T.R.
BOLU ABANT İZZET BAYSAL UNIVERSITY
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DEPARTMENT OF BIOLOGY

DETERMINATION OF SEASONAL VARIATION IN ALKALOID CONTENT, MORPHOLOGICAL CHARACTERIZATION AND EVALUATION OF BIOLOGICAL ACTIVITIES OF *LEUCOJUM AESTIVUM* L. (SUMMER SNOWFLAKE) POPULATIONS GROWING IN DIFFERENT LOCATIONS OF TURKEY

DOCTOR OF PHILOSOPHY

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APPROVAL OF THE THESIS

DETERMINATION OF SEASONAL VARIATION IN ALKALOID CONTENT, MORPHOLOGICAL CHARACTERIZATION AND EVALUATION OF BIOLOGICAL ACTIVITIES OF *LEUCOJUM AESTIVUM* L. (SUMMER SNOWFLAKE) POPULATIONS GROWING IN DIFFERENT LOCATIONS OF TURKEY submitted by **Sina Cafer DEMİR** and defended before the Examining Committe Members listed below in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** in **Department of Biology, Institute of Graduate Studies of Bolu Abant Izzet Baysal University in 27.07.2021** by

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ABSTRACT

DETERMINATION OF SEASONAL VARIATION IN ALKALOID CONTENT, MORHOLOGICAL CHARACTERIZATION AND EVALUATION OF BIOLOGICAL ACTIVITIES OF *LEUCOJUM AESTIVUM* L. (SUMMER SNOWFLAKE) POPULATIONS GROWING IN DIFFERENT LOCATIONS OF TURKEY
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Leucojum aestivum L. (summer snowflake), bulbous plant of Amaryllidaceae family, is a well-known producer of pharmaceutically important alkaloid galanthamine for the treatment of Alzheimer's disease. Another striking alkaloid lycorine has strong antiviral activity especially against SARS-CoV-2 (COVID-19). Objective of the study was to determine the best population of *L. aestivum* in regard to galanthamine and lycorine content by correlating the growing seasons of the plant, soil micro and macro elements, and monthly average temperature and precipitation. Morphological measurements, total phenol-flavonoid content, antioxidant capacity and antibacterial potential were also evaluated. Bulbs and leaves of *L. aestivum* were collected from 6 different locations in Turkey (Gölcük-Bolu, Yeniçağa-Bolu, Kaynarca-Sakarya, Delmece-Yalova, Uluabat-Bursa and Terkos-İstanbul) at 3 different growing periods (vegetative, reproductive and ripening). Sample collection also continued after leaf senescence for two locations in Bolu. Bulbs and leaves were quantitatively analyzed for their alkaloid content with HPLC-DAD. Generally, vegetative and ripening periods were efficient in alkaloid production and higher temperature increased the alkaloid content of the bulbs. Neutral to slightly alkaline soils with high organic matter content were effective in enhancing the alkaloid content. In the numerical evaluation of all taxa, principal component and cluster analysis were used, and two locations in Bolu were the most similar than other locations. Higher antioxidant capacity was observed with leaves in relation with the highest total phenolic content and tested bacteria were not susceptible to the extracts. The locations of Yalova and Bolu-Gölcük provided galanthamine rich population and optimal growing conditions for the cultivation of this species.

KEYWORDS: Antioxidant, Galanthamine, *Leucojum aestivum*, Lycorine, Phenol

ÖZET

TÜRKİYE'DE FARKLI LOKASYONLARDA YETİSEN *LEUCOJUM AESTİVUM* L. (GÖLSOĞANI) POPÜLASYONLARININ ALKALOİD İÇERİGİNİN MEVSİMSEL VARYASYONUNUN BELİRLENMESİ, MORFOLOJİK KARAKTERİZASYONU VE BİYOLOJİK AKTİVİTELERİNİN DEĞERLENDİRİLMESİ
DOKTORA TEZİ
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BİYOLOJİ ANABİLİM DALI
(TEZ DANIŞMANI: DOÇ. DR. İSMAİL EKER)
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Amaryllidaceae familyasının soğanlı bitkisi olan *Leucojum aestivum* L. (gölsoğanı), Alzheimer hastalığının tedavisi için farmasötik açıdan önemli olan alkaloid galantaminin iyi bilinen bir üreticisidir. Bir başka çarpıcı alkaloit likorin, özellikle SARS-CoV-2'ye (COVID-19) karşı güçlü antiviral aktiviteye sahiptir. Çalışmanın amacı, bitkinin büyüme mevsimi, toprak mikro ve makro elementleri ve aylık ortalama sıcaklık ve yağış arasında ilişki kurarak galantamin ve likorin içeriği açısından *L. aestivum*' un en iyi popülasyonunu belirlemektir. Çalışmamızda morfolojik ölçümler, toplam fenol-flavonoid içeriği, antioksidan kapasite ve antibakteriyel potansiyel de değerlendirilmiştir *L. aestivum*' un soğan ve yaprakları Türkiye'de 6 farklı lokasyondan (Gölcük-Bolu, Yeniçağa-Bolu, Kaynarca-Sakarya, Delmece-Yalova, Uluabat-Bursa ve Terkos-İstanbul) 3 farklı yetiştirme döneminde (vejetatif, reprodüktif ve olgunlaşma) toplanmıştır. Bolu'da iki lokasyon için yaprak senesensinden sonra da numune toplanmaya devam edilmiştir. Yumrular ve yapraklar, HPLC-DAD ile alkaloit içerikleri için kantitatif olarak analiz edilmiştir. Genel olarak vejetatif ve olgunlaşma dönemleri alkaloit üretiminde verimli olmuştur ve yüksek sıcaklık soğanların alkaloit içeriğini artırmıştır. Yüksek organik madde içeriğine sahip nötr ila hafif alkali topraklar, alkaloid içeriğini arttırmada etkili olmuştur. Tüm taksonların sayısal olarak değerlendirilmesinde temel bileşen ve küme analizi kullanılmış olup, Bolu'daki iki lokasyon diğer lokasyonlara göre en benzer olarak görülmüştür. En yüksek toplam fenolik içerikle ilişkili olarak yapraklarda daha yüksek antioksidan kapasite gözlemlenmiştir ve test edilen bakterilerin ekstraktlara duyarlı olmadığı görülmüştür. Yalova ve Bolu-Gölcük lokasyonları, bu türün yetiştirilmesi için galantamin bakımından zengin popülasyon ve optimal büyüme koşulları sağlamıştır.

ANAHTAR KELİMELER: Antioksidan, Galantamin, *Leucojum aestivum*, Likorin, Fenol

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LIST OF ABBREVIATIONS AND SYMBOLS

AChE	**:** Acetylcholinesterase
Ach	**:** Acetylcholine
AD	**:** Alzheimer's Disease
AF	**:** After flowering
APG	**:** Angiosperm Phylogeny Group
BChE	**:** Butyrylcholinesterase
BF	**:** Before flowering
DPPH	**:** 2,2-Diphenyl-1-picrylhydrazyl
F	**:** Flowering
FDA	**:** Food and Drug Administration
F-C	**:** Folin – Ciocalteau Method
GAE	: Gallic acid equivalents
GAL	**:** Galanthamine
HPLC	**:** High Performance Liquid Chromatography
LY	**:** Lycorine
OTUs	**:** Operational taxonomic units
PCA	**:** Principle component analysis
QE	**:** Quercetin equivalents

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1. INTRODUCTION

Throughout history, mankind has benefited from existing nutrients in order to ensure their nutrition, then chose to produce foods in a planned way, thus seeking methods of self-protection against disease factors. Human beings first recognized the wild plants in their immediate surroundings, determined what they could and not eat, the poisonous ones by trial and error, and then began to benefit from the plants in their immediate vicinity to cure diseases (Ceylan, 1995).

The oldest written records documenting the use of medicinal plants in Anatolia are included in the Hittite tablets found in Hattusa, the capital of the Hittites. From these records, prescriptions of thuja, mustard, licorice, almond, mandrake, garlic, willow and olive were found (Kan and Arslan, 2001).

Information about the use of medicinal plants in Anatolia during the Ottoman Empire period is found in the studies of Ibn Battuta (1304-1369) and Evliya Çelebi (1611-1685). Interest in medicinal plants decreased with the industrial revolution in the 1900s. However, due to reasons such as high side effects of synthetic drugs, cheaper and easier supply of herbal drugs, herbal products having more than one effect, medicinal plants and biologically active compounds obtained from them have become the focus of attention again. Studies on Anatolia have become prominent in the Republic period and thus, researches and publications on nature and human relations have started (Baytop, 1999; Kendir and Güvenç, 2010; Badem, 2019). Ord. Prof. Dr. A. Heilbronn (1885-1961) ushered in medicinal plant research in the field of pharmacognosy in Turkey (Baytop, 2003). In an article prepared by Sadıkoğlu and Alpınar (2004) covering the Republican period (1928-1997), 765 publications on Turkish ethnobotanics were examined. It has been determined that 466 of these publications are related to the medicinal uses of plants.

Turkey is located at the intersection of the Mediterranean and Near Eastern gene centers and situated at the crossroads of Europe-Siberia, Mediterranean and Iran-Turan phytogeographic regions. Because of these reasons, our country has a special position in terms of plant genetic resources (Vavilov, 1994). Factors such as being surrounded by seas on three sides, having different elevations, slope conditions, and having different climatic and edaphic characteristics have increased the plant diversity of our country (Saya and Güney, 2014; Gözcelioğlu, 2016).

The floristic studies in Turkey were initiated by French botanist Tournefort. He conducted researches in Western Anatolia, North Anatolia and Eastern Anatolia Regions and published the collected plants in 1702 (Burtt, 2001). The first major study "*Flora Orientalis*" in Turkey that include 6000 plant specimens was published between 1867 and 1888 by the Swiss botanist Pierre Edmond Boissier. The most important floristic research was carried out between 1965-1988 under the editorship of British botanist Peter Hadland Davis. "Flora of Turkey and the East Aegean Islands" was written in 9 volumes. The first additional volume was published in 1988. Including this volume, the total number of plants was 8.575. 2.651 of them are endemic. The endemism rate was found to be 30.9% (Davis et al., 1965-1985; Davis et al., 1988). After 1988, a second supplementary volume containing new plants was published. With this additional volume, the number of species has reached 8988. 2991 species are endemic and the percentage of endemism is approximately 33% (Güner et al., 2000). The third supplementary list was published by Özhatay and Kültür in 2006. There are 295 taxa in this list. 175 taxa with the fourth list published by Özhatay et al. in 2009. The fifth list and 249 taxa in 2011. In 2013, 228 taxa were added to the sixth list. The number of taxa in Turkey reached 11707 and the rate of endemism reached 31.82% in the checklist titled "*Türkiye Bitkileri Listesi-Damarlı Bitkiler*" written by Turkish botanists (Güner et al., 2012). The first volume of "*Resimli Türkiye Florası*" was published by Turkish botanists in 2014 under the auspices of Presidency of Republic of Turkey. In the first volume, information about the geographical, geological, edaphic, vegetative and climatic diversity of Turkey is given. In addition, the botanical history of Anatolia, cultivated plants, ethnobotanical information, Turkish plant terminology are included in this study (Güner and Ekim, 2014). The second volume of the work was published in 2018, and includes some of the plants in our country (Güner et al., 2018).

Turkey as well as in terms of other plants has also quite rich in terms of geophytic flora. Most of the geophytes which spend most of the year in the form of bulbs, tubers and rhizomes under the ground, bloom in the first days of spring and some of them in autumn. The diversity of geophytes in Anatolia which are used as ornamental, food and medicinal plants have an important place economically and have attracted the attention of foreign researchers since the 16th century (Demir and Eker, 2015). Bulbous plants have an important place among the plants exported abroad for about a century. Especially, since the 1960s, the trade of bulbous plants has reached very high levels, thus many species collected from nature have been severely damaged (Ekim et al., 1991).

Some of the bulbous plant species traded belongs to the Amaryllidaceae family. According to the APG IV (*Angiosperm Phylogeny Group IV*) system; Amaryllidaceae family within the order of Asparagales from monocotyledons, consists of herbaceous, bulbous and perennial plants. *Leucojum aestivum* L. (summer snowflake) which is the only natural species of the genus *Leucojum* L. in our country belongs to the Amaryllidaceae family and is one of the bulbous plants that are traded. Summer snowflake is very important medicinal plant and also a valuable ornamental plant. Both bulbs and fresh green leaves of the species contain many important chemicals, especially galanthamine alkaloid (Çiçek et al., 2013). It is a valuable export material in Turkey exporting 6 000 000 pieces of bulbs every year for galanthamine production to pharmaceutical industry (Resmi gazete, 2020). This species spreads naturally between the sea level and 1100 m altitudes, mostly in the North Anatolian coastal areas, around Marmara, Beyşehir (Konya) and Erzurum (Mill, 1984).

One of the most important features of geophytes is that they are used for therapeutic thanks to the active ingredients that contained in bulbs, tubers and rhizomes (Işık, 2006). Alkaloids are used in the treatment of Alzheimer's, MS, myasthenia gravis, myopia, glaucoma (Orhan et al., 2011). Amaryllidaceae alkaloids are mostly isolated from *Galanthus* L., *Leucojum* and *Narcissus* L. species. Galanthamine isolated from *Leucojum* species is used in the treatment of polio and Alzheimer's disease, and lycorine is used as a pesticide against the fungus formed in tomatoes (Şener and Orhan, 2005). Both bulbs and fresh green leaves of the species contain many important chemicals, mainly galanthamine alkaloids. These chemicals (lectins, chelidonic acid, etc.) contain versatile pharmacological effects (Eichorn 1998). In addition, it has been shown that ethanol obtained from the plant is effective against viruses (Hudson et al. 2000).

Today, drugs used for the treatment of cognitive dysfunction related to Alzheimer's disease act through two main mechanisms: These drugs either strengthen the weakened cholinergic neurotransmission in the brains of

Alzheimer's patients or weaken the increased glutaminergic neurotransmission. Acetylcholinesterase (AChE) inhibitors which are frequently used drugs in the treatment of Alzheimer's disease are drugs that enhance cholinergic neurotransmission by increasing the amount of acetylcholine (ACh) in cholinergic synapses. Tacrine, donepezil, rivastigmine and galanthamine are cholinesterase inhibitors developed for use in the treatment of Alzheimer's disease and approved by the US Food and Drug Administration (FDA). Galanthamine is an effective, selective, reversible and competitive inhibitor of acetylcholinesterase and an allosteric modulator of the neuronal nicotinic receptor for acetylcholine. AChE is responsible for degradation of acetylcholine at the neuromuscular junction in peripheral and central cholinergic synapses. Galanthamine has the ability to cross the blood-brain barrier and act in the central nervous system. Galanthamine hydrobromide has superior pharmacological profiles and higher tolerance compared to the original AChE inhibitors, physostigmine or tacrine. Galanthamine is therapeutic for mild to moderate stages of Alzheimer's. Galanthamine was approved by the FDA in 2001 and was first isolated from *Galanthus woronowii* Losinsk. (Sosa-Ortiz et al. 2012).

1.1 Studies about Amaryllidaceae Alkaloids

Gorinova et al. (1993) found a relationship between the galanthamine content of *L. aestivum* and the chemical composition of the soil and inferred that galanthamine biosynthesis can be controlled by the soil fertility level. Hudson et al. (2000) evaluated a total of 16 ethanol extracts of Turkish medicinal plants for antiviral activities against herpes simplex virus (HSV) and Sindbis virus (SINV). Elgorashi et al. (2002) investigated the distribution and seasonal variation of alkaloids (lycorine, 1-O-acetyllycorine, crinine, powelline, crinamine, crinamidine, 3-O-acetylhamayne, 1-epideacetylbowdensine and cherylline) in *Crinum macowanii* Baker. Ayan et al. (2004) indicated that bulb yield of *L. aestivum* was much more in *in vitro* grown system under shaded conditions and they suggested that shading would be a very useful practice to enhance bulb yield in the cultivation of *L. aestivum* under field conditions. Nikolova and Gevrenova (2005) determined the phenolic acids in Amaryllidaceae species by HPLC. Diop et al. (2006) determined the galanthamine content in both *in vitro* and *in vivo* extracts of *L. aestivum* at different stages of morphogenesis. Georgieva et al. (2007) reported the alkaloid variability of *L. aestivum* bulb extracts collected from

18 Bulgarian populations and found that bulbs mainly contain galanthamine, epinorgalanthamine, narwedine, and lycorine. They suggested that genetic factor plays an important role in the alkaloid biosynthesis of *L. aestivum*. Guo et al. (2007) concluded that high temperature could enhance the accumulation of some alkaloids in *Catharanthus roseus* (L.) G. Don. that is related with the treatment time. Pavlov et al. (2007) displayed that light raised the galanthamine production in the shoot-clump of *L. aestivum*. Berkov et al. (2009) reported that shoot-clump cultures of *L. aestivum* have a higher ability for galanthamine production. Jansen et al. (2009) showed that seed alkaloid content of *Lupinus angustifolius* L. cultivars ascended with increasing temperature. Sahoo et al. (2012) investigated the variations in the total alkaloid and phenol during different seasons in some medicinal plants [*Barleria prionitis* L., *Boerhavia diffusa* L., *Citrullus colocynthis* (L.) Schrad. and *Grewia tenax* (Forssk.) Fiori.]. Karuncula (2013) investigated the alkaloids of the *L. aestivum* and determined the AChE and BChE inhibition activities. Lubbe et al. (2013) reported that bulbs of *Narcissus pseudonarcissus* cv. Carlton had higher concentration of galanthamine before flowering (April). In addition, leaves had approximately same levels of galanthamine until full flowering (May) followed by a decrease after flowering. Demir (2014) investigated medical resource value appraisal for *L. aestivum* in Turkey. Kaya et al. (2014) determined galanthamine and lycorine content in *Galanthus elwesii* Hook.f. Ločárek et al. (2015) investigated the effect of ethanol extract of *L. aestivum* bulbs against some bacteria. Petruczynik et al. (2016) compared different HPLC systems for analysis of galanthamine and lycorine in various species of Amaryllidaceae family. Quantification of galanthamine in *Narcissus tazetta* L. and *Galanthus nivalis* L. populations growing wild in Iran was investigated and maximum galanthamine content was determined in some populations by Khonakdari et al. (2018). Hundur et al. (2018) reported the antibacterial activity of ethanol extracts of below ground parts of *L. aestivum* against *E. coli and S. typhimurium*. Arslan et al. (2020) showed the seasonal variation of galanthamine and lycorine. They suggested that positive correlation was observed between lycorine and galanthamine content of the bulbs and temperature. Ateş (2021) investigated the effect of salt stress on alkaloid content (galanthamine and lycorine) and antioxidant activities (enzymatic and non-enzymatic) in *L. aestivum*.

1.2 Biological Informations

1.2.1 Amaryllidaceae Family

Amaryllidaceae is a monocotyledonous family consisting of 79 highly cosmopolitan genera native to Central Asia and North America, found in all the biomes of the world (Sabiu et al. 2019). It spreads in temperate and subtropical regions, they are bulbous, rhizomatous and tuberous perennial herbaceous plants, rarely shrubs and trees (Meerow et al., 1999). Many taxa are grown as ornamental plants in gardens and pots. The leaves are simple, usually deciduous, fleshy, often flat and linear to elliptic at the base. The flowers are hermaphrodite and actinomorphic or zygomorphic. Perianth usually fused (basally to completely) or free; sometimes a conspicuous corona (e.g. *Narcissus*), inconspicuous (e.g. short rim of scales) or absent. Stamen filaments free or fused, usually attached to perianth. Usually 6 but can be (2-)3 or 9-18 (e.g. *Gethyllis* L.); anthers dorsifixed or basifixed, introrse; staminodes 3(-4) (e.g. *Gilliesia* Lindl.). Ovary superior or inferior, rarely part-inferior; carpels fused, septal nectaries present; locules (1-)3; ovules 1-many per locule; placentation axile, basal or apical. Fruit a loculicidal capsule or berry (Byng 2014). There are six genera of Amaryllidaceae in Turkey. These are *Leucojum*, *Galanthus*, *Sternbergia* Waldst. & Kit., *Pancratium* Dill. ex L., *Narcissus* and *Allium* L. (Mill, 1984; Govaerts, 2021).

1.2.2 *Leucojum aestivum* L.

The genus *Leucojum* includes two species and two varieties (three taxa) in the world and represented by one species in Turkey (Govaerts, 2021). *L. aestivum* is a bulbous perennial plant, a member of Amaryllidaceae (Ptak, 2014). It is native to the Balkans, South Europe, the Caucasus, Northern Iran, the Mediterranean regions, Southern Africa, Western Asia, and Turkey. *L. aestivum* was first featured in the book "Garden Pleasant Flowers" by the English botanist John Parkinson in 1629 as the "great bulbous violet" (Karuncula, 2013). It is known as "summer snowflake" in English and "gölsoğanı, akçabardak, çan çiçeği, sümbül, kabalak, sarıklı kökü" among people in Turkey (Kutbay and Kılınç, 1993).

Bulb 25-45 mm diameter. Leaves broadly linear, 22-62 cm × 7-14 mm. Scape (8-) 25-60 (-90) cm, equaling or exceeding leaves, stout, hollow, compressed, 2-winged, wings with narrow hyaline remotely denticulate margin. Umbel 2-5 flowered. Spathe (20-) 25-40 × 3.5-8 mm, 1-valved. Pedicels (8-)10-65 mm, longest equaling or exceeding spathe. Perianth segments white with a green blotch just below thickened apex, 12-20 mm. Anthers c. 4 mm. Style slightly clavate longer than stamens. Seeds black, 5-7 mm, strophiole absent. It spreads in moist meadows up to 1100 meters above sea level (Mill, 1984).

1.2.3 Alkaloids

Alkaloids are made up of amino acids, but terpenes and steroids are also often other precursor compounds that make up the alkaloid skeleton. Alkaloids play a role in the defense of plants against herbivores and pathogens. According to Cordell et al. (2001), the total number of alkaloids found in monocotyledons, dicotyledons and gymnosperms is 21120. Alkaloids are used as pharmacological, stimulant, narcotic and poison (Zulak et al., 2006). Biochemical studies of alkaloids in plants started in 1806 with the isolation of morphine. Due to the stereo-chemical complexity of the morphine molecule, its structure could not be explained until 1952. For more than half a century, alkaloid biosynthesis in plants has been tried to be understood through chemical, biochemical and molecular research (Facchini and Bird, 1997). The intake amounts of alkaloids are also important. Alkaloids taken in small amounts can be beneficial, while alkaloids taken in large amounts can be lethal (Önmez, 2007).

Amaryllidaceae family includes different types of alkaloids with an extensive of biological activities (Petruczynik et al., 2016). This family is one of the 20 most important alkaloid-containing families, and these alkaloids are considered as isoquinoline alkaloids (Ünver, 2007). Up to now, about 500 structurally different alkaloids have been isolated from Amaryllidaceae (Jin, 2005).

Figure 1.1 *Leucojum aestivm* L. (by Sina Cafer Demir, 2019)

1.2.4 Galanthamine

Among the Amaryllidaceae alkaloids, one of the most common and remarkable is galanthamine alkaloid. Galanthamine was first isolated in 1952 from *Galanthus woronowii* (a Caucasian snowdrop plant) (Heinrich and Teoh, 2004) which is native to the Caucasus Mountain range from Russia and Eastern parts of Turkey to Iran. Then it was extracted from *Galanthus nivalis* (Sweeney et al., 1989) which is a closely related plant to *G. woronowii* being native to the Mediterranean shores and over the northern temperate zone. Today, galanthamine is produced from *L. aestivum* (Halpin et al., 2010). Galanthamine is a reversible competitive acetylcholinesterase inhibitor and potentially improves cholinergic function in the brain through two mechanisms of action. The first occurs by inhibiting the enzyme, the second by affecting acetylcholine receptors. In Eastern Europe, galanthamine is used in anesthetic applications to awaken the patient because it neutralizes the anesthetic effect of tubocurarine. It has also been observed that galanthamine has a mild strengthening and analgesic effect like morphine. It is known to reduce the pressure inside the eye in eye surgeries (Scott and Goa, 2000; Karuncula, 2013). Galanthamine has been used for decades for different indications like treatment of poliomyelitis, myasthenia gravis and other neuromuscular disorders (Klosi et al., 2016). The use of galanthamine, usually under the Nivalin® trade name, was mostly limited to Bulgaria, Italy, France and Germany during the 1960s through the 1980s. Galanthamine was launched in the USA and Europe for the symptomatic treatment of Alzheimer’s disease, originally as Reminyl®, which was later changed to Razadyne® in the USA (Mucke, 2015).

1.2.5 Lycorine

Lycorine, a pyrrolophenanthridine alkaloid, is the major alkaloid that found in certain parts of all Amaryllidaceae taxa (Ilavenil et al. 2010). This alkaloid was first isolated in 1877 from *Narcissus pseudonarcissus* L. and its structure was explained by Nagakawa et al. in 1956 (Wang et al. 2017). Lycorine has important biological activities such as anti-inflammatory, antiviral, antibacterial, antifungal, antiprotozoal. Also, it has cytotoxic effects against numerous tumor cell lines e.g., leukemia, cervical and prostate cancer (De Andrade et al., 2012; Khalifa et al., 2018). Lycorine known as an immunosuppressor and can be useful in suppression of the immune systems of mammals for the treatment of autoimmune diseases, complex immune syndromes, allergic and rheumatic conditions, as well as for prophylaxis against transplant rejections (Dickneite et al. 1987; Refaat et al. 2013; Khalifa et al., 2018). Lycorine has also shown suppressive activity on *in vitro* viral replication of flaviviruses such as Japanese encephalitis, yellow fever, dengue-4, bunya, Punta Toro, Rift Valley fever, herpes simplex 1 and poliomyelitis viruses (Renard-Nozaki et al., 1989; Gabrielsen et al., 1992; Hwang et al., 2008). Moreover, lycorine was reported to inhibit diverse coronavirus infections such as SARS-CoV, MERS-CoV, HCoV-NL63, and HCoV-OC43 both *in vitro* and *in vivo* (Li et al., 2005; Shen et al., 2019). SARS-CoV-2 is an infection associated with the outbreak of viral pneumonia ongoing worldwide. Lycorine inhibited this infection in cell culture (Zhang et al., 2020). Jin et al. (2020) suggested that lycorine may be more effective against SARS-CoV or SARS-CoV-2 infections than in MERS-CoV infections, and that lycorine is more effective than remdesivir, which is currently the most promising anti-SARS-CoV-2 drug.

1.2.6 HPLC Method

In the high-performance liquid chromatography (HPLC) method, which is a special application of the liquid chromatography method, the particle size used as the stationary phase is significantly reduced, resulting in the stationary phase surface area interacting with the mobile phase increasing and thus the efficiency of the column is increased. A pressure must be applied in order for the mobile phase to pass through the very tightly filled column at a certain speed. These high efficiency columns and extremely high pressures HPLC, where it is used, is the most widely applied type of chromatography in element speciation (Samanidou et al 2003).

Today, HPLC is recognized as an indispensable tool for separation and analysis in fields including chemistry, biochemistry, biotechnology, pharmacology, medical chemistry, plant chemistry, agriculture and chemical engineering. It is particularly suitable for separation and analysis of compounds not suitable for other chromatographic techniques (Cai et al. 2006).

1.2.7 Alzheimer’s Disease

Alzheimer's disease firstly described by a German doctor named Alois ALZHEIMER in 1907. It is a progressive brain disease in which various mental functions such as memory, speaking, finding directions, recognizing people, problem solving weaken over time, their ability to perform daily tasks decreases and behavioral disorders can be seen. Alzheimer's disease, which has a long treatment period because it is a chronic disease, is one of the diseases with the highest cost of treatment following heart diseases and cancer. Alzheimer's disease has become one of the most important diseases of our age, especially in developed countries, due to the increase in living standards and the increase in the elderly population due to the development of health services (Burns, 1992).

Since the pathogenesis and pathophysiological events that cause Alzheimer's disease have not been fully elucidated, there is currently no complete preventive treatment for Alzheimer's disease. The main problem in Alzheimer's disease is neuron loss and reduction in the cholinergic system. Although neuron loss is not reversible, increasing the cholinergic effect improves the patient's quality of life and the course of the disease (Burns, 1992).

1.2.8 Acetylcholinesterase (AChE) Inhibitory Activity

The main mechanism in the development of Alzheimer's disease symptoms is neurotransmitter changes. The decrease in the amount of ACh indicates Alzheimer's disease. Some neurotransmitter systems, especially the cholinergic system, are selectively affected in patients with Alzheimer's disease. The presence of ACh at the synapse depends on the activity of the choline acetyltransferase and AChE enzymes. ACh also has a deep relationship with learning and memory, and thus there are ACh-containing neurons in the brain. ACh is synthesized in certain neurons by the choline acetyltransferase enzyme, from choline and acetyl-CoA. After completing its task by being released into the synaptic space, ACh is destroyed by converting it into choline and acetate (acetic acid salt) with the help of AChE enzyme. ACh molecules produced in the presynaptic neuron are stored in specialized vacuoles at presynaptic terminals, and these vacuoles pour their contents into the synaptic space when a nerve impulse comes to the neuron. Most of the ACh molecules released into the synapse gap immediately bind to postsynaptic receptors, and molecules that cannot bind to the receptors while transmitting the stimulus are inactivated by AChE. ACh molecules attached to the postsynaptic receptors are separated from the receptor after the nerve impulse is transmitted to the other neuron, it is destroyed by AChE, and the remaining choline from the molecule is returned to the presynaptic neuron for reuse. Cholinesterases are enzymes with a wide distribution in cholinergic and noncholinergic tissues, also in plasma and other body fluids (Kent, 2000; Brenner and Stevens, 2006).

The reaction catalyzed by AChE takes place enzymatically in two steps. In the first step the enzyme plays the role of a powerful nucleophile. In the second step, the enzyme functions as an excellent cleavage group via the nucleophilic hydroxyl group of a specific serine residue. The main function of AChE is to terminate cholinergic neurotransmission. Neuron and axon loss with the onset of Alzheimer's disease causes lower levels of ACh release. At lower levels of neurotransmitters, the continuity of nerve impulses and consequently the transmission of information become more difficult. One of the methods to correct this situation is to give ACh-like substances to nerve cells. Another method to increase the ACh level is to suppress the AChE enzyme, which breaks down ACh. The aim of keeping acetylcholine longer in the synaptic space is the most commonly used strategy in the treatment of the disease today. Cholinesterase enzyme inhibitors are mostly used for this purpose (Temel, 2008). AChE inhibitory activities are measured by applying the spectrophotometric method (Ellman et al. 1961).

1.2.9 Antioxidant Activity

The development of technology, environmental pollution and many other factors cause us to be exposed to various toxic substances. Due to these toxic substances, the number of diseases (such as heart, cancer, premature aging, etc.) is increasing day by day. Remedies for these diseases can be accomplished by controlling free radicals, which are the main causes of these diseases. As people's defense mechanisms weaken as age progresses, the free radical balance of the body deteriorates. Because the production of endogenous enzymes that are the body's natural antioxidants decrease. Therefore, it is important to take natural foods containing antioxidants in order to restore balance (Çelik and Ayran, 2020).

Antioxidants can be divided into two categories such as synthetic and natural. The main targeted site of free radicals’ damage and defensive approach of antioxidants in the body is at the cellular level. Based up on this, these antioxidants can also be classified as enzymatic and nonenzymatic antioxidants. Enzymatic antioxidants primarily include glutathione peroxidase, catalase, and superoxide dismutase (Anwar et. al., 2012). Nonenzymatic antioxidants contain several subdivisions mainly vitamins such as A, C, D, and vitamin E, enzyme cofactors (Q10), peptides and some minerals such as zinc and selenium (Carocho and Ferreira, 2013).

1.2.10 Non-Enzymatic Antioxidant Activity

DPPH is one of the few stable organic nitrogen radicals and its color is dark violet. This method is based on the scavenging of the DPPH radical due to a redox reaction by antioxidants. The dark violet color of the methanolic DPPH solution is opened and the decrease in absorbance is measured by spectrophotometer. The absorbance value after mixing of the extract with DPPH solution shows that the extract has antioxidant activity (Brand-Williams et al. 1995)

Phenolic acids are hydroxylated that derive from cinnamic and benzoic acids. Hydroxybenzoic acid is mainly present in the form of glucosides in foods, while hydrocinnamic acid, such as p-coumaric, caffeic acid, and ferulic acid, is mostly found in food as simple esters (Mattila & Kumpulainen, 2002). Phenolic compounds also have protective effects on humans when the plants are consumed as food (Nićiforović et al. 2010). The antioxidant capacity of phenols in plant extracts is effective at low concentrations. It is associated with the prevention of cardiovascular disease and cancer in humans. (Chaves et al. 2020). Total phenolic acids can be determined by adhering to the method proposed by Slinkard and Singleton (1977), which is known as the Folin–Ciocalteu phenol reagent technique.

Flavonoids are widely distributed in plants, and they consist of a large group of polyphenolic compounds that can be characterized by benzo-y-pyrone structure. Flavonoids are low molecular phenolics, and they can be divided into several subclasses, such as flavones, flavanones, isoflavones, anthocyanins, flavanols, and flavonols. The most important function of flavonoids is their antioxidant activity. Flavonoids can scavenge various oxidizing species, including superoxide anion, hydroxyl or peroxyl radicals by quenching the singlet oxygen. Flavonoids can also act as an inhibitor in oxidation of low-density lipoprotein (LDL), anti-bacterial, and anti-fungal, besides preventing malaria (Harborne and Williams, 2000; Subramanian et al. 2007).

1.2.11 Antibacterial Activity

Drugs from plants have played an important role in pharmaceutical care for the treatment of various diseases. Antibiotic resistance has become a growing problem in recent years due to infections have been on the rise (Austin et al.

1999). Microorganisms are the most important pathogens causing severe morbidity and fatal infections in humans. Natural products of plants may provide a new source of antimicrobial agents with potentially novel mechanisms of action (Nostro et al. 2000). Secondary metabolites from plants serve as defense agents against invading microorganisms (Fabry et al. 1998). The plants selected for evaluation as sources of antimicrobial agents are often those used in traditional medicine for the treatment of infectious and other diseases (Ahmad et al. 1998). Disc diffusion methods are extensively used to investigate the antibacterial activities of natural antimicrobial substances and plant extracts (Bauer et al., 1966).

2. AIM AND SCOPE OF THE STUDY

L. aestivum is a well-known producer of pharmaceutically important alkaloids especially galanthamine. Another alkaloid, lycorine, has been getting a lot of attention lately. It was found as inhibitor to new coronavirus (SARS-CoV-2) and more effective than remdesivir that is the only FDA-approved drug for the treatment of SARS-CoV-2 (COVID-19) patients (Ates et al. 2021). *L. aestivum* is an endangered perennial bulbous plant because of gathering them from natural habitats for pharmaceutical industry causing reduction in wild populations. Although this plant grows naturally in Turkey, it is forbidden to collect it from nature. Only leaves of *L. aestivum* from the natural habitat were allowed to collect and export in Turkey. This plant has been cultivated in our country in the licensed fields for the exportation of bulbs to pharmaceutical industries (Resmi Gazete, 2020). The discovery of population that is rich in galanthamine and lycorine can be a guide to obtain the best genotype in terms of alkaloid content for the cultivation of this species. The discovery of the population that is rich in alkaloid content by correlating the growing seasons of the plant, soil micro and macro elements, and monthly average temperature and precipitation will lead to obtain more alkaloids from the same number of plants in the cultivation of this plant.

Objective of this study to evaluate and compare alkaloid content (galanthamine and lycorine), morphological characterization, total phenol and flavonoid content, and biological activities (antioxidant, antibacterial) of *L. aestivum* bulbs and leaves collected from 6 different natural environments at different growing season (before flowering, flowering and after flowering stages), as well as to correlate them with soil structure and chemical composition besides monthly average temperature and precipitation.

3. MATERIALS AND METHODS

3.1 Collection of *L. aestivum*

Bulbs and leaves of *L. aestivum* were collected from six different locations (Gölcük-Bolu, Yeniçağa-Bolu, Kaynarca-Sakarya, Delmece-Yalova, Uluabat-Bursa and Terkos-İstanbul) in 2019. Collection time started in March for each locality. Plants from each location were harvested throughout the growing season. Ten plants were harvested at three time-points: before flowering (vegetative period), during full flowering (reproductive period) and after flowering (ripening period) for all locations. Plants continued to be collected for both locations in Bolu (Gölcük and Yeniçağa) during ripening and shoot senescence period between March and October. For morphological characterization, 10 flower and 10 fruit samples were collected once from each population. The collected plants were identified using "Flora of Turkey and the East Aegean Islands" (Mill, 1984) and voucher specimens were deposited in the Bolu Abant Izzet Baysal University Herbarium.

3.2 Extract Preparation

Bulbs and leaves of *L. aestivum* collected from all localities were freeze-dried (Christ®) at - 65 °C and then grounded into fine powder prior to the extraction. Extractions were carried out in water bath for 18 h by using methanol and then methanol was concentrated using rotary evaporator to obtain the crude extract. Extract yields were calculated with the formula: Yield (%) = Weight of extract (g) / powdered plant material (g) × 100

3.3 Determination of Alkaloid Content

Quantitative analysis of methanol extracts was determined by HPLC system (VWR-Hitachi LaChrom Elite®) equipped with a Hitachi L-2455 diode array detector (DAD), Hitachi L-2130 Pump and Hitachi L-2200 autosampler. Two alkaloid standards [galanthamine hydrobromide (TCI America®) and lycorine (Sigma®)] were used as reference (in 1% TFA) and calibration curve of them was determined. The HPLC operating conditions were applied and analyses were performed using an isocratic elution as in previously described method by Arslan et al. (2020). A reverse phase C18 column (250 mm X 4.6 mm, 5 µm particle size, Venusil Technology®) was used in all HPLC analyses. Using an isocratic eluting solvent mixture, TFA: water: acetonitrile (0.01:90:10 v/v/v), as mobile phase. An injection volume of 20 µL, flow rate of 1ml/min and column temperature at 25ºC were applied during the analysis. Detector was run at 290 nm wavelength. In the quantitative evaluation of the components, the standard mixture prepared at known concentrations of each component was used. Quantitative analysis was carried out by integrating the peak areas of samples and standards run under the same conditions.

Mobile phase prepared before HPLC injection was filtered with 0.45 µm hydrophilic polypropylene membrane filter (47 mm) (Pall®). Stock solutions of all standards were prepared as 1000 mg/l concentration in 0.1% trifluoroacetic acid (TFA), then 6 different concentrations of standards (6.25, 12.5, 25, 50, 100 and 200 mg/l) were used to obtain the standard calibration curves. All extracts and standards were transferred to 2 ml HPLC vials (Isolab®) by filtration with 0.2 µm GHP Acrodisc (25 mm) (Pall®) and the whole was repeated 3 times for the analyzed extract.

3.4 Non-Enzymatic Antioxidant Activity

Free radical scavenging activity of *L. aestivum* methanol extracts were determined by DPPH (1,1-diphenyl-2-picrylhydrazyl) assay as described by Yıldırım (2020). Quercetin was used as an antioxidant standard.

Total phenolic and flavonoid content of methanolic extracts were determined by using Folin-Ciocalteu and aluminum chloride colorimetric method, respectively as described by Turker et al. (2021). Gallic acid and quercetin were used as a reference phenol and flavonoid, respectively to prepare calibration curves.

3.4.1 DPPH Free Radical Scavenging Activity

In this method, stock solutions were prepared from each extract as 1000 µg/ml concentration. The extracts were diluted with methanol (12.5, 25, 50, 100 and 200 µg/ml) were prepared from these stock solutions. Quercetin (1000 µg/ml concentration) as an antioxidant was used and a standard curve was prepared by various concentrations of quercetin (12.5, 25, 50, 100 and 200 µg/ml). In addition, 0.13 mM solution of DPPH that used as radical substance was prepared daily to obtain 1.4 absorbance units at 517 nm. 0.1 ml of the prepared dilutions (extract or quercetin) or methanol (control DPPH) was taken and mixed with 1.4 ml of DPPH solution. These mixtures were kept in the dark for 30 minutes and then the absorbance values were measured by spectrophotometer (Hitachi U-1900, UV-

Vis) at 517 nm. Absorbance values of the extracts were measured versus blank control (methanol). The DPPH radical scavenging activity of *L. aestivum* was calculated according to following formula. Then, the IC_{50} value of each extract was calculated using the % inhibition values.

% DPPH INHIBITION VALUE = $[A_{Control} - A_{Sample}] / A_{Control} \times 100$

$A_{Control}$: Absorbance of Control

A_{Sample}: Absorbance of Sample

3.4.2 Determination of Total Phenolic Content

In the total phenol method, stock solutions of 5000 μg/ml in methanol were prepared from *L. aestivum* extracts. 2500 and 1000 μg/ml dilutions were obtained from the prepared stock solutions. Standard gallic acid stock solution was prepared at 1000 μg/ml concentration and solutions were obtained at concentrations of 0, 12.5, 25, 50, 100, 150, 250, 500 μg/ml to form a calibration curve.

Measuring process: 20 μL extract, gallic acid or methanol (blank), 1.58 ml of distilled water and 100 μL of Folin-Ciocalteu solution were added individually and mixed thoroughly. After 2 minutes, 300 μL of sodium carbonate solution was added to each tube and mixed again and kept at room temperature. The absorbance values of the samples were measured at 765 nm (Hitachi U-1900, UV-Vis Spectrophotometer 200V, Japan) against the blank solution within 2 hours, and the total phenol amounts were given as the equivalent gallic acid amount (mg GA/g methanol extract) using the standard gallic acid curve. All analyses were made in triplicate.

3.4.3 Determination of Total Flavonoid Content

In the total flavonoid method, 5000 μg/ml stock solutions of *L. aestivum* extracts were prepared with methanol. 2500 and 1000 μg/ml dilutions were obtained from the prepared stock solutions. A stock solution of standard quercetin at a concentration of 1000 μg/ml was prepared and solutions at concentrations of 0, 12.5, 25, 50, 100, 150, 250, 500 μg/ml were obtained to form the calibration curve. In addition, 5% $NaNO_2$ (w/v), 10% Aluminum chloride ($AlCl_3$) and 1M NaOH solution were prepared in methanol.

Measuring process: 500 μL of extract, quercetin or methanol (blank), 2 ml of distilled water and 100 μL of $NaNO_2$ were added individually and mixed thoroughly. After 5 minutes, 150 μl $AlCl_3$ at 6th min 1000 μL NaOH were added to mixture. Then the solution was completed to 5 ml with 1200 μL of water. Its absorbance was measured at 410 nm using the spectrophotometer (Hitachi U-1900, UV-Vis Spectrophotometer 200V, Japan). The total flavonoid amounts of the samples were given as the equivalent amount of quercetin (mg Quercetin/g methanol extract) using the standard quercetin curve.

3.5 Antibacterial Bioassay

The antibacterial effect of *L. aestivum* leaf and bulb extract against ten human pathogenic bacteria was determined by using disc diffusion method as described by Turker et al. (2021). Gram positive [*Streptococcus pyogenes* (ATTC 19615), *Staphylococcus aureus* (ATTC 25923), and *S. epidermidis* (ATCC 12228)] and Gram negative [*Escherichia coli* (ATCC 25922), *Enterobacter cloacae* (ATCC 23355), *Salmonella typhimurium* (ATCC 14028), *Serratia marcescens* (ATCC 8100), *Klebsiella pneumoniae* (ATCC 13883), *Proteus vulgaris* (ATCC 13315) and *Pseudomonas aeruginosa* (ATCC 27853)] bacteria (Becton Dickinson Laboratories, France®) were used for the experiment. Six antibiotic discs were used for positive control [ampicillin (10 μg), erythromycin (15 μg), penicillin (1 μg), piperacillin-tazobactam (30/6 μg), tetracycline (30 μg) and trimethoprim/sulfamethoxazole (25 μg)].

Bacterial disks were transferred to test tubes containing 2 ml TSB (Tryptic Soy Broth) and incubated for 3 hours at 37 °C. After 3 hours, each microorganism was inoculated into TSA (Tryptic Soy Agar). At the end of 2 days, a single colony was separated and inoculated into a new TSA containing petri dish. It was waited for 2 days at 37 °C and thus obtains the pure culture of each bacterium. The concentration of each bacterial culture for the anti-bacterial assay was adjusted to 0.5 McFarland standard using saline (0.9% NaCl). The 0.5 McFarland standard is known to be equivalent to 10^8 cfu/ml of bacterial suspension. All bacterial suspensions to be used in this manner were at the same concentration. Sterile cotton sticks were immersed in bacterial cultures and then inoculated into petri dishes containing Mueller-Hinton agar. After the extracts were sterilized using a 0.22 μm filter, 11 μl extracts were placed on filter paper disks and then placed in inoculated petri dishes. There were filter paper disks containing 4 extracts in each petri dishes. Two petri dishes were used for each microorganism. Petri dishes were kept in a 37 °C in incubator for 16-18 hours and then the diameter of the inhibition zones was measured. Water was used as negative control. The experiment was repeated three times.

3.6 Soil Analysis

Soil analyzes were performed by Bolu Directorate of Provincial Agriculture and Forestry. Soil texture, electrical conductivity (EC), total salt (%), pH in soil, calcium carbonate ($CaCO_3$), organic matter (%), electrical conductivity (EC) and the levels of Phosphorous (P), Potassium (K), Manganese (Mn), Zinc (Zn), Copper (Cu), Iron (Fe), Calcium (Ca), Sodium (Na), Magnesium (Mg) were analyzed for each month and location.

3.7 Data Analysis

Data analysis was performed using analysis of variance (ANOVA) and Duncan's multiple range tests using SPSS version 26 (IBM Corp, NY, USA). All data in the tables were presented as a mean number ± standard error (SE). Pearson correlation analysis was performed for the determination of relationship.

3.8 Morphological Analysis

In the research areas, a total of 18 field studies were conducted between March to June in 2019 and a total of 180 specimens were collected from 6 populations (Table 3.1).

Table 3.1 Informations of locations

Locations	Collection Number	GPS	Habitat and Altitude	
Sakarya-Kaynarca	LEU1006	41°05'16"N 30°26'39" E	Wet meadows	50 m
Bursa-Uluabat	LEU1007	40°10'15"N 28°43'24" E	Wet meadows	8 m
Yalova-Delmece	LEU1008	40°34'07"N 29°00'38" E	Wet meadows	730 m
İstanbul-Arnavutköy	LEU1009	41°17'11"N 28°40'02" E	Wet meadows	25 m
Bolu-Yeniçağa	LEU10010	40°47'23"N 32°02'50" E	Wet meadows	1005 m
Bolu-Gölcük	LEU10011	40°39'37"N 31°37'28" E	Wet meadows	1126 m

The definition of taxa was made according to measurements and examinations on fresh and dried plants. All of the quantitative characters and qualitative characters were measured/examined on dried plants. During the field work, the color characteristics of the plants were observed and photographs were taken from the natural habitats of the species. Measurements/observations of microscopic structures were taken with a ruler under the stereo-microscope while measurements/observations of macroscopic structures were taken with a ruler. Two systems were applied for evaluation of the taxa: traditional taxonomy and numerical taxonomy in morphological studies. For the numerical taxonomic analyses, 10 specimens from each population were sampled randomly. The mean values of each character were calculated. The raw measurements of each character were obtained for each ten individuals from locations. Then, mean values of the raw measurements were calculated for every character from locations according to Sneath and Sokal (1973), and Abbott et al. (1985). 26 quantitative, 23 qualitative characters were used. Quantitative and qualitative characters used in the plant descriptions are listed in Table 3.2 and 3.3.

Thereafter, according to the modified Whisker's graph (Massart et al., 2005), the mean values of each character with their standard errors were plotted on one group graph by using GraphPad program. When means and standard error lines of each quantitative characters belonging to different populations have overlapped with each other, they were grouped and coded as the same state of the character in ascending order (Almeida and Bisby, 1984). After all quantitative characters were coded in this technique, a taxonomic matrix was generated (Appendix 1).

MINITAB (Meyer and Krueger, 2004) and SPSS (Levesque, 2007) statistical package programs were used for principal component analysis (PCA). In cluster analysis, for measuring the distance between OTUs in n-dimensional space, Average Linkage between groups was used because of strengthening the tree diagrams geometrically. Euclidean Block Method was used for analysis of clusters and establishing of dendrogram. Cluster analysis techniques can group the OTUs according to the distances measured among the OTUs and their similarities or dissimilarities. But real places of the OTUs in n-dimensional space, the group structures and their connection levels cannot be shown easily by cluster analysis. For these reasons, the PCA was performed to overcome these restrictions of cluster analysis. During the PCA, two-dimensional case factor scatter-plots were generated by using most appropriate components which are the components 1 and 2. The two-dimensional plotted graphs of OTUs were created according to the components 1 and 2 which gave the most meaningful groups of OTUs.

Table 3.2 Quantitative characters

No	Abbreviation of character	Explanation of character
1	SN	Scape Number
2	FN	Flower Number*
3	GYN	Gynoecium Length*
4	OL	Ovary Length*
5	OW	Ovary Width*
6	SPL	Spatha Length*
7	SPW	Spatha Width*
8	SCL	Scape Length*
9	SCW	Scape Width*
10	BL	Bulb Length*
11	BW	Bulb Width*
12	LN	Leaf Number*
13	LL	Leaf Length*
14	LW	Leaf Width*
15	PL	Pedicel Length*
16	TL	Tepal Length*
17	TW	Tepal Width*
18	CAL	Cataphyll Length*
19	CAW	Cataphyll Width*
20	SL	Seed Length*
21	SW	Seed Width*
22	FL	Fruit Length*
23	FW	Fruit Width*
24	PW	Pedicel Width
25	AL	Anther Length
26	AW	Anther Width

* Quantitative characters used in numerical analyses

Table 3.3 Qualitative characters

No	Abbreviation of character	Explanation of character
1	SPC	Spathe Color
2	SPS	Spathe Surface Pubescence
3	SIS	Scape Inner Surface Pubescence
4	SC	Scape Color
5	SOS	Scape Outer Surface Pubescence
6	SWI	Scape Wing Direction
7	LC	Leaf Color
8	LD	Leaf Direction
9	LSH	Leaf Shape
10	LES	Leaf Spots
11	LS	Leaf Surface Pubescence
12	OC	Ovary Color
13	OS	Ovary Surface Pubescence
14	PC	Pedicel Color
15	PS	Pedicel Surface Pubescence
16	PSB	Perianth Segment Blotch Presence
17	PSC	Perianth Segment Color
18	TC	Tunic Color
19	BS	Bulb Shape
20	SCO	Seed Color
21	SS	Seed Shape
22	FC	Fruit Color
23	FS	Fruit Shape

4. RESULTS AND DISCUSSION

This study was carried out to determine the variation of galanthamine and lycorine (Table 4.1 and 4.2), total phenol and flavonoid content and antioxidant activities (Table 4.5 and 4.6), and antibacterial activities (Table 4.7) in the bulbs and leaves of *L. aestivum* obtained from 6 different localities in Turkey at different growing seasons. Plants were collected considering 3 different growing seasons (before flowering, flowering and after flowering) for all localities. It was observed that vegetative growing season (before flowering) was March for 4 locations (Kaynarca-Sakarya, Terkos-İstanbul, Uluabat-Bursa, Delmece-Yalova) but it was March and April for both Bolu locations (Gölcük and Yeniçağa). Similarly, although April was reproductive growing season (flowering) and May was ripening season (after flowering) for 4 localities, they were May and June respectively for both Bolu locations. Growing season differences of Bolu locations from other 4 places were due to average temperatures and precipitations in each month (Figure 4.1). Plant samples of *L. aestivum* were collected between March and May (3 months) for 4 locations. On the other hand, sample collection was performed between March and October (8 months) for both Bolu locations.

Bulbs and leaves were extracted with methanol and the percentage yields of the extracts were calculated (Table 4.1 and 4.2). Generally, leaf extracts gave higher percentage yields than bulb extracts because of having more kinds of metabolites such as phenolic compounds and chlorophylls.

In the morphological analyses, 26 quantitative characters were measured and 23 qualitative characters were observed. However, of these measured/observed characters, only 22 quantitative characters were encoded. No significant differences were observed between populations for all qualitative characters and 4 quantitative characters, and therefore 22 quantitative characters were used in the numerical analyses (Table 4.12).

4.1 HPLC-DAD Analysis of Galanthamine and Lycorine

Methanolic extracts of the bulbs and leaves were quantitatively analyzed using HPLC-DAD system. The chromatogram of galanthamine and lycorine was presented in Figure 4.4. Galanthamine content ranged from 0 to 29.53 mg/g in the bulbs and from 0 to 10.37 mg/g in the leaves for all locations. Lycorine content ranged from 4.20 to 26.17 mg/g in the bulbs and from 0.50 to 15.80 mg/g in the leaves for all locations (Table 4.1 and 4.2).

Table 4.1 Quantitative analyses of 2 alkaloids (galanthamine and lycorine) in the bulb and leaf extract of *L. aestivum* collected from 4 different locations (Sakarya, Yalova, Bursa and İstanbul) with the evaluation of 3 months with HPLC-DAD analysis. Before flowering (BF): Vegetative growing season; Flowering (F): Reproductive growing season; After flowering (AF): Ripening growing season.

Locations	Months	Extraction yield (%)		Alkaloid amounts (mg/g dry extract)			
		Bulb	Leaf	Bulb		Leaf	
				G	L	G	L
Sakarya	Mar^{BF}	5.9	20	9.40 ± 0.15^{f}	26.17 ± 0.19^{a}	5.03 ± 0.15^{d}	13.53 ± 0.17^{b}
	Apr^{F}	4.7	23	8.10 ± 0.35^{g}	8.70 ± 0.50^{d}	5.53 ± 0.12^{d}	3.30 ± 0.00^{f}
	May^{AF}	8	17.2	0.40 ± 0.06^{j}	4.20 ± 0.25^{e}	0.53 ± 0.13^{g}	0.90 ± 0.10^{i}
Yalova	Mar^{BF}	8.8	16.6	17.70 ± 0.10^{b}	8.93 ± 0.07^{d}	10.37 ± 0.07^{a}	15.80 ± 0.10^{a}
	Apr^{F}	6.7	20.6	13.73 ± 0.09^{d}	8.17 ± 0.03^{d}	9.15 ± 0.03^{b}	9.35 ± 0.03^{c}
	May^{AF}	5.5	18.9	27.03 ± 0.09^{a}	19.40 ± 0.60^{b}	7.67 ± 0.07^{c}	8.77 ± 0.22^{cd}
Bursa	Mar^{BF}	8.4	20.3	7.17 ± 0.09^{i}	8.80 ± 0.06^{d}	4.93 ± 0.15^{d}	8.03 ± 0.92^{d}
	Apr^{F}	3.2	27.4	7.97 ± 0.19^{gh}	5.60 ± 0.80^{e}	2.83 ± 0.03^{f}	1.90 ± 0.30^{gh}
	May^{AF}	2.3	25.9	16.87 ± 0.19^{c}	11.27 ± 0.73^{c}	2.60 ± 0.10^{f}	0.50 ± 0.06^{i}
İstanbul	Mar^{BF}	9.9	18.1	9.13 ± 0.27^{f}	7.60 ± 1.34^{d}	5.13 ± 0.13^{d}	6.77 ± 0.18^{e}
	April^{F}	3.8	22.8	7.50 ± 0.00^{hi}	8.30 ± 0.06^{d}	2.43 ± 0.07^{f}	2.07 ± 0.41^{g}
	May^{AF}	2	20.1	10.07 ± 0.15^{e}	10.73 ± 0.13^{c}	3.90 ± 0.68^{e}	1.03 ± 0.17^{hi}

Table 4.2 Quantitative analyses of 2 alkaloids (galanthamine and lycorine) in the bulb and leaf extract of *L. aestivum* collected from 2 different locations (Gölcük and Yeniçağa) in Bolu with the evaluation of 8 months with HPLC-DAD analysis. Before flowering (BF): Vegetative growing season; Flowering (F): Reproductive growing season; After flowering (AF): Ripening growing season including leaf senescence.

Locations	Months	Extraction yield (%)		Alkaloid amounts (mg/g dry extract)			
		Bulb	Leaf	Bulb		Leaf	
				G	L	G	L
Bolu-Gölcük	Mar^{BF}	10	18	11.20 ± 0.10^{h}	8.47 ± 0.28^{l}	4.60 ± 0.00^{d}	6.40 ± 0.40^{b}
	Apr^{BF}	9.7	16.6	29.53 ± 0.03^{a}	16.57 ± 0.03^{c}	5.27 ± 0.07^{c}	4.87 ± 0.17^{c}
	May^{F}	10.2	19.5	24.90 ± 0.10^{c}	9.83 ± 0.07^{j}	6.75 ± 0.03^{b}	2.25 ± 0.03^{de}
	June^{AF}	5.6	23.8	16.93 ± 0.07^{f}	10.83 ± 0.03^{h}	4.63 ± 0.03^{d}	1.63 ± 0.03^{f}
	July^{AF}	3.9	18.4	22.87 ± 0.07^{d}	18.73 ± 0.09^{b}	5.33 ± 0.28^{c}	2.50 ± 0.10^{d}
	Aug^{AF}	4.6	NC	27.60 ± 0.06^{b}	20.90 ± 0.06^{a}	NC	NC
	Sept^{AF}	6.7	NC	17.70 ± 0.00^{e}	18.93 ± 0.03^{b}	NC	NC
	Oct^{AF}	7	NC	15.83 ± 0.07^{g}	14.90 ± 0.10^{f}	NC	NC
Bolu-Yeniçağa	Mar^{BF}	9.9	20.2	0.23 ± 0.03^{m}	5.40 ± 0.15^{m}	8.23 ± 0.22^{a}	7.43 ± 0.13^{a}
	Apr^{BF}	10.8	18.4	0.00	10.83 ± 0.03^{h}	0.30 ± 0.00^{e}	5.10 ± 0.00^{c}
	May^{F}	6.2	21.8	1.10 ± 0.00^{k}	10.50 ± 0.00^{i}	0.00	2.27 ± 0.03^{de}
	June^{AF}	3.6	23.1	1.00 ± 0.00^{k}	9.00 ± 0.00^{k}	0.00	1.70 ± 0.00^{f}
	July^{AF}	2.9	20.6	0.70 ± 0.00^{l}	15.20 ± 0.00^{e}	0.00	1.90 ± 0.21^{ef}
	Aug^{AF}	3.2	NC	1.80 ± 0.00^{i}	15.70 ± 0.00^{d}	NC	NC
	Sept^{AF}	4.2	NC	1.40 ± 0.00^{j}	11.20 ± 0.00^{g}	NC	NC
	Oct^{AF}	6.4	NC	1.30 ± 0.00^{j}	9.90 ± 0.00^{j}	NC	NC

If we consider the amount of galanthamine in the bulbs for all localities, the best galanthamine levels were obtained in Gölcük-Bolu (April) at vegetative growing season (29.53 mg/g) (Figure 4.5) and then in the same location (August) at the ripening period (27.69 mg/g). The bulbs obtained from Yalova (May) was also effective in galanthamine content at ripening period (27.03 mg/g). Similarly, leaves obtained from Yalova had the most galanthamine amount (10.37 mg/g) at vegetative growing season. In regard to lycorine amounts, bulbs obtained from Sakarya (26.17 mg/g) and leaves collected from Yalova (15.80 mg/g) had the highest amount of lycorine at vegetative growing period. Other three locations (Bursa, İstanbul and Yeniçağa-Bolu) were not as effective as Gölcük-Bolu, Yalova and Sakarya considering galanthamine and lycorine content. It was observed that bulb and leaves contained more lycorine than galanthamine for Sakarya and Yeniçağa-Bolu. Only leaves had more lycorine than galanthamine for Yalova and Bursa. On the other hand, bulbs included more galantamine than lycorine for Gölcük-Bolu (Table 4.1 and 4.2).

When the locations were evaluated among themselves in terms of growing season, vegetative growing season in the bulbs was the best for Sakarya and Gölcük-Bolu in galanthamine content and only Sakarya in lycorine content. Vegetative growing season of the leaves gave the most galanthamine and lycorine content in Sakarya, Yalova, Bursa, İstanbul and Yeniçağa-Bolu. This vegetative growing season was the best only in lycorine content in the leaves for Gölcük-Bolu location. Reproductive season was not efficient in terms of galathamine and lycorine content in the bulbs and leaves. Only leaves obtained from Gölcük-Bolu showed the most quantity of galanthamine at this season. Contrary to the result of Gorinova et al. (1993), the concentrations of galanthamine in the bulbs were higher than that in the leaves at the flowering stage in all locations (Table 4.1 and 4.2).

If we consider after flowering periods, maximum amount of galanthamine in the bulbs was obtained in Yalova, Bursa, İstanbul and Yeniçağa-Bolu. In addition to these locations, Gölcük-Bolu had the most lycorine content in the bulbs at this season. After reproductive period, leaves did not accumulate more galanthamine and lycorine in Gölcük-Bolu compared to other seasons.

The accumulation of natural products is influenced by growing conditions such as temperature, light regime, and nutrient supply. Stress and other environmental factors have an effect on the metabolic pathways that lead to the accumulation of secondary plant products. (Bohnert, 1995; Selmar and Kleinwächter 2013). In general, increasing temperature caused an increase in alkaloid content in the bulbs for 5 locations (positive correlation). Only the bulbs obtained from Sakarya had the highest galanthamine and lycorine contents in the coldest month, March (negative correlation). Correlation coefficient (r) values between tested alkaloids (galanthamine and lycorine) in the bulbs and monthly average temperatures were 0.99 and 0.67 for Bursa, 0.86 and 0.96 for Yalova, 0.74 and 0.76 for Yeniçağa, 0.63 and 0.99 for İstanbul, 0.25 and 0.44 for Gölcük-Bolu and - 0.99 and - 0.81 for Sakarya, respectively. Relation of alkaloid levels with monthly average temperatures were evaluated for 8 months in Gölcük-Bolu and Yeniçağa-Bolu (Figure 4.2 and 4.3). Weak and moderate correlations were observed with Gölcük-Bolu and Yeniçağa-Bolu, respectively. Lycorine was affected more by monthly average temperature than galanthamine. There was significant habitat difference between two Bolu locations. The plants were collected from within or near the water in Gölcük-Bolu, however, plants were not grown in waterbed in Yeniçağa-Bolu. Increase in the amounts of alkaloids with increasing temperature was not observed in the leaves. Generally, galanthamine and lycorine content was the highest with cold months in the leaves in all localities. Especially monthly average temperatures showed strong negative correlations with galanthamine content in the leaves obtained from Yalova (r = - 0.97) and Sakarya (r = - 0.95), and with lycorine content in the leaves obtained from Yeniçağa-Bolu (r = - 0.98) and Gölcük-Bolu (r = - 0.94). Arslan et al. (2020) found a similar tendency for alkaloid quantities in the bulbs and leaves of *L. aestivum*. They investigated the monthly variations of alkaloids (galanthamine and lycorine) in *L. aestivum* cultivated in a greenhouse in Bolu. Galanthamine and lycorine quantities in the bulbs showed a positive correlation with temperature. But, both alkaloids in the leaves did not demonstrate a relationship with temperature (Arslan et al. 2020). Parallel to the results of our study, many investigations were reported about drought stress related increase in the concentrations of alkaloids (Selmar and Kleinwächter 2013). Sahoo et al. (2012) showed the variations in the total alkaloid and phenol during different seasons in some medicinal plants and indicated that the lowest amount of total alkaloids and phenols were observed in rainy season, while the highest in summer. Jansen et al.

(2009) reported that seed alkaloid content of *Lupinus angustifolius* L. cultivars increased with temperature. Guo et al. (2007) indicated the improvement of the accumulation of some alkaloids with high temperature in *Catharanthus roseus* (L.) G. Don (Madagascar periwinkle). In the case of *Papaver somniferum* L. (opium poppy), *Conium maculatum* L. (wild hemlock) and *Cinchona officinalis* L. (cinchona), alkaloid concentrations were generally higher during dry periods than during wet periods (Timmermann et al., 1984).

Correlation was also evaluated between monthly total precipitation and alkaloid content in the bulbs and leaves, and no general trend was observed (Figure 4.2 and 4.3)

Because of the restricted supply of the natural source and a high cost of synthetic production, harvesting period and different naturally growing populations of *L. aestivum* with the most effective galanthamine and lycorine content should be investigated. Khonakdari et al. (2018) determined maximum galanthamine content in naturally growing populations of *Galanthus nivalis* L. and *Narcissus tazetta* L. in Iran. Georgieva et al. (2007) reported the alkaloid variability of *L. aestivum* bulb extracts collected from 18 Bulgarian populations. They suggested that genetic factor plays important role in the alkaloid biosynthesis of *L. aestivum*. In consistent with galanthamine content results in the bulbs and leaves obtained from Sakarya, Lubbe et al. (2013) indicated that higher galanthamine concentration was observed at before flowering (April) period in the bulbs of *Narcissus pseudonarcissus* cv. Carlton. Leaves also had same level of galanthamine until full flowering (May) followed by a decline after flowering. Elgorashi et al. (2002) found a significant organ-to-organ variation, but no significant seasonal variation in bulb lycorine content. We also determined significant variations of lycorine in regard to bulb and leaves. On the other hand, seasonal variation was also observed with bulb lycorine content in our study (Table 4.1 and 4.2).

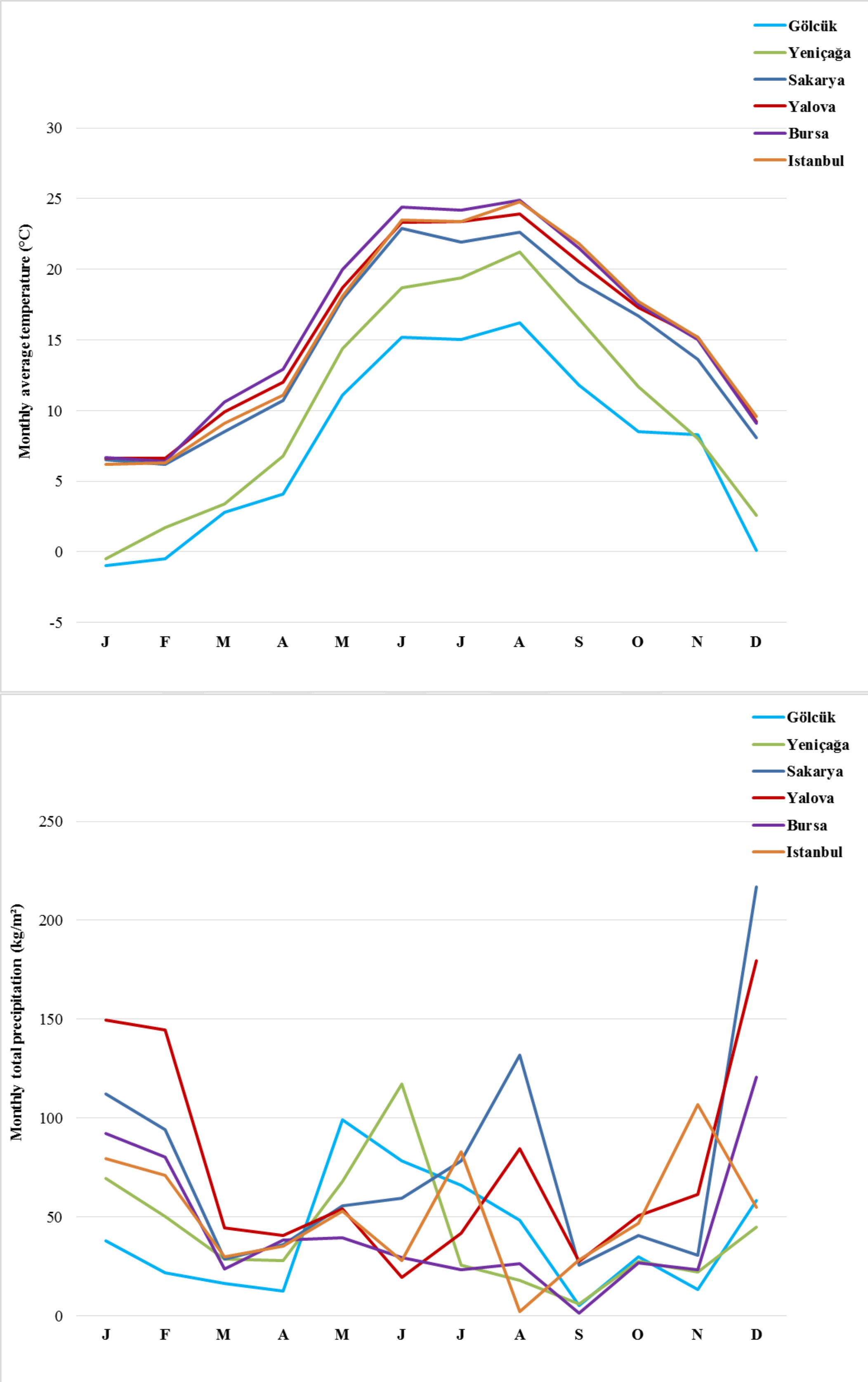

Figure 4.1 Monthly distribution of monthly average temperature (above) and monthly total precipitation (below) in 6 different localities.

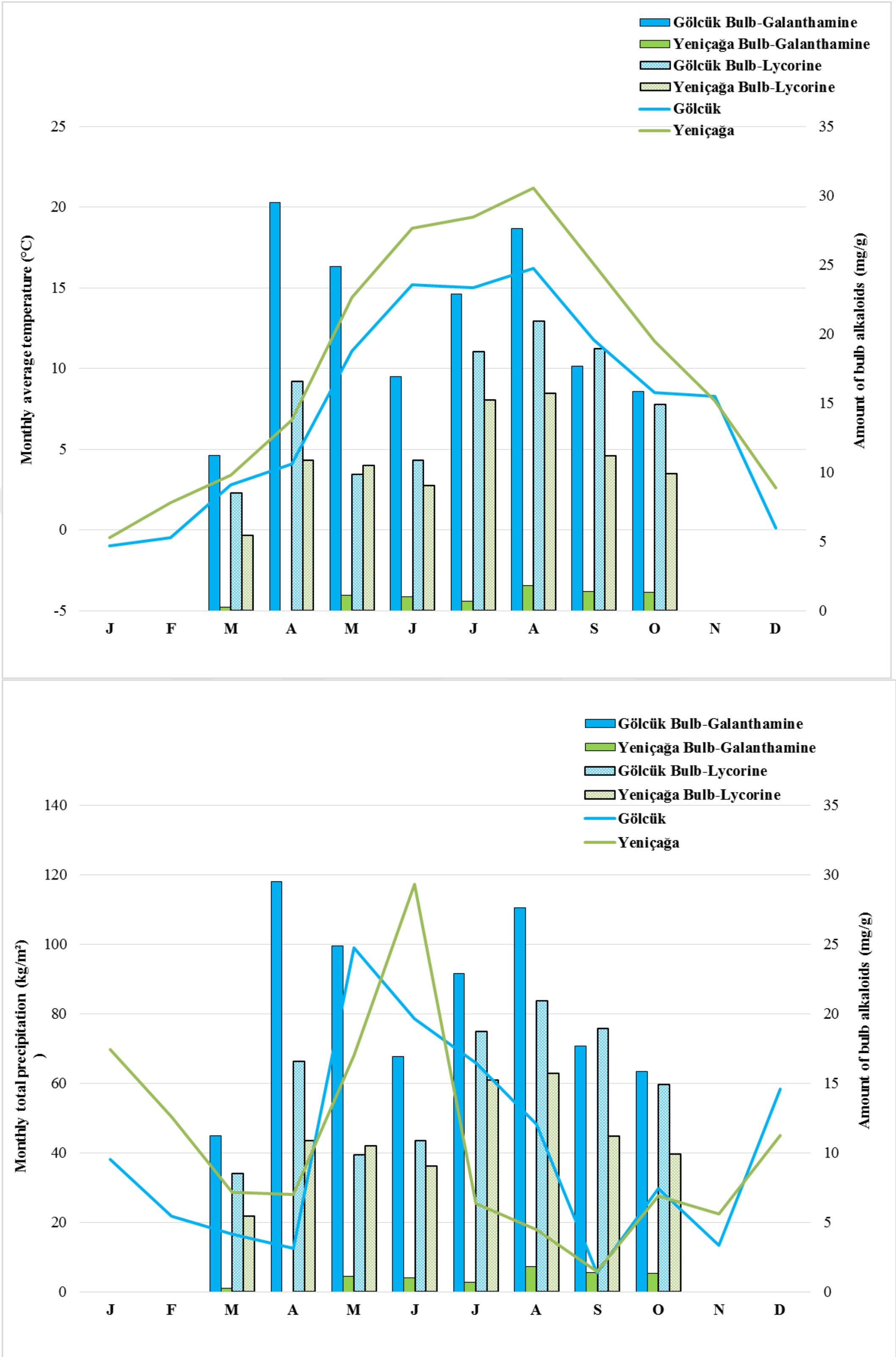

Figure 4.2 Correlation of bulb galanthamine and lycorine levels (mg/g) of *L. aestivum* collected from Bolu and Yeniçağa with monthly average temperature (above) and monthly total precipitation (below).

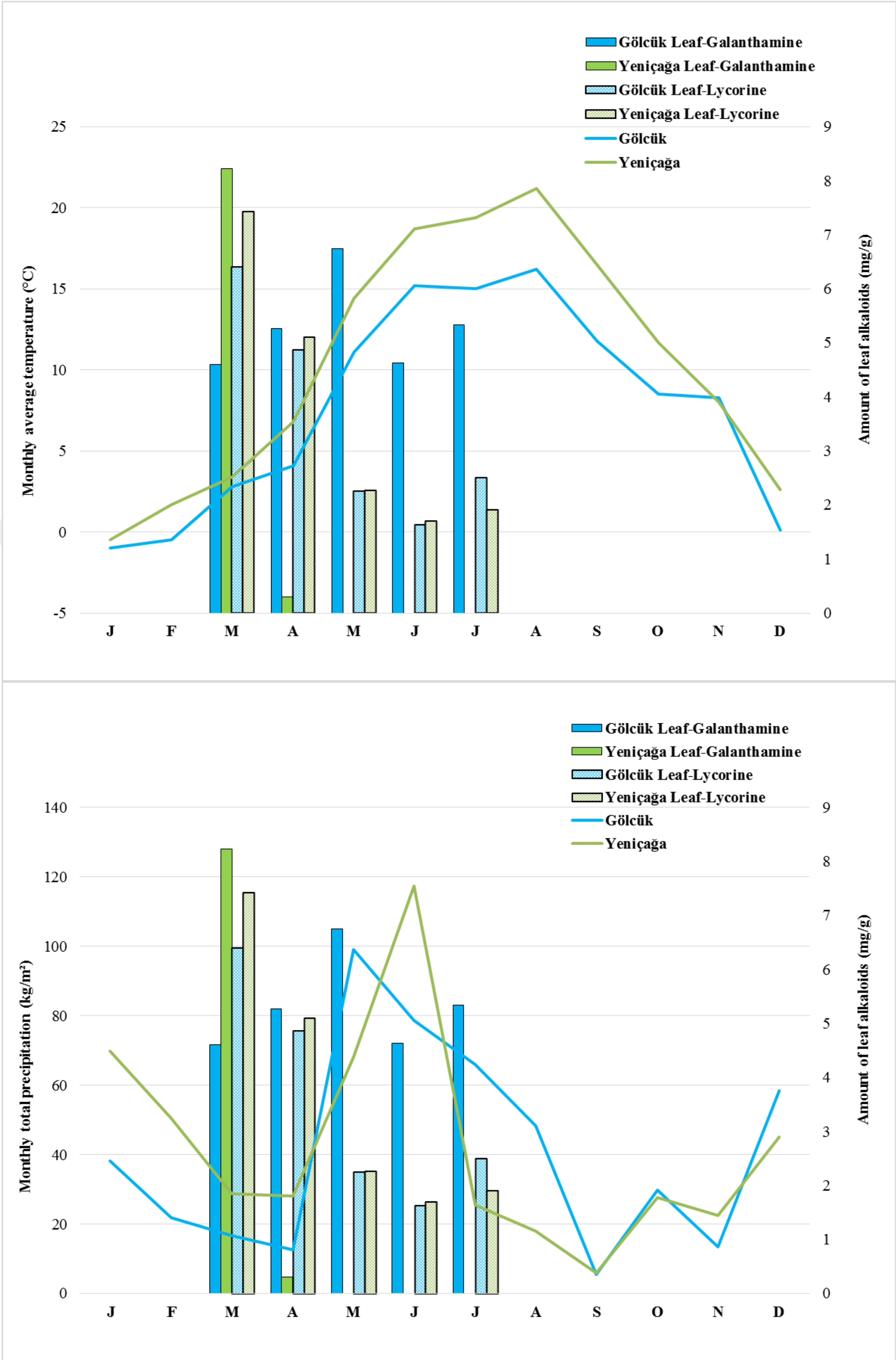

Figure 4.3 Correlation of leaf galanthamine and lycorine levels (mg/g) of *L. aestivum* collected from Bolu and Yeniçağa with monthly average temperature (above) and monthly total precipitation (below).

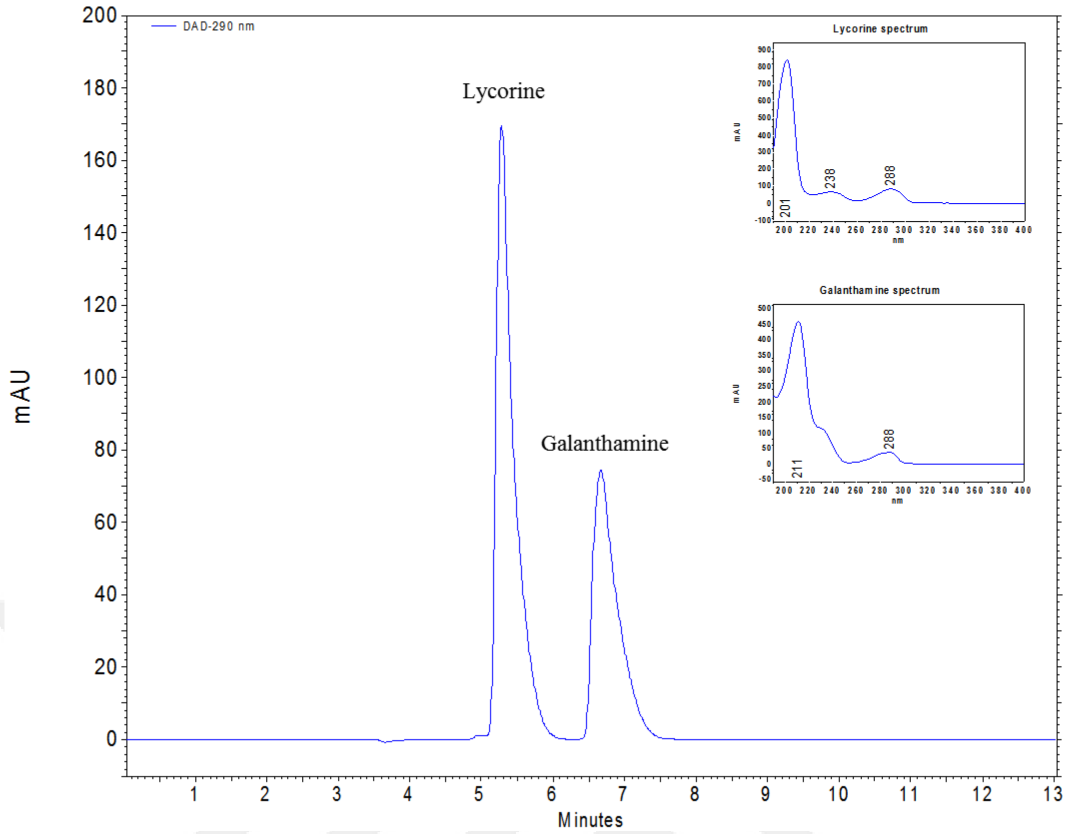

Figure 4.4 HPLC chromatogram of lycorine and galanthamine with their spectrums.

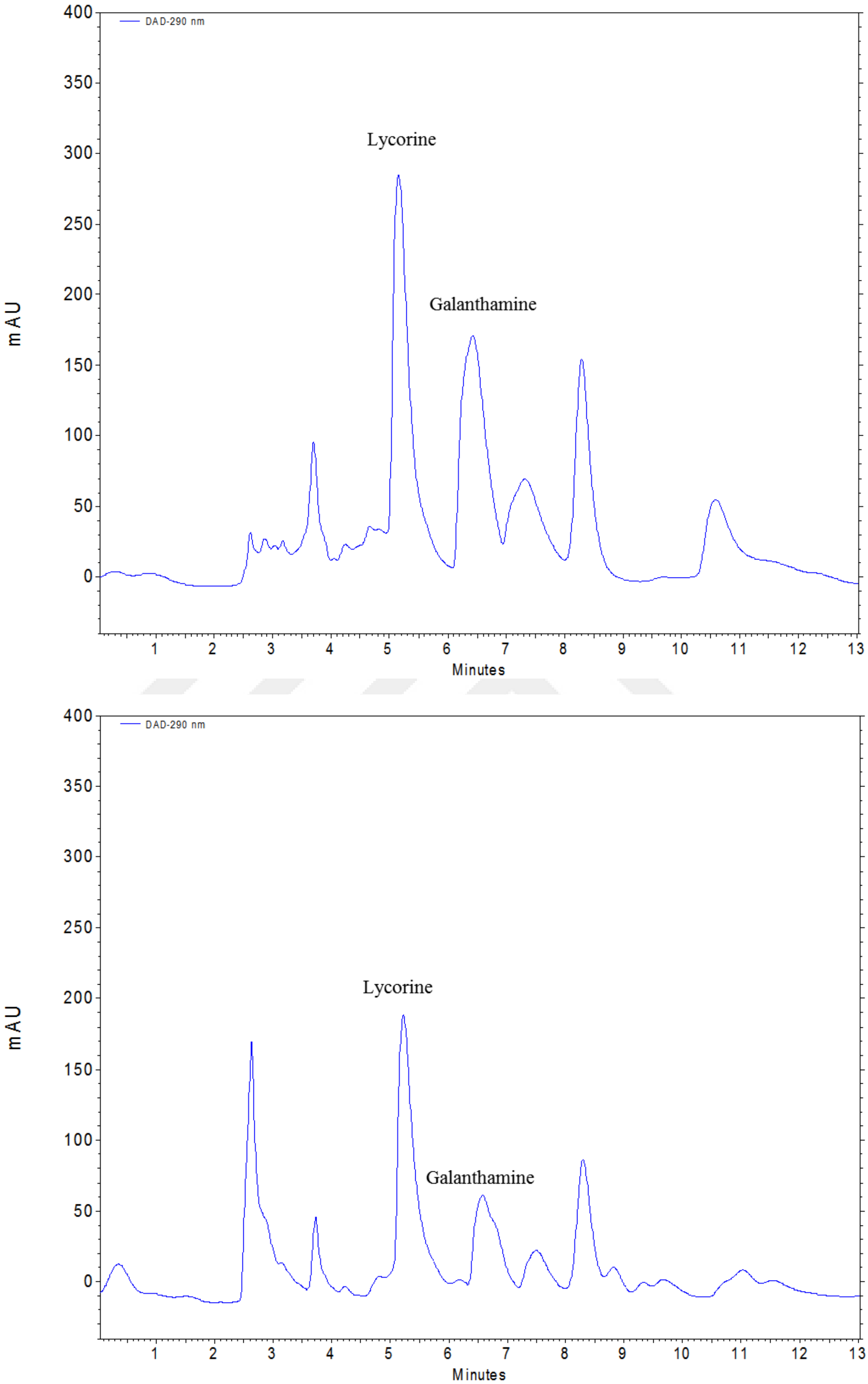

Figure 4.5. HPLC chromatogram of the bulbs (above) and leaves (below) obtained from Gölcük.

Soil samples were taken for each location, along with plant samples, each month, and then all soil samples were analyzed (Table 4.3 and 4.4). Gorinova et al. (1993 and 1995) found a connection between the galantamine content of *L. aestivum* and the chemical composition of the soil, implying that soil fertility levels regulate galantamine biosynthesis. They reported that soils near neutral in pH and rich in organic substances were the most suitable for the growth of *L. aestivum*, and galanthamine levels were the highest in areas where the soils had a good supply of N, K, Mg, B, Mo, Zn, Fe, and Cu. Similarly, neutral to slightly alkaline soils with high content of organic matter were effective to increase the alkaloid levels of *L. aestivum* in our study (Table 4.3 and 4.4). A good correlation (r = 0.81) was observed between organic matter and bulb galanthamine content in Gölcük-Bolu location with the evaluation of 8 months (Table 4.3). A very strong correlation (r = 0.99) was observed between K levels and bulb galanthamine content in Yalova (Table 4.4). Mean number of galanthamine quantities in bulbs in descending order in 3 months were Gölcük-Bolu (21.88 mg/g), Yalova (19.49 mg/g), Bursa (10.67 mg/g), İstanbul (8.90 mg/g), Sakarya (5.97 mg/g) and Yeniçağa-Bolu (0.44 mg/g). Whereas, mean number of soil K levels in ascending order in 3 months were Yalova (7.50 mg/L), Gölcük-Bolu (9.40 mg/L), Bursa (18.89 mg/L), Yeniçağa-Bolu (22.35 mg/L), İstanbul (24.48 mg/L) and Sakarya (40.63 mg/L) (Table 4.4). Yalova, one of the localities with the highest bulb galantamine content had the lowest level of K in soil. On the other hand, Sakarya with low level of bulb galanthamine content had the highest K level in soil. Soil K level may be related to the galanthamine content in *L. ae*stivum. The highest bulb galanthamine contents were observed with the lowest soil K levels. Gorinova et al. (1993) reported that *L. aestivum* had a K requirement and thus produced the most galanthamine only when K levels in the soil were high (82 mg / 100 mg). The sudden increase in total salt content in Gölcük-Bolu (0.421%) in August compared to other months may have caused an increase in the bulb galanthamine and lycorine levels (Table 4.3). Similar to this result, an enhancement in the amounts of galanthamine and lycorine in *L. aestivum* bulbs and leaves under salt stress (4 g/L $CaCl_2$) was observed by Ates et al. (2021). Among the all localities, the highest Cu concentration level in soil was found in Gölcük-Bolu that is the most efficient locality in terms of bulb galanthamine quantity. Sakarya location with the highest K, Mn, Zn and the lowest Fe, Na and Mg comparing with other 5 locations showed low efficiency in bulb galanthamine level.

Table 4.3 Soil texture, EC, total salt (%), pH, $CaCO_3$, organic matter (%) in the soils that were collected every month from 6 different locations while collecting plant samples.

Locations	Months	Soil Texture	E.C (ms/cm)	Total salt (%)	pH	$CaCO_3$ (%)	Organic matter (%)	
Sakarya	**Mar**^{BF}	Clay	0.92	0.0589	7.67	0	3.97	Fine
	Apr^{F}	Clay	1.877	0.12	7.85	0	3.48	Fine
	May^{AF}	Heavy clay	1.977	0.177	6.66	2.39	4.91	High
Yalova	**Mar**^{BF}	Clay	0.611	0.035	7.63	2.87	4.56	High
	Apr^{F}	Clay loam	0.58	0.026	7.68	5.59	3.66	Fine
	May^{AF}	Clay loam	0.302	0.013	6.98	0	4.73	High
Bursa	**Mar**^{BF}	Clay	1.092	0.06	6.2	1.11	4.43	High
	Apr^{F}	Clay	1.282	0.08	7.2	10.06	3.21	Fine
	May^{AF}	Clay	1.209	0.074	7.36	1.27	4.58	High
İstanbul	**Mar**^{BF}	Clay	1.673	0.124	7.26	4.79	0.76	Slightly
	April^{F}	Heavy clay	1.337	0.145	7.6	19.97	4.95	High
	May^{AF}	Heavy clay	1.926	0.208	4.49	17.57	4.83	High
Bolu-Gölcük	**Mar**^{BF}	Heavy clay	1.65	0.14	7.47	7.99	4.49	High
	Apr^{BF}	Clay loam	0.83	0.02	7.76	0	5.08	High
	May^{F}	Clay	1.1	0.07	7.18	0	5.01	High
	June^{AF}	Clay	1.13	0.07	7.44	4.15	4.06	High
	July^{AF}	Clay	0.97	0.06	7.79	16.3	4.64	High
	Aug^{AF}	Clay	0.72	0.42	7.76	9.58	5.07	High
	Sept^{AF}	Heavy clay	0.92	0.06	7.91	15.98	4.06	High
	Oct^{AF}	Heavy clay	1.39	0.09	7.86	1.59	4.21	High
Bolu-Yeniçağa	**March**^{BF}	Clay	0.79	0.05	7.9	3.99	1.77	Low
	April^{BF}	Clay	1.47	0.08	7.75	15.18	4.35	High
	May^{F}	Clay	0.88	0.05	7.96	2.39	2.14	Medium
	June^{AF}	Heavy clay	1.81	0.14	7.73	15.18	2.9	Medium
	July^{AF}	Clay	1.65	0.12	7.79	14.38	4.35	High
	Aug^{AF}	Clay	1.12	0.09	7.65	11.8	4.58	High
	Sept^{AF}	Clay loam	1.12	0.04	7.75	11.66	3.48	Fine
	Oct^{AF}	Clay	0.72	0.03	7.82	12.3	5.22	High

Table 4.4 Macro and micro elements in soils that were collected every month from 6 different locations while collecting plant samples.

		Elements found in soil (mg/L)								
Locations	**Months**	**P**	**K**	**Mn**	**Zn**	**Cu**	**Fe**	**Ca**	**Na**	**Mg**
Sakarya	**Mar^{BF}**	0.0258	58.5	47.60	5.09	4.91	55.54	1077	0.089	9.76
	Apr^{F}	0.0068	20.8	322.0	0.22	0	0	979.2	0.227	7.40
	May^{AF}	0.0213	42.6	133.9	10.91	6.84	41.37	750.9	0.087	7.14
Yalova	**Mar^{BF}**	0.0006	6.87	60.52	1.09	2.12	17.93	979.2	4.85	26.03
	Apr^{F}	0.0013	5.99	131.9	4.94	6.31	119	733.5	1.58	14.19
	May^{AF}	0.0011	9.63	103.0	8.18	7.68	97.41	528.8	0.13	2.70
Bursa	**Mar^{BF}**	0.0017	21.41	23.12	1.97	6.07	43.59	263.9	1.01	14.07
	Apr^{F}	0.0081	15.53	55.35	5.60	18.42	135.30	331.5	1.86	21.77
	May^{AF}	0.0105	19.73	24.56	2.87	9.30	88.99	969.8	1.39	35.19
İstanbul	**Mar^{BF}**	0.0041	21.54	35.28	2.48	6.07	52.67	934.4	2.81	46.28
	April^{F}	0.0050	24.8	30.27	2.55	6.61	48.50	1113	3.98	68.61
	May^{AF}	0.0086	27.09	45.75	4.31	7.66	67.94	1135	10.79	23.48
Bolu-Gölcük	**Mar^{BF}**	0.0085	10.43	131.4	1.58	131.4	70.8	1372	11.21	27.96
	Apr^{BF}	0.0062	13.00	53.18	2.51	53.18	66.49	1052	3.54	55.95
	May^{F}	0.0054	4.76	18.47	1.44	18.47	28.96	1205	9.50	31.45
	June^{AF}	0.0032	13.72	63.88	2.65	63.88	45.52	1123	5.34	66.97
	July^{AF}	0.0029	16.12	58.71	2.57	58.71	37.81	972.6	0.26	18.98
	Aug^{AF}	0.0148	18.48	26.95	2.22	26.95	46.11	768.5	5.27	48.50
	Sept^{AF}	0.0061	13.51	20.44	1.59	20.44	31.78	877.1	0.75	15.32
	Oct^{AF}	0.0114	20.49	91.68	2.05	91.68	42.26	1006	0.81	23.92
Bolu-Yeniçağa	**March^{BF}**	0.0130	22.99	33.30	2.26	8.60	73.54	1042	1.34	11.09
	April^{BF}	0.0018	12.82	40.75	2.46	7.86	51.63	872.1	0.76	29.50
	May^{F}	0.0131	31.23	25.51	2.39	8.11	58.03	1104	6.48	29.35
	June^{AF}	0.0024	21.02	55.00	2.56	9.09	64.34	964.1	0.70	38.40
	July^{AF}	0.0043	17.02	41.34	0.94	5.90	29.11	789.5	3.46	41.91
	Aug^{AF}	0.0096	28.35	28.19	2.60	8.68	63.87	995.1	5.20	65.39
	Sept^{AF}	0.0088	17.83	27.30	2.83	6.57	55.09	1020	2.49	38.50
	Oct^{AF}	0.0770	13.92	47.00	0.01	0.06	0.135	759	12.4	26.70

4.2 Phenolic Content and Antioxidant Potential

Phenolics and flavonoids are powerful scavengers of free radicals due to their hydroxyl groups. Total phenolic content of *L. aestivum* bulb and leaf extracts was calculated from the gallic acid calibration curve (R^2 = 0.9999). Phenolic content ranged from 2.51 to 30.24 mg GAE/g in the bulbs and from 10.47 to 21.03 mg GAE/g in the leaves for all locations. Yeniçağa-Bolu was the best location for phenolic content in the bulb at ripening period and in the leaves at vegetative period. Bulbs obtained at ripening periods in Yeniçağa-Bolu (30.24 mg GAE/g), Gölcük-Bolu (29.26 mg GAE/g), Yalova (28.03 mg GAE/g) and Bursa (27.49 mg GAE/g) had the best total phenolic contents. Total phenolic content of the leaves obtained from Yeniçağa-Bolu at vegetative period (21.03 mg GAE/g), Bursa at reproductive period (19.66 mg GAE/g) and İstanbul at ripening period (19.36 mg GAE/g) had the highest quantities (Table 4.5 and 4.6).

Total flavonoid content of *L. aestivum* extracts was calculated from the quercetin calibration curve (R^2 = 0.9999). Flavonoid content ranged from 3.10 to 70.70 mg QE/g in the bulbs and from 71.49 to 170.85 mg QE/g in the leaves for all locations. Bulbs at vegetative period obtained from Sakarya (70.70 mg QE/g), and the leaves at vegetative period obtained from Gölcük-Bolu (170.85 mg QE/g) and Yeniçağa-Bolu (168.84 mg QE/g) had the highest total flavonoid contents (Table 4.5 and 4.6).

On the contrary to our result, Resetar et al. (2017) found lower total phenolic content in the bulbs and leaves as 4.9 ± 1.52 mg GAE/g and 15.93 ± 2.47 mg GAE/g, respectively. Furthermore, Ates et al. (2021) reported total phenolic content of methanol extract of *L. aestivum* bulbs and leaves in pot culture as 19.02 ± 0.08 GAE/g and 11.46 ± 0.03 GAE/g, and total flavonoid content as 8.22 ± 0.02 mg QE/g and 41.50 ± 0.04 mg QE/g, respectively. Interestingly, Hundur et al. (2018) recorded higher total phenolic content in the bulbs (58.92 mg GAE/g) and leaves (53.93 mg GAE/g), and flavonoid content in the bulb (85 mg QE/g) but lower total flavonoid content (68.33 mg QE/g) than our study.

Antioxidant potential of methanolic extracts of *L. aestivum* bulb and leaves collected from different locations were evaluated determining IC_{50} (half maximal inhibitory concentration) values of the extracts (Table 4.5 and 4.6). The best antioxidant activity was observed with leaves obtained from İstanbul at after flowering period (2.51 mg GAE/g) and then Yeniçağa-Bolu at vegetative period (2.68 mg GAE/g). On the other hand, bulbs obtained from Bursa at flowering period (3.15 mg/ml) and then Sakarya at vegetative period (3.54 mg/ml) demonstrated the best antioxidant potential. The highest antioxidant potentials of the leaves obtained from İstanbul and Yeniçağa-Bolu should be related to the highest total phenolic contents (19.36 mg GAE/g and 21.03 mg GAE/g, respectively) in these leaves. Similarly, the highest antioxidant potentials of the bulbs obtained from Bursa and Sakarya were accompanied by the highest total flavonoid contents (62.86 mg QE/g and 70.70 mg QE/g, respectively) in these bulbs. Generally, IC_{50} values of the bulbs and leaves showed a negative correlation with total phenol and flavonoid content. For example, strong negative correlation was observed with total phenol in the leaves (r = - 0.97) and total flavonoid in the bulbs (r = - 0.99) in Bursa. It can be said that the increase in total phenol and flavonoid content caused an increase in the antioxidant capacity.

All *L. aestivum* bulbs and leaves did not show strong antioxidant capacity, and the best IC_{50} values of bulbs and leaves were observed as 3.15 mg/ml and 2.51 mg/ml, respectively in our study. Similar to our result, different studies showed intermediate antioxidant capacity. Hundur et al. (2018) reported IC_{50} values of naturally grown *L. aestivum* bulbs and leaves methanol extract as 317µg/mL and 345 µg/mL, respectively. Nikolova et al. (2011) indicated IC_{50} value as > 200 µg/mL for methanol extract of naturally grown *L. aestivum* leaves. Ates et al. (2021) reported IC_{50} value of methanol extract of *L. aestivum* bulbs and leaves in pot culture as > 20 mg/ml. Resetár et al. (2017) reported moderate antioxidant capacity of native *L. aestivum* bulbs and leaves using different radical scavenging activity method (ABTS*). Genotypic variation, culture conditions and differences in the method applied may be the reasons of obtaining various results about antioxidant potential and total phenol-flavonoid content in *L. aestivum* bulbs and leaves.

It is noticeable that alkaloid and total phenol accumulation in the bulbs showed similar tendency. For example, the highest temperature (May) in Yalova, Bursa and İstanbul provoked the highest total phenolic content and alkaloid levels (galanthamine and lycorine) in the bulbs. Higher levels of total phenol and flavonoid contents in the bulbs and leaves were observed at the vegetative period like galanthamine and lycorine levels in Sakarya. In parallel to the results of tested alkaloid contents, dry months with the highest temperatures (July and August) in Bolu locations (Gölcük and Yeniçağa) induced the higher content of total phenol in the bulbs. A relationship was observed between monthly average temperature and total phenolic content in the bulbs for Gölcük-Bolu and Yeniçağa-Bolu with the evaluation of 8 months as $r = 0.49$ and $r = 0.51$, respectively. The rise in salt content in August in Gölcük-Bolu may have caused a rise in total phenol content in the bulbs. Similarly, Ates et al. (2021) reported that salt stress caused by some concentrations of $CaCl_2$ or NaCl enhanced the antioxidant activity and total phenol-flavonoid content in the bulbs and leaves. Soil in Sakarya with the highest K level in March comparing with other 5 locations had better antioxidant capacity in the bulbs and higher total phenol-flavonoid content in the bulbs and leaves. These rises may be related to K level in the soil. Higher Fe level in the soil in Bursa may be related to the best antioxidant capacity in the bulbs and total phenol and flavonoid contents in the bulbs and leaves.

Table 4.5 DPPH radical scavenging activity and total phenol-flavonoid content of *L. aestivum* in the bulb and leaf extract of *L. aestivum* collected from 4 different locations (Sakarya, Yalova, Bursa and İstanbul) with the evaluation of 3 months.

Locations	Months	*L. aestivum* extracts: IC$_{50}$ DPPH Inhibition (mg/ml)		Total Phenol (mg GAE/g extract)		Total Flavonoid (mg QE/g extract)	
		Bulb	Leaf	Bulb	Leaf	Bulb	Leaf
Sakarya	**Mar^{BF}**	3.54±0.02^{c}	5.04±0.04^{g}	21.14±0.77^{b}	17.88±0.12^{b}	70.70±3.00^{a}	151.28±8.03^{a}
	Apr^{F}	13.26±0.08^{i}	6.26±0.01^{k}	12.65±3.43^{de}	15.00±0.37^{cd}	22.37±2.61^{f}	86.06±2.98^{d}
	May^{AF}	8.86±0.02^{fg}	9.63±0.06^{m}	8.60±0.12^{f}	12.95±0.20^{e}	22.54±0.61^{f}	151.65±2.89^{a}
Yalova	**Mar^{BF}**	>20	3.90±0.02^{c}	10.03±1.22^{ef}	16.15±0.33^{c}	3.10±0.72^{h}	105.71±4.65^{c}
	Apr^{F}	18.59±0.09^{j}	4.50±0.02^{f}	2.51±0.27^{h}	10.72±0.36^{f}	23.02±1.88^{f}	151.63±4.59^{a}
	May^{AF}	5.33±0.02^{e}	5.75±0.04^{i}	28.03±0.33^{a}	14.07±0.59^{de}	35.35±1.19^{d}	142.63±1.65^{a}
Bursa	**Mar^{BF}**	8.73 ± 0.06^{f}	5.54±0.02^{h}	8.19±0.09^{f}	10.82±0.18^{f}	25.61±3.58^{ef}	73.25±1.94^{e}
	Apr^{F}	3.15 ± 0.03^{b}	8.97±0.02^{l}	15.65±0.20^{cd}	19.66±0.21^{a}	62.86±0.87^{b}	117.61±4.12^{bc}
	May^{AF}	5.18 ± 0.01^{d}	5.84±0.02^{j}	27.49±0.43^{a}	13.81±0.40^{de}	49.04±3.84^{c}	114.28±1.37^{c}
İstanbul	**Mar^{BF}**	13.24±0.08^{i}	4.31±0.01^{e}	4.33±0.25^{gh}	16.01±0.25^{c}	12.10±2.16^{g}	71.49±3.33^{e}
	April^{F}	13.05±0.04^{h}	4.21±0.01^{d}	6.58±3.21^{fg}	18.88±1.02^{ab}	32.26±1.55^{de}	128.73±4.42^{b}
	May^{AF}	8.91±0.03^{g}	2.51±0.02^{b}	16.55±1.42^{c}	19.36±0.48^{a}	33.44±2.22^{d}	114.42±3.61^{c}
Quercetin		0.022 ± 0.00^{a}					

Table 4.6 DPPH radical scavenging activity and total phenol-flavonoid content of *L. aestivum* in the bulb and leaf extract of *L. aestivum* collected from 2 different locations in Bolu (Gölcük and Yeniçağa) with the evaluation of 8 months.

Locations	Months	*L. aestivum* extracts: IC$_{50}$ DPPH Inhibition (mg/ml)		Total Phenol (mg GAE/g extract)		Total Flavonoid (mg QE/g extract)	
		Bulb	Leaf	Bulb	Leaf	Bulb	Leaf
Bolu-Gölcük	**Mar^{BF}**	8.68±0.04^{l}	5.37±0.01^{d}	13.35±1.06^{f}	12.92±0.17^{bc}	14.96±3.73^{i}	75.46±2.95^{ef}
	Apr^{BF}	14.24±0.02^{o}	4.50±0.05^{c}	19.00±0.09^{de}	14.78±0.25^{b}	42.10±8.85^{c}	170.85±4.87^{a}
	May^{F}	16.32±0.05^{p}	7.00±0.01^{f}	13.66±0.53^{f}	12.94±0.16^{bc}	40.98±1.07^{cd}	145.93±4.47^{c}
	June^{AF}	9.52±0.02^{m}	20.80±0.01^{j}	15.69±0.38^{f}	10.90±0.32^{bc}	33.54±0.99^{cdef}	146.28±2.16^{c}
	July^{AF}	4.10±0.03^{b}	20.10±0.10^{i}	29.26±1.24^{a}	12.08±0.47^{bc}	35.32±1.27^{cde}	157.04±4.64^{b}
	Aug^{AF}	7.10±0.01^{i}	NC	26.22±0.13^{b}	NC	22.68±1.92^{ghi}	NC
	Sept^{AF}	6.71±0.01^{h}	NC	21.69±0.51^{c}	NC	22.31±1.15^{ghi}	NC
	Oct^{AF}	8.50±0.02^{j}	NC	24.51±0.65^{b}	NC	14.36±0.65^{i}	NC
Bolu-Yeniçağa	**March^{BF}**	5.68±0.03^{e}	6.19±0.03^{e}	22.37±1.45^{c}	13.25±0.31^{bc}	18.41±1.89^{hi}	85.38±4.00^{de}
	April^{BF}	8.12±0.02^{j}	2.68±0.02^{b}	13.86±0.39^{f}	21.03±1.42^{a}	25.86±0.89^{fgh}	168.84±2.79^{a}
	May^{F}	8.46±0.02^{k}	7.33±0.06^{g}	17.98±0.49^{e}	12.34±0.26^{bc}	39.47±1.37^{cd}	87.89±2.91^{d}
	June^{AF}	5.13±0.003^{c}	7.03±0.03^{f}	21.48±0.19^{c}	18.66±3.39^{a}	59.31±1.40^{a}	80.33±2.68^{def}
	July^{AF}	5.83±0.02^{f}	12.18±0.05^{h}	20.58±0.23^{cd}	10.47±0.52^{c}	32.38±0.77^{def}	73.50±2.74^{f}
	Aug^{AF}	5.43±0.01^{d}	NC	30.24±1.48^{a}	NC	49.96±0.85^{b}	NC
	Sept^{AF}	6.60±0.01^{g}	NC	20.60±0.41^{cd}	NC	27.93±0.99^{efg}	NC
	Oct^{AF}	13.11±0.02^{n}	NC	15.00±0.52^{f}	NC	22.55±1.17^{ghi}	NC
Quercetin		0.022 ± 0.00^{a}					

4.3 Antibacterial Activity

Antibacterial activity of 28 bulb extracts and 22 leaf extracts obtained from all localities were evaluated against 10 bacterial pathogens and none of the extracts showed antibacterial potential. Six different antibiotics (ampicillin, erythromycin, penicillin, piperacillin-tazobactam, tetracycline and trimethoprim/ sulfamethoxazole) were also tested as positive controls. Generally, antibacterial activity of antibiotics were observed against all tested bacteria (Table 4.9). The highest inhibition zones were 40.66 ± 0.33 mm for trimethoprim/sulfamethoxazole against *S. marcescens*, 32.33 ± 0.33 mm for piperacillin-tazobactam against *S. aureus* and *P. vulgaris*, and 30.83 ± 0.17 mm for erythromycin against *S. aureus* (Table 4.9). Similar to our result, Ločárek et al. (2015) did not observe the effect of ethanol extract of *L. aestivum* bulbs against *S. aureus* and *E. coli*. On the other hand, Hundur et al. (2018) reported the antibacterial activity of ethanol extracts of below ground parts of *L. aestivum* against *E. coli and S. typhimurium*.

Table 4.7 Antibacterial activity of *L. aestivum* in the bulb and leaf extract of *L. aestivum* collected from 6 different locations (Sakarya, Yalova, Bursa and İstanbul) with the evaluation of 3 months, in Bolu (Gölcük and Yeniçağa) with the evaluation of 8 months.

Locations	Periods	Mean Diameter of Inhibitory Zones (mm ± SE) *S. auerus*	*S. epidermidis*	*S. pyogenes*	*S. marcescens*	*S. typhimurium*	*P. aeruginosa*	*P. vulgaris*	*K. pneumonia*	*E. cloacae*	*E. coli*
Sakarya	BF	-	-	-	-	-	-	-	-	-	-
	F	-	-	-	-	-	-	-	-	-	-
	AF	-	-	-	-	-	-	-	-	-	-
Yalova	BF	-	-	-	-	-	-	-	-	-	-
	F	-	-	-	-	-	-	-	-	-	-
	AF	-	-	-	-	-	-	-	-	-	-
Bursa	BF	-	-	-	-	-	-	-	-	-	-
	F	-	-	-	-	-	-	-	-	-	-
	AF	-	-	-	-	-	-	-	-	-	-
İstanbul	BF	-	-	-	-	-	-	-	-	-	-
	F	-	-	-	-	-	-	-	-	-	-
	AF	-	-	-	-	-	-	-	-	-	-
Bolu-Gölcük	BF	-	-	-	-	-	-	-	-	-	-
	F	-	-	-	-	-	-	-	-	-	-
	AF	-	-	-	-	-	-	-	-	-	-
Bolu-Yeniçağa	BF	-	-	-	-	-	-	-	-	-	-
	F	-	-	-	-	-	-	-	-	-	-
	AF	-	-	-	-	-	-	-	-	-	-
Ampicillin (10 µg)		21.83 ± 0.44^{d}	16.33 ± 0.17^{d}	24.16 ± 0.17^{ab}	8.16 ± 0.17^{d}	18.16 ± 0.17^{d}	-	12.33 ± 0.17^{d}	-	20.66 ± 0.33^{d}	-
Erythromycin (15 µg)		30.83 ± 0.17^{b}	26.50 ± 0.29^{a}	18.16 ± 0.17^{d}	-	8.16 ± 0.17^{c}	-	8.16 ± 0.17^{c}	8.16 ± 0.17^{d}	-	8.16 ± 0.17^{c}
Penicillin (1 µg)		24.33 ± 0.33^{c}	-	24.5 ± 0.29^{a}	-	-	-	-	-	-	-
Piperacillin-Tazobactam (30/6 µg)		32.33 ± 0.33^{a}	21.33 ± 0.33^{c}	23.33 ± 0.17^{bc}	26.33 ± 0.33^{b}	19.33 ± 0.17^{c}	26.33 ± 0.17^{a}	32.33 ± 0.33^{a}	30.66 ± 0.33^{b}	25.66 ± 0.33^{b}	17.83 ± 0.44^{ab}
Tetracycline (30 µg)		30.66 ± 0.17^{b}	8.16 ± 0.17^{c}	22.66 ± 0.66^{c}	18.16 ± 0.17^{c}	24.33 ± 0.33^{b}	10.50 ± 0.29^{b}	24.33 ± 0.33^{c}	24.33 ± 0.33^{b}	22.33 ± 0.17^{b}	17.16 ± 0.17^{b}
Trimethoprim/sulfamethoxazole (25 µg)		32.66 ± 0.33^{a}	24.33 ± 0.44^{b}	-	40.66 ± 0.33^{a}	30.66 ± 0.33^{a}	-	30.66 ± 0.33^{b}	30.66 ± 0.33^{a}	26.50 ± 0.29^{a}	18.66 ± 0.17^{a}

4.4 Morphologic Examination

Bulb 1.9–4.2 × 1.9–4.7 cm, brown, sub-spherical. Leaf number 4–7, 15.7–52.2 cm × 8–16 mm, broadly linear, grassy green, spotless, glabrous. Cataphyll 6.5–20.5 × 1–2.1 cm. Scape 21.1–53.8 cm × 4–9 mm, equalling or exceeding leaves stout, hollow, pale, glabrous, 2-hyaline winged, sometimes with second scape. Umbel 3–7 flowered. Spathe 2.1–4.1 cm × 4–7 mm, pale, glabrous, 1-valved. Pedicels 2.1–6.9 cm x c. 1 mm, pale, glabrous. Tepals 1.2–2 cm × 4–9 mm, white with a green blotch just below thickened apex. Anther c. 4 × 1 mm. Gynoecium 1.2–1.9 cm. Ovary 3–9 × 3–7 mm, green, glabrous. Fruit 1.9–2.9 × 1.8–2.8 cm, pale, sub-spherical. Seed 5–7 × 4–6 mm, black, spherical. Odorless. Flowering from March to May. Fruting from May to July. Populations are under threatened in all locations due to picnic areas, camping and grazing.

In the numerical evaluation of all taxa, principal component and cluster analyses were used to analyses the variation of 22 morphological characters measured for 6 OTUs. The character states are listed in (Table 4.9). In PCA analyses, the first two components gave a result which contained 62.6% of the variations (Table 4.8). Therefore, first and second components are graphed as a result of PCA and it was interpreted (Figure 4.6). In PCA graph, LEU1010 and LEU1011 were clearly separated from other populations. However, LEU1006 was separated from LEU1007, LEU1008 and LEU1009. LEU1007 and LEU1009 were similar to each other.

Table 4.8 Principal component analyses for all OTUs

Component	Total	% of Variance	Cumulative %
1	7.762	35.3	35.3
2	6.005	27.3	62.6
3	3.629	16.5	79.1
4	2.698	12.3	91.3
5	1.905	0.8	1

Table 4.9 Selected characters and character states used in principal component and cluster analysis

CHARACTERS AND CHARACTER STATES FOR ALL TAXA																							
CH		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**
OTUs		**FN**	**GYN**	**OL**	**OW**	**SPL**	**SPW**	**SCL**	**SCW**	**BL**	**BW**	**LN**	**LL**	**LW**	**PL**	**TL**	**TW**	**CAL**	**CAW**	**SL**	**SW**	**FL**	**FW**
1	**LEU1006**	2	2	2	3	2	2	3	1	2	2	2	2	2	2	1	1	2	2	1	2	1	1
2	**LEU1007**	1	2	2	2	1	2	2	1	2	2	2	2	2	1	1	2	2	2	2	2	2	2
3	**LEU1008**	1	2	2	2	1	2	2	1	1	1	1	2	1	1	2	1	1	2	2	2	3	2
4	**LEU1009**	2	1	2	2	1	2	1	1	2	2	2	1	1	1	2	2	2	2	1	2	2	1
5	**LEU1010**	1	2	3	2	1	1	4	2	2	2	1	3	2	3	2	2	2	2	1	1	2	2
6	**LEU1011**	1	1	1	1	1	1	4	1	2	1	1	3	2	2	2	1	2	1	1	1	2	2

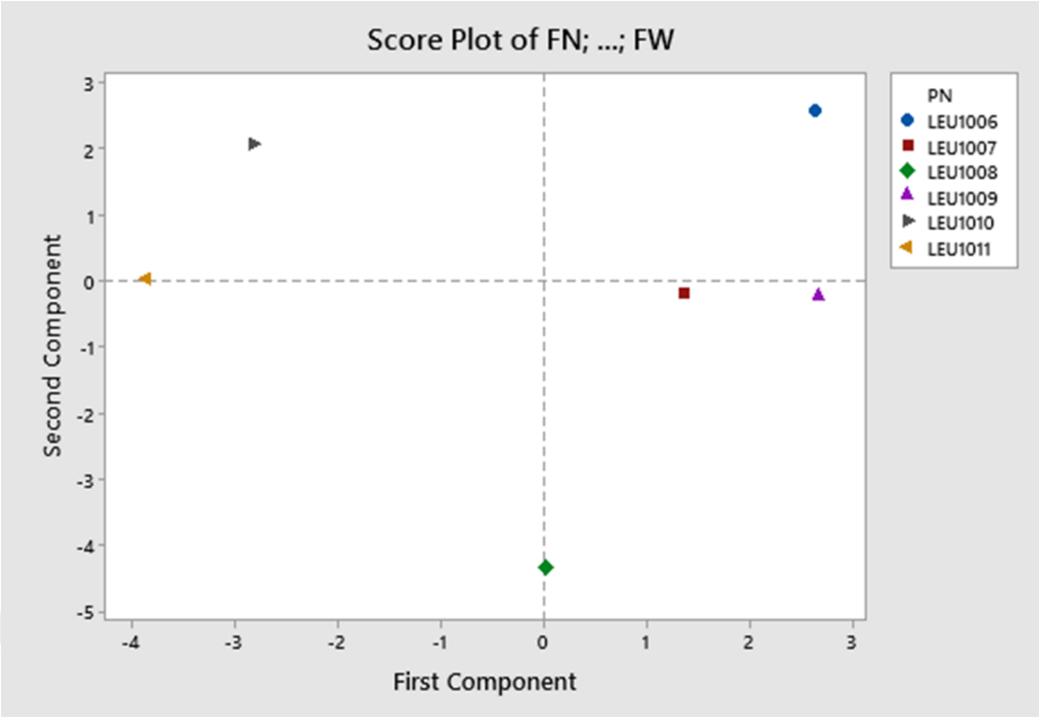

Figure 4.6 The results of principle component analysis of all OTUs

The results of cluster analysis can be explained as follows (Figure 4.7): All clusters were generated in two main groups at the similarity levels of 14.36–44.53%. At 44.53%, LEU1007 and LEU 1009 were highly similar to each other but the LEU1008 showed some differences and was joined to this group at 38.29%. Then, LEU1006 merged with this group at 29.86%. Other distinct populations LEU1010 and LEU1011 were similar to each other at 34.95%. After that, LEU1010 and LEU1011 were linked to them at 14.36%.

Ecological and geographic conditions are important factors for morphological similarities. According to our numerical results, the most similarity is between Gölcük-Bolu and Yeniçağa-Bolu populations. These two locations exist in the same city and have similar climatic conditions and altitudes (Figure 4.1, 4.2, 4.3 and Table 3.1). On the other hand, İstanbul, Bursa and Sakarya have similar altitudes but Sakarya has different climatic conditions for total precipitation and mean temperature than others. Also, İstanbul, Bursa and Yalova have similar have climatic conditions for total precipitation and mean temperature but Yalova has high altitude than others. According to cluster and principal component analyses, İstanbul and Bursa populations show more morphological similarity than the other two locations (Yalova and Sakarya). The Yalova population is morphologically close to these two populations (İstanbul and Bursa) compared to the Sakarya population. In light of this information, geographical proximity, altitude and climatic conditions can be considered effective in determining morphological proximity or distance. On the other hand, no significant effect of soil structure on morphological character was observed.

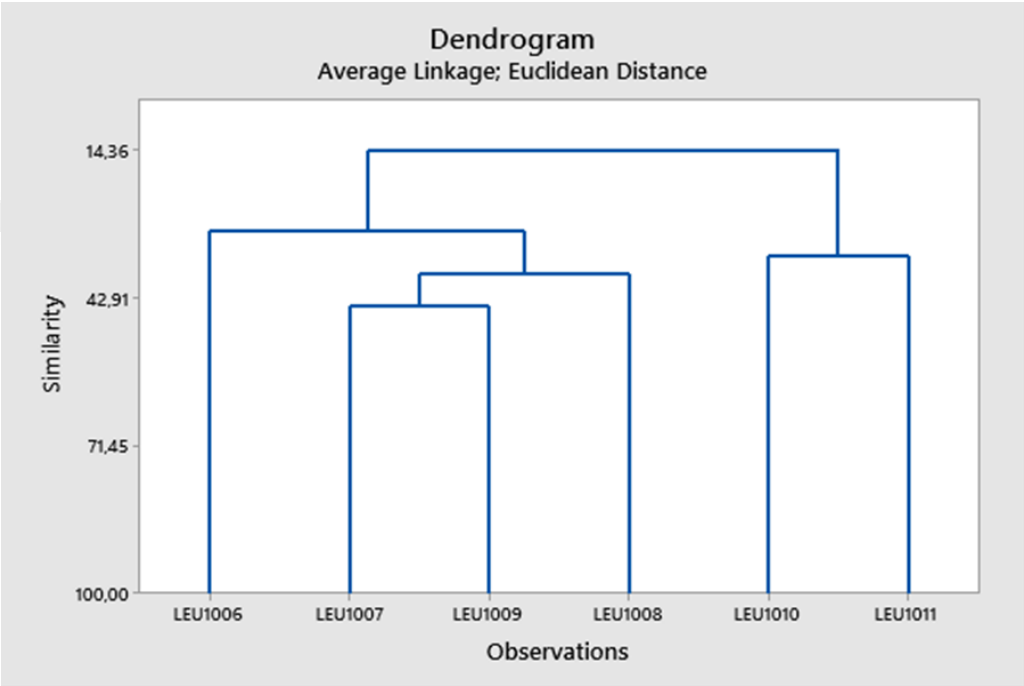

Figure 4.7 Cluster analyses of all OTUs

5. CONCLUSIONS AND RECOMMENDATIONS

Galanthamine is a highly valuable alkaloid in the pharmaceutical industry for the treatment of the symptoms of Alzheimer's disease. Lycorine has also recently attracted attention due to its Anti-COVID-19 effect. This study was conducted for the revealing of the galanthamine rich populations in Turkey. Genetic factor is important in the alkaloid biosynthesis of *L. aestivum* and the most efficient population in terms of galanthamine and lycorine was revealed with this study for the first time among the locations studied. *L. aestivum* bulbs obtained from Gölcük-Bolu was the most efficient in terms of galanthamine and lycorine levels. Bulbs and leaves obtained from Yalova were also productive in regard to these alkaloids. Collection of plants at different growing season showed that full flowering season (reproductive period) was not effective in alkaloid content. Plants use all their power for reproduction at this period. Vegetative period followed by ripening periods were the most productive in terms of alkaloid accumulation. It can be concluded that leaves had more galanthamine and lycorine at vegetative season. Leaves can be the best collected as they open during the spring months and alkaloid contents drops as they start to bloom. Bulbs were efficient in terms of galanthamine and lycorine content both before flowering and after flowering periods at all tested locations. Both seasons were efficient once the plant has drawn its vitality back below the ground.

Generally higher temperatures in the bulbs and lower temperatures in the leaves supported the galanthamine and lycorine production. General trend in correlation between monthly total precipitation and alkaloid content in the bulbs and leaves were not observed.

Soils that were neutral to slightly alkaline with a high organic matter concentration were effective in increasing the alkaloid content of *L. aestivum*. Gölcük-Bolu and Yalova had the lowest soil potassium (K) level and the highest bulb galanthamine content. These two locations provided galanthamine rich population and optimal growing conditions for the cultivation of this species.

Comparison of antioxidant potential and total phenol-flavonoid content demonstrated that phenolic content accumulation was not related to the growing seasons in *L. aestivum*. Yeniçağa-Bolu was the most efficient in terms of phenolic content in the bulbs and leaves. Bulbs from Sakarya and leaves from Gölcük-Bolu had the most total flavonoid content. Bulbs from Bursa and leaves from İstanbul had the best antioxidant capacity.

All tested bulb and leaf extracts of *L. aestivum* did not show antibacterial activity.

According to morphological analysis, climatic conditions and geographic proximity were the most important factor for similarities. It was supported by the similarity of Gölcük and Yeniçağa populations in Bolu. On the other hand, both methods used in morphometric analyze the samples and characters will be an important source for further research.

Bulbs collected from each location were planted in research and application field of bulbous plants in Bolu Abant İzzet Baysal University in order to provide guidance for future studies.

Impact of soil elemental levels in alkaloid accumulation was noteworthy and future studies should be focused on the effect of macro and micro elements especially K and Cu on alkaloid production.

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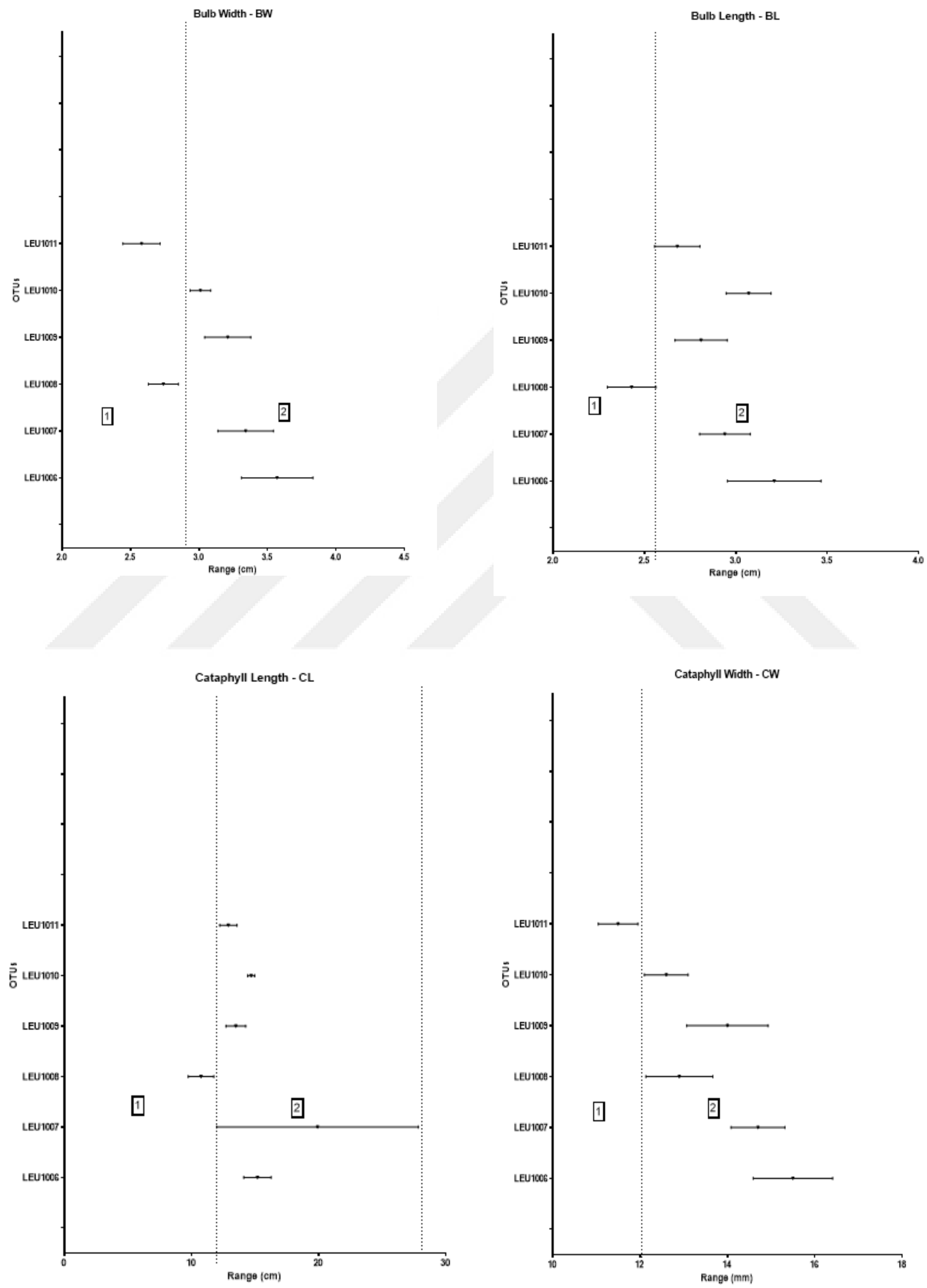
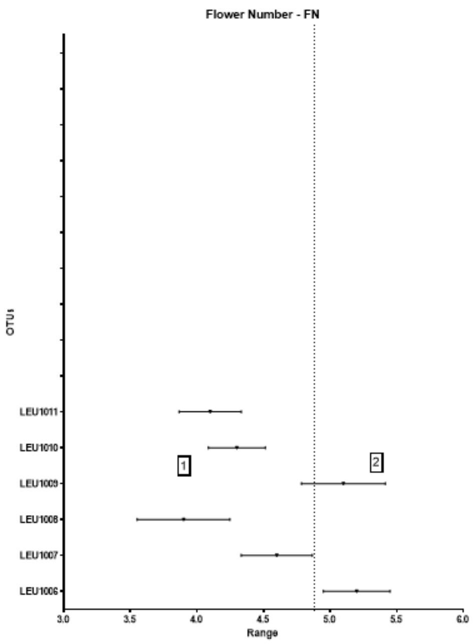
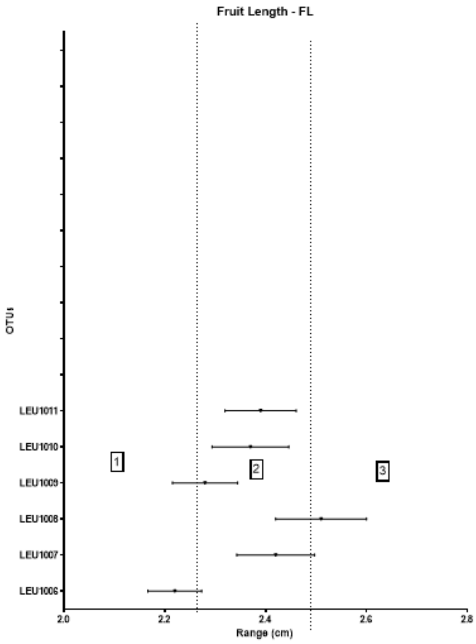
7. APPENDICES

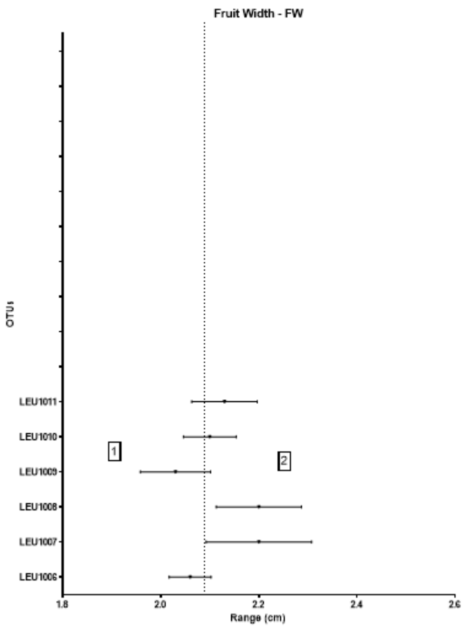
Appendix 1 Codified Quantitative Characters in Graphpad Program (Means with Standart Error Plots).

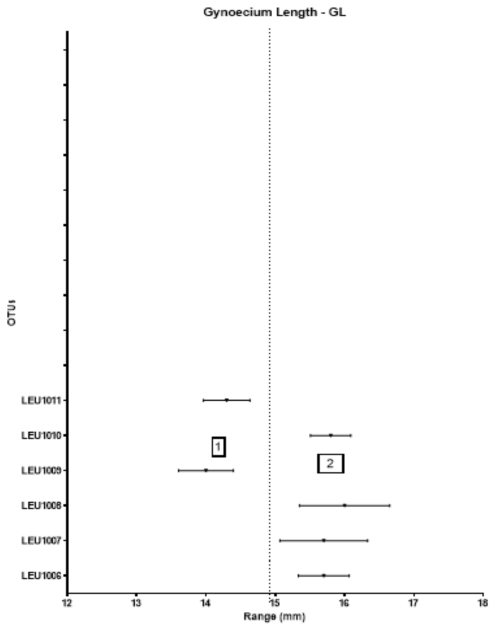


Appendix 1 (continued)

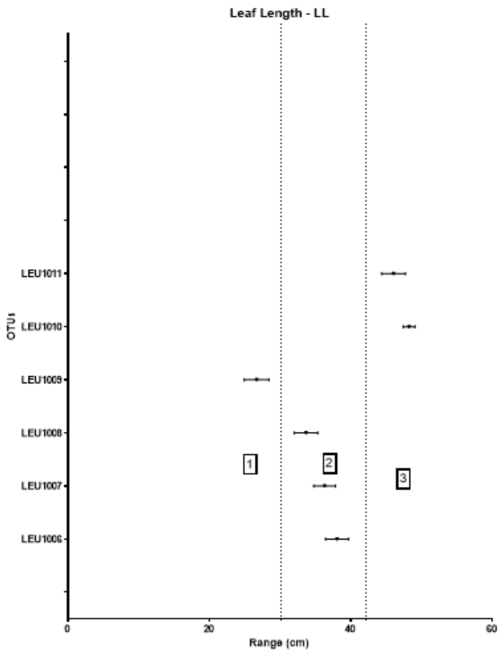


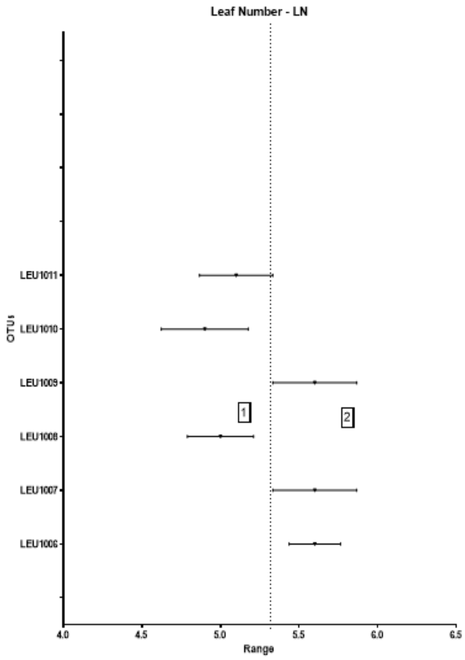


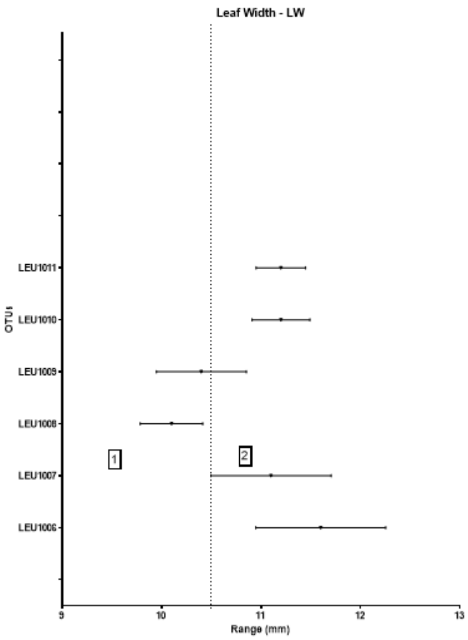


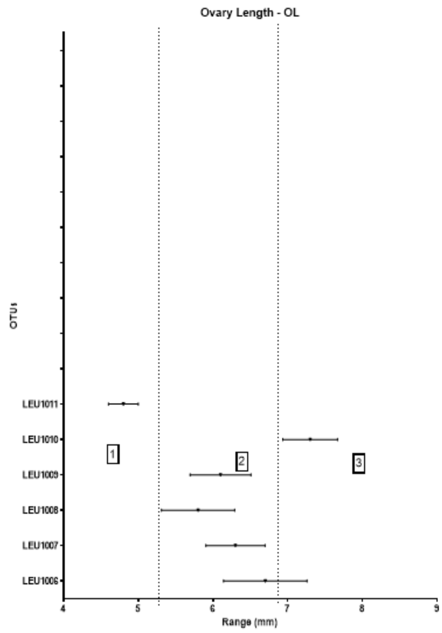


Appendix 1 (continued)









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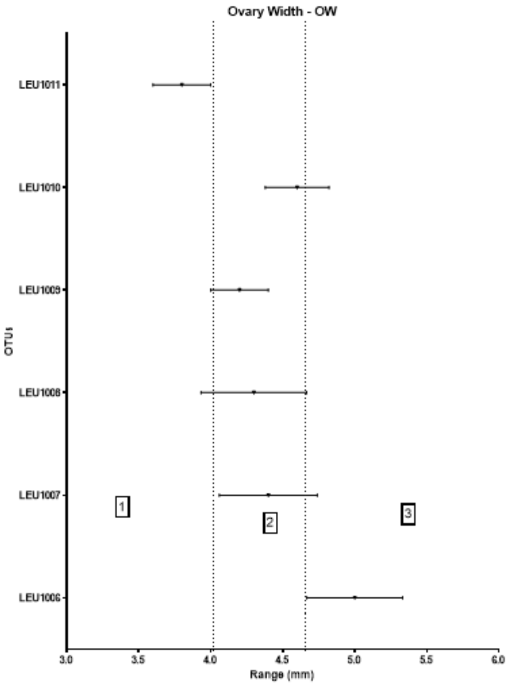

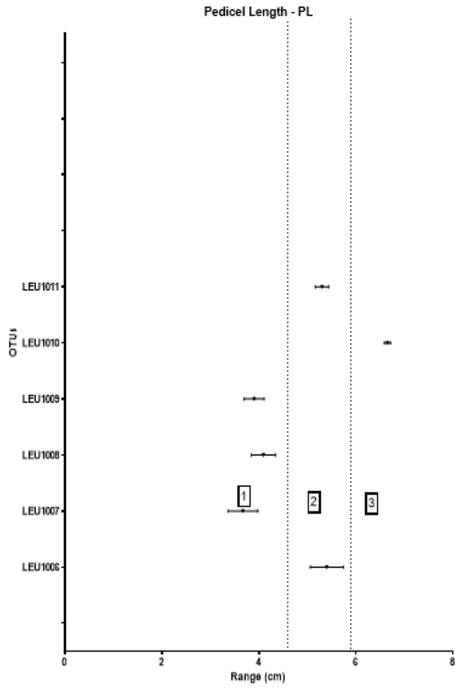

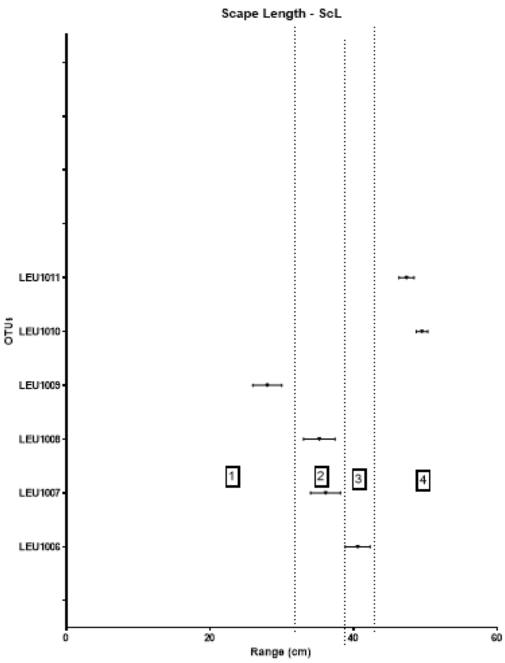

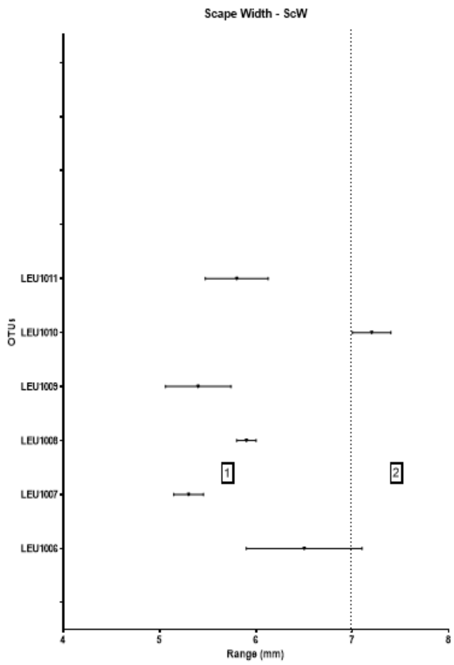

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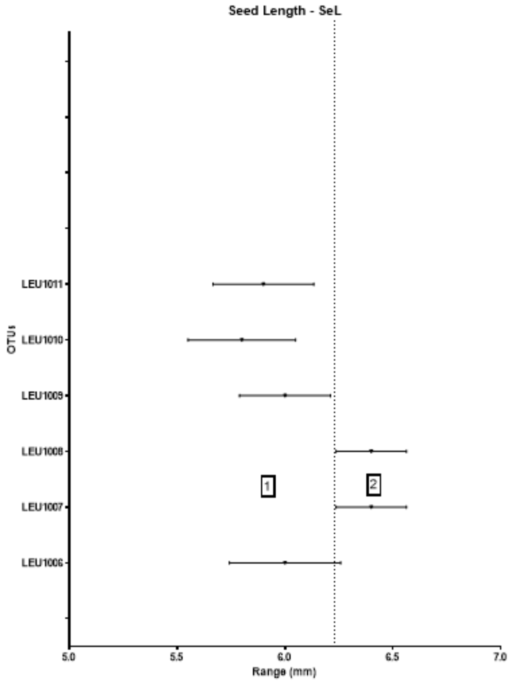

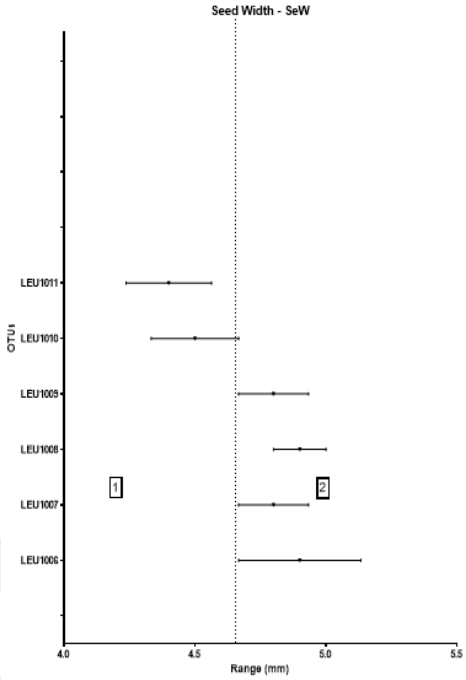

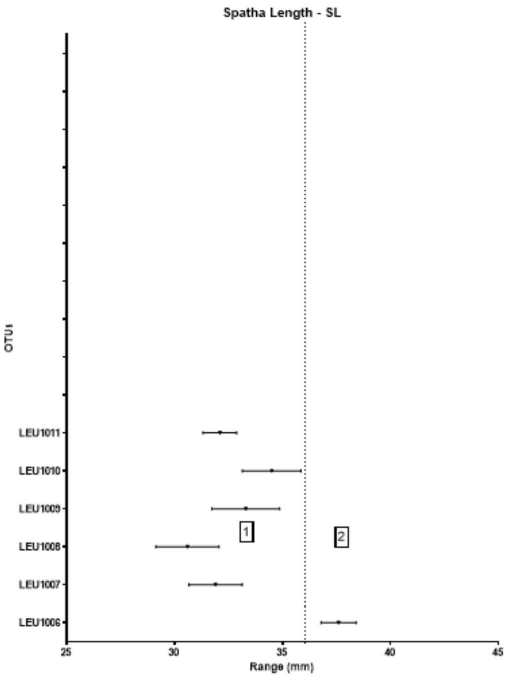

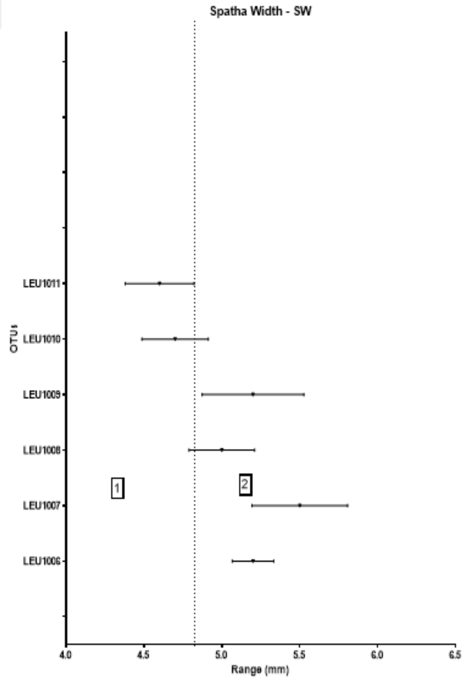

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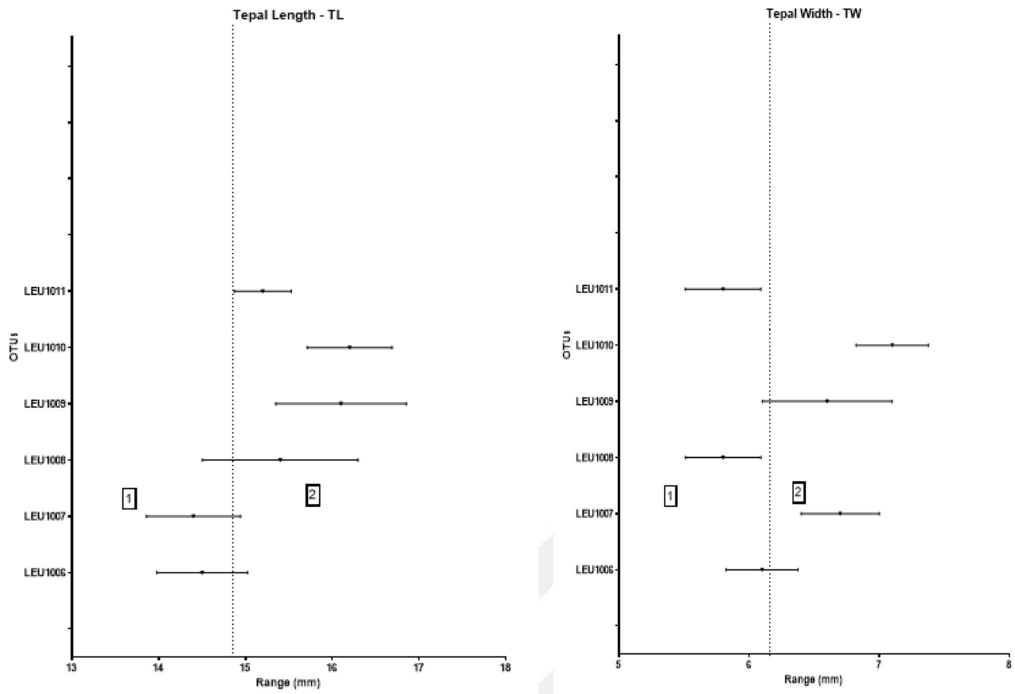
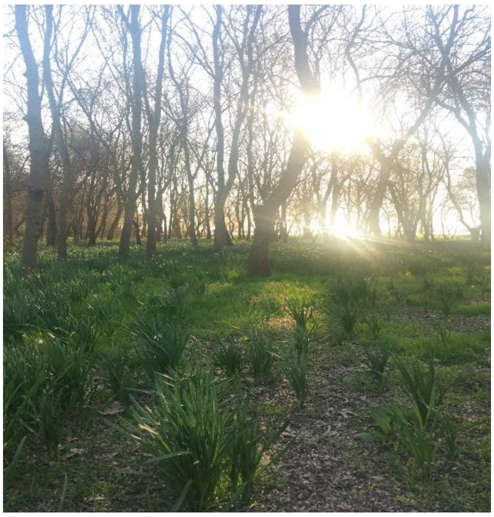


Appendix 2 Research areas Delmece-Yalova (by Sina Cafer Demir, 2019)

Appendix 3 Research areas Kaynarca-Sakarya (by Sina Cafer Demir, 2019)

Appendix 4 Research areas Terkos-İstanbul (by Sina Cafer Demir, 2019)

Appendix 5 Research areas Uluabat-Bursa (by Sina Cafer Demir, 2019)



Appendix 6 Research areas Gölcük-Bolu (by Sina Cafer Demir, 2019)

Appendix 7 Research areas Yeniçağa-Bolu (by Sina Cafer Demir, 2019)