 0.069 cb | 20.675 a | 21.950 ab |
| **Harvesting at 5 cm height** | 37.210 b | 44.400 b | 0.082 b | 0.076 b | 15.225 d | 21.800 ab |
| **Harvesting at 0 cm surface level** | 52.070 a | 75.738 a | 0.094 a | 0.096 a | 19.750 b | 19.800 c |
| **Control** | 47.845 a | 69.375 a | 0.079 b | 0.066 cd | 20.775 a | 23.400 a |
| **General Average** | 38.793 | 50.876 | 0.080 | 0.074 | 19.508 | 21.945 |

** The differences between the mean values were statistically significant at the 0.01 level.
* The differences between the mean values were statistically significant at the 0.05 level.

100% output was observed in all of the planted bulbs. Every two years; number of leaves, fresh leaf weight, galanthamine percentage in leaves, leaf length, and second year; number of bulbs, bulb weight, one bulb weight, galanthamin percentage in bulbs obtained from the experiment. Variance analysis results, mean values and duncan tests are given below (Table 1).

The maximum (68.0) and minimum (55.5) number of bulbs was obtained once the leaves were harvested at height of 10 cm and at the ground level respectively. These treatments showed numerical variability in average number and that did not show any statistically significant difference (Table 1).

The differences between average bulb weight and single bulb weight values were found significantly different ($p<0.01$). The maximum weight was obtained in control treatments during both years (498.600 g and 8.085 g). The minimum bulb weight (349.125 g) was obtained when the leaves were harvested at the ground level (Table 1).

It was found that the differences between the mean values of bulb galanthamine percentage were significantly different ($p<0.01$) and the highest percentage was obtained from control treatment (0.376%). It was seen that the differences between the average values obtained from other treatments were not significantly different (Table 1).

The differences between the average number of leaves were significantly different ($p<0.01$) in first year, and not significant in second year. The maximum values in the first and second year were noted as 3.600 and 5.500 respectively in control treatments (Table 1).

The differences between the average values of fresh leaf weight were significantly different ($p<0.01$) for two years. The maximum value was obtained when the leaves were harvested at the ground level in each years (52.070 g in the first year and 75.738 g in the second year). However, the differences between control averages were not significantly different (Table 1).

The differences between the average values of galanthamine obtained from the leaves were found significantly different ($p<0.01$) during both years. In both years; the maximum galanthamine percentage was obtained when the leaves were harvested at the ground level (0.094% during the first year, 0.096% in the second year), and the least galanthamine percentage was obtained when the leaves were harvested at 15 cm above soil level (0.67% during the first year and 0.063% in the second year) (Table 1).

The differences between the mean values of leaf height were significantly different ($p<0.01$) during both years. The maximum leaf height of (20,775 cm and 23,400 cm) was obtained from the control treatments in the first and second years respectively.

Comparing results of all treatments; it is seen that the bulb formation is less in the control treatment; whereas the control treatment had larger bulbs, and highest bulb weight was obtained. It was noted that when the leaves were harvest at the ground level the plants lacked photosynthesis that affected both the number of bulbs and bulb weight negatively. The bulb weight increased proportionally with each increase in leaf length at the time of harvest from soil level to 5 cm and 10 cm. The bulb weight and number of bulbs increased in direct proportion to the increase in the length of leaves remaining above the ground level.

It is seen that the percentage of galanthamine tend to increase (excluding control treatment) with increase in length of leaves. It was noted that the amount of galanthamine in the leaf was not high in the control treatment. This situation can be explained; when the leaves are not harvested, with the drying of the leaves, galanthamine moves from leaf to bulb and accumulates in the bulb [30]. The amount of galanthamine in the bulb was higher, when the leaves were not harvested (control treatment). At the same time the maximum galanthamine amount after control treatment was obtained at 15 cm harvesting lengths of leaves, but there were no differences among the other treatments mean values.

According to Ayan et al. [27] ; $GA_3$ and NAA treatments were carried out together in shade and light environment on summer snowflake that showed improvement of leaf length to 38.07 cm and 47.10 cm, the number of leaves and number of bulblets that ranged 5.28- 6.20 and between 0.63-1.33 in the same order. They found that the length of the plants obtained from $GA_3$ treatment was longer compared to the plants obtained after NAA treatment. The leaf heights in this experiment was shorter compared to the leaf heights noted by other researchers in their experiments.

Berkov et al. [19] found that galanthamine content in summer snowflake populations collected from different regions of Bulgaria was 0.003-0.08% in the northern regions and 0.42% in the southern regions and increased up to 0.65% in some single plant samples. According to geographic distributions, the presence of a large number of chemotypes was mentioned and they indicated that the amount of galathamine varies greatly among chemotypes. The findings of the researchers are in parallel with the findings in this research.

Yildirim et al. [24] have determined that hormone application has an effect on summer snowflake growth and yield criteria. Most galanthamine was determined in bulbs, followed by leaves and roots. They also stated that the leaves can be used to obtain galanthamine. Galanthamine general mean values were determined as 0.062 in roots, 0.089 in leaves and 0.245 in bulbs. While the rate of galanthamine in the leaf was similar, the amount of galantamine in the bulbs were found to be higher in our research.

Many researchers have reported that amount of galanthamine in the leaves of summer snowflake is very important source for obtaining and production of raw material in the pharmaceutical industry with 0.30% percentage in bulbs and 0.34% in leaves [26, 31-33].

Kreh [30], in a study performed on a daffodil species and their fresh bulb weight measurements noted that the amount of galanthamine content (in November) decreased slightly immediately after planting. Furthermore they foıund that the amount of galanthamine increased by 400% three months post planting, and the amount of galanthamine reached to the peak from mid-April to the beginning of June. Thereafter, it decreased with the aging of leaves. In the same study, it was noted that the amount of galanthamine increased dramatically in the above-ground parts before flowering, it fell slowly until the end of flowering and then remained stable for a certain time and reached the least level when the leaves were completely dry.

In another study conducted in daffodils, it was noted that galanthamine content varied according to plant type, harvest period and weather conditions. Galanthamine was found to be the maximum between 0.2% and 0.5% in leaves of eight daffodils. They found the highest percentage of galanthamine when the growth rate was at the top of the bud formation stage [26, 34, 35].

Galanthamine content in dry leaves of summer snowflake varies from low to 0.5% (usually 0.1-0.3%) according to population and geographical regions [15], and in another study, galanthamine content ranged 0.9-2.6 mg $g^{-1}$ [36]. The galanthamine values we obtained from the leaves were lower than those obtained from these studies. It can be said that these differences are caused by ecological factors, a complex physiological cycle of the plant and different chemotypes [24].

## 4. Conclusion

According to the results; it has been determined that the moving of the leaves of the summer snowflake has an effect on the bulb weight and galanthamine content both in leaves and bulbs. The control treatment stands out in terms of galanthamine yield in bulbs (since bulb weight and galanthamine percentage were higher). Excluding control treatment, harvesting of leaves at 10 and 15 cm could be recommended for bulbs to gain weight. The leaves harvesting at the ground level may be recommended when obtain galanthamine from leaves (every two years fresh leaf yield was highest when the leaves were harvested at the ground level). However, when the leaves were harvested at the ground level it had negative impact on both bulb induction and weight. In this case, the development bulbs was negatively influenced when the leaves were harvested at the ground level. It is vital importance that, in order to avoid such problems instead of harvesting the leaves at the ground level, it would be appropriate to harvested them at a height 10 or 15 cm. Inappropriate height of leaves at the time of harvest could have negative effects on bulb development. Because the leaves of the plant have important role in photosynthesis and consequently on the development of the bulbs. Therefore they must be cut at appropriate length/height to allow photosynthesis for a long time.

It is presumed that this study will significantly influence in designing of future studies. And also; studies should be made for determining and detection of different summer snowflake chemotypes with high galanthamine content to achieve high value products. This research will make an important contribution to the studies of summer snowflake.

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