BOLU ABANT IZZET BAYSAL UNIVERSITY
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

EFFECT OF SALT STRESS ON ALKALOID CONTENT (GALANTHAMINE AND LYCORINE) AND ANTIOXIDANT ACTIVITIES (ENZYMATIC AND NON-ENZYMATIC) OF SNOWFLAKE (*LEUCOJUM AESTIVUM* L.)

MASTER OF SCIENCE

MUHAMMED TURAL ATEŞ

BOLU, AUGUST 2019

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APPROVAL OF THE THESIS

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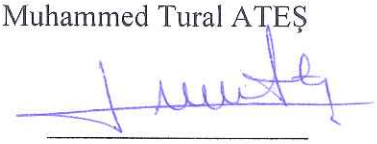
Director of Graduate School of Natural and Applied Sciences

To my lovely son Ali Yusuf and my wife Huri

DECLARATION

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Muhammed Tural ATEŞ



ABSTRACT

EFFECT OF SALT STRESS ON ALKALOID CONTENT (GALANTHAMINE AND LYCORINE) AND ANTIOXIDANT ACTIVITIES (ENZYMATIC AND NON-ENZYMATIC) OF SNOWFLAKE (*LEUCOJUM AESTIVUM* L.)
MSC THESIS
MUHAMMED TURAL ATES
BOLU ABANT IZZET BAYSAL UNIVERSITY GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF BIOLOGY
(SUPERVISOR: PROF. DR. ARZU TÜRKER)
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BOLU, AUGUST 2019

Summer snowflake (*Leucojum aestivum* L.) is a bulbous plant belongs to Amaryllidaceae family. *L. aestivum* is a Euro-Mediterranean region plant and is a well-known source of pharmacologically important alkaloids. Galanthamine is the major bioactive compound that is widely used in the treatment of neurological disorders, including Alzheimer's disease. Another alkaloid lycorine has antitumor, antimalarial, hepatoprotective, antiviral, antifungal and antiparasitic activities. Objective of this study is to determine the effect of different salt stress treatments on galanthamine and lycorine amounts, growth and development, non-enzymatic antioxidant activity (free radical scavenging activity-DPPH and total phenol-flavonoid content) and enzymatic antioxidant activity (SOD and CAT) in *L. aestivum*. The plant was cultivated for 3.5 months watering with 3 different concentrations (2, 4 and 8 g/L) of 2 different salt types (NaCl and $CaCl_2$). Obtained leaves and bulbs were extracted with methanol and analyzed with HPLC-DAD system. Galanthamine and lycorine amount were enhanced with 4 g/L $CaCl_2$ in the bulbs. Galanthamine in the leaves was also improved with 8 g/L NaCl. Salt stress treatments did not change the shoot length, bulb size and water content percentage significantly. Salt stress caused by 4 g/L $CaCl_2$ increased the antioxidant activity only in the leaves and total phenol-flavonoid content in the bulbs. Antioxidant enzymes also elevated with some salt stress treatments in the bulbs and leaves.

KEYWORDS: Antioxidant, Galanthamine, HPLC, *Leucojum aestivum*, Lycorine, Salt stress, Summer snowflake.

ÖZET

TUZLULUK STRESININ GÖL SOĞANININ (*LEUCOJUM AESTIVUM* L.) ALKALOID IÇERIĞINE (GALANTAMIN VE LIKORIN) VE ANTIOKSIDAN AKTIVITESINE (ENZIMATIK VE ENZIMATIK OLMAYAN) ETKILERI
YÜKSEK LISANS TEZI
MUHAMMED TURAL ATEŞ
BOLU ABANT İZZET BAYSAL ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ
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BOLU, AĞUSTOS - 2019

Göl soğanı (*Leucojum aestivum* L.) Amaryllidaceae familyasına ait soğanlı bir bitkidir. *L. aestivum* bir Avrupa-Akdeniz bölgesi bitkisidir ve farmakolojik olarak iyi bilinen alkaloidlerin kaynağıdır. Galantamin yaygın olarak Alzheimer hastalığı olmak üzere nörolojik bozuklukların tedavisinde kullanılan başlıca biyoaktif bileşiktir. Diğer alkaloid likorindir antitümör, antimalaryal, hepatoprotektif, antiviral, antifungal ve antiparazitik aktivitelere sahiptir Bu tezin amacı *L. aestivum*'da farklı tuz stresi uygulamalarının galantamin ve likorin miktarları, büyüme ve gelişme, enzimatik olmayan antioksidant aktivite (serbest radikal süpürme aktivitesi-DPPH ve toplam fenol-flavonoid içeriği) ve enzimatik antioksidan aktivite (SOD ve CAT) üzerine etkilerinin belirlenmesidir. *L. aestivum* 3.5 ay boyunca 2 farklı tuz çeşidinin (NaCl ve $CaCl_2$) üç farklı konsantrasyonu (2, 4 ve 8 g/L) ile sulanarak yetiştirilmiştir. Elde edilen yaprak ve yumrular metanol ile özütlenmiş ve HPLC-DAD systemi ile analiz edilmiştir. Galantamin ve likorin miktarları yumrularda 4 g/L $CaCl_2$ ile arttırılmıştır. Yapraklardaki galantamin de 8 g/L NaCl ile çoğaltılmıştır. Tuz stresi uygulamaları gövde boyu, yumru büyüklüğü ve su içerik yüzdesini önemli ölçüde değiştirmemiştir. 4 g/L $CaCl_2$ ile oluşturulan tuz stresi yapraklarda antioksidan aktiviteyi ve yumrularda toplam fenol-flavonoid miktarını arttırmıştır. Antioksidan enzimler de bazı tuz stress uygulamaları ile yumru ve yapraklarda artmıştır.

ANAHTAR KELİMELER: Antioksidan, Galantamin, Gölsoğanı, HPLC, *Leucojum aestivum*, Likorin, Tuz stresi.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

LIST OF ABBREVIATIONS AND SYMBOLS

ACCA **:** Aluminium Chloride Colorimetric Assay

ACh **:** Acetylcholine

AChE **:** Acetylcholinesterase

AD **:** Alzheimer's Disease

CAT **:** Catalases

CITES **:** Convention on International Trade in Endangered Species

DPPH **:** 2,2-Diphenyl-1-picrylhydrazyl

F-C **:** Folin – Ciocalteau Method

F-D **:** Folin – Denis Method

GAL **:** Galanthamine

HPLC **:** High Performance Liquid Chromatography

LY **:** Lycorine

ROS **:** Reactive Oxygen Species

SOD **:** Superoxide Dismutase

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1. INTRODUCTION

The history of medicinal plants is parallel to the history of humanity. Firstly, the Sumerians and Assyrians used the medicinal plants (B.C. 5000–3000). In the following years, the Greeks, the Egyptians, and the Hittites used these plants. As known, there are about one million plant species in the world today. However, the number of plants used for the treatment has increased since ancient times (Karancula, 2013).

Alkaloids are utilized in the treatment of Alzheimer disease (AD), multiple sclerosis, myasthenia gravis, myopia and glaucoma (Orhan et al., 2011). Amaryllidaceae alkaloids are mostly isolated from *Galanthus*, *Leucojum* and *Narcissus* sp.

1.1 *Leucojum aestivum* L.

Leucojum aestivum L. (English; summer snowflake, Turkish; gölsoğanı) is a perennial plant and a bulbous member of Amaryllidaceae (Nergisgiller) family (Ptak, 2014). It is native to the Balkans, South Europe, Caucasia, Northern Iran, Mediterranean regions, South Africa, Western Asia and Turkey. The natural habitats of *L. aestivum* are in Thrace, between North Anatolian and East Marmara regions, and around Beyşehir in Turkey (Fig. 1.1). *L. aestivum* plant grows in humid areas like marsh, forests, valleys and wetland communities, and grows in high areas between sea level and 1100 meters (Cicek et al., 2007; Demir, 2014).

L. aestivum first appeared in 1629 by the English botanist John Parkinson's book (A Garden of Pleasent Flowers). In this book, the name of this plant was called Big Bulb Violet (Karancula, 2013).

In the past, snowflake has been used for foliage plant in Europe. Today, bulbs and leaves of *L. aestivum* are used as medicinally. *L. aestivum* has valuable alkaloids such as galanthamine and lycorine. Especially, galanthamine has been used to Alzheimer's disease (Cicek et al., 2007).

The genus *Leucojum* includes eleven species in the world (Tutin et al., 1980). The genus *Leucojum* is represented by only one species in Turkey (Davis, 1984).

1.1.1 Botany of *Leucojum aestivum* L.

L. aestivum is a perennial bulbous geophyte. The bulb is sub-spherical (more than six cm in diameter) with a brown tunic, which leads the plant to resist dry period in the summer; it includes the basal plate, between 1 and 4 shoots, fleshy scales, and occasionally lateral buds. Mature bulbs were found to be 10.7 ± 1.2 cm below the soil area. The bulb exhibits a sympodial branching system, each part of which is constituted of six or eight foliage leaves and a lingulate scale, and terminates in an inflorescence. Roots which its system is not ramified grow usually at the end of the summer/beginning of autumn. Contractile roots which are about a third of roots have existed only in subadult plants. The root system radius was 9.6 ± 2.9 cm. The leaves are extensively linear, amplexicaule with a lamina 5-20 mm wide and 10–110 cm long during the vegetation period; the bases of foliage leaves, which surround the axis and by stages enlarge, serve as a food storage organ. The scape which compressed with two serrulated hyaline margins is stout and hollow. The inflorescence is a single helicoidal cyme subtended by a spathe formed of a single two-keeled leaf which is 2–8 pendulous flowers. Each plant can generate up to three peduncle or flowering scapes, but only one can produce more commonly. Spatha bracts are fused entirely in one side, 7–11 mm wide and 3–5 cm long. Pedicels are between two and seven centimeter long and the longest can pass the spatha. The perianth is constituted by 13–22 mm long, 3 + 3 tepals with a green spot just below the apex. The style is slightly club-shaped (or clavate) and longer than the 3 + 3 orange stamens; the anthers dehisce by terminal pores. The gynoecium which includes 10–40 bitegmic crassinucleate ovules, supported by a marginalcentral placenta is 3–14 mm long. The fruit which is fleshy capsule 1-2 cm wide and 2-4 cm long, is a subspherical. The seeds are spherical, black and large, without strophiole and with a spongy testa which lets them to float (Tutin et al., 1980; Parolo et al., 2011; Mori et al., 1991).

Snowflakes have elegant white flowers above dark green leaves. They can be between 30 and 60 cm, and the number of bell-shaped flowers per plant usually varies from 1 to 5. Their linear leaves are about 3–6 mm wide (Demir, 2014; Kahraman and Akcal, 2016) (Fig. 1.2).

Snowflakes can be grown with bulblet and seeds. But, the development of plant until flowering from seeds can take at least 5 years (Kahraman and Akcal, 2016).

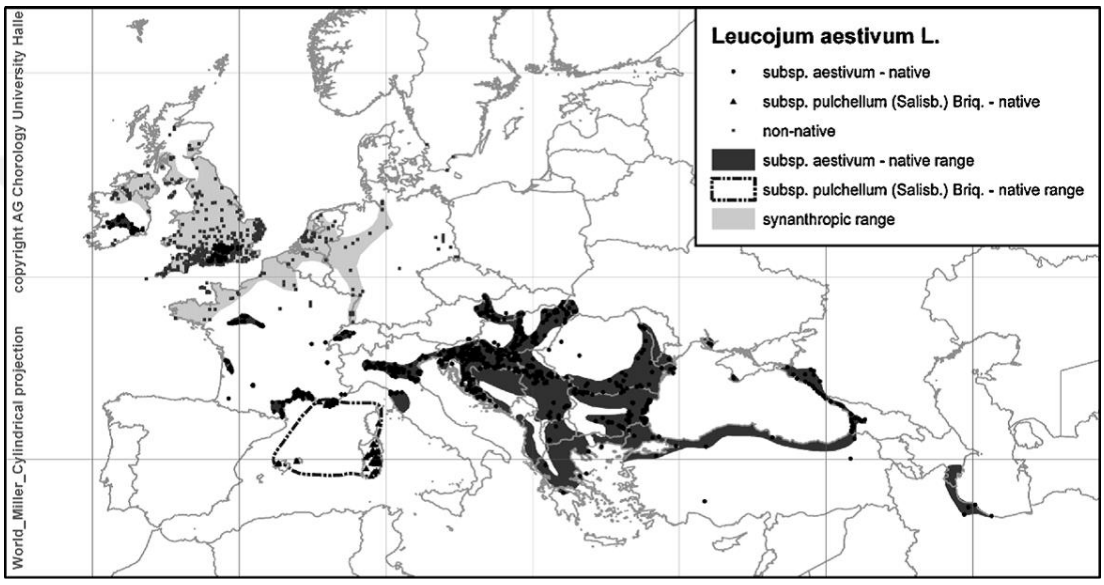

Figure 1. 1 Distribution of *Leucojum aestivum* (Parolo et al., 2011)

Figure 1. 2 Pictures of *Leucojum aestivum* (by Sina Cafer Demir)

1.1.2 Active Constituents

Alkaloids which are nitrogen-containing compounds of plant origin are defined as pharmacologically active. And they can inhibit enzymes, interfere with neurotransmission, block ion channels, loss of coordination, producing hallucinations, convulsions, vomiting, and death (Tiwari and Rana, 2015).

Amaryllidaceae family plants which include different types of alkaloids with an extensive of biological activities have many medicinal properties. (Petruczynik et al., 2016). Amaryllidaceae alkaloids are a specific type of special isoquinoline alkaloids to the family. They are structurally diverse and classified into nine basic skeleton groups: tazettine-, arciclasine-, homolycorine-, norbelladine-, crinine-, montanine-, haemanthamine-, galanthamine- and lycorine- type alkaloids. Lycorine and galanthamine are potential plant growth-inhibitors and exhibit cytotoxic, anti-inflammatory, antitumor, anti-malarial, anti-bacterial, anti-viral, and acetylcholinesterase inhibitory activities (Ghane et al., 2018).

In plants, the functions of alkaloids are not yet detected. Firstly, alkaloids have been proposed as waste products of metabolic processes of plants. According to the evidence, they have been shown to serve certain biological functions. In some plants, increasing the concentration of alkaloids just prior to seed formation and then decreasing the concentration of alkaloids when seed ripening was observed. This process shows that alkaloids may play an important key role. Alkaloids may preserve destruction of some plants by insect species (Encyclopaedia Britannica, Alkaloid).

1.1.2.1 Galanthamine

Galanthamine is an important alkaloid found in Amaryllidaceae species. This alkaloid was first isolated from *Galanthus woronowii* (English; Snowdrop, Turkish; Kardelen). But now, it is isolated from *Leucojum* and *Narcissus* (Heinrich and Teoh, 2004). Galanthamine is a selective, reversible, long acting, and competitive acetylcholinesterase (AChE) inhibitor. AChE inhibitors, commonly used in the treatment of Alzheimer's disease, are drugs that enhance the cholinergic neurotransmission by increasing the amounts of acetylcholine (ACh) in cholinergic synapses. Treatment of Alzheimer's disease is provided by drugs that increase ACh levels such as galanthamine. Galanthamine is used in the treatment of mild and moderate cases of Alzheimer's disease (Kaya et al., 2014). Galanthamine has been used for decades for different indications like treatment of poliomyelitis, myasthenia gravis and other neuromuscular disorders (Klosi et al., 2016).

According to some other known AChE inhibitors, galanthamine which is also a positive allosteric modulator of nicotinic receptor is more advantageous in clinical usage. Another activity of galanthamine could modulate cholinergic and therefore cognitive function (Colovic et al., 2013).

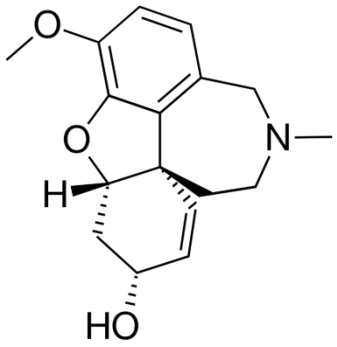

Figure 1. 3 Galanthamine structure

1.1.2.2 Lycorine

Lycorine firstly was isolated from *Narcissus pseudonarcissus* (English; Wild Daffodil, Turkish; Yabani Nergis) in 1877 and its structure was elucidated by Nagakawa et al. in 1956. Over the past decades, lycorine has attracted a special interest owing to its outstanding biological properties as analgesic, antiinflammatory, antioxidant antiviral, antibacterial, antifungal, antiprotozoal, hepatoprotective as well as wide-ranging cytotoxic effects against numerous tumor cell lines, e.g., leukemia, cervical cancer, prostate cancer, lymphoma, carcinoma, multiple melanoma and myeloma (Petruczynik et al., 2016; Khalifa et al., 2018; Roy et al., 2018). Lycorine-type alkaloids, which exhibit a unique pyrrolophenanthridine skeleton, represent the most common alkaloidal group, of which lycorine is the major alkaloid commonly found in the leaves and bulbs of all Amaryllidaceae plants.

Lycorine is most commonly encountered alkaloid in Amaryllidaceae species and it has been further identified from different genera of family Amaryllidaceae, such as *Ammocharis*, *Boophane*, *Brunsvigia*, *Crinum*, *Galanthus*, *Haemanthus*, *Hippeastrum*, *Hymenocallis*, *Leucojum*, *Lycoris*, *Narcissus*, *Sternbergia*, and *Zephyranthes* (Khalifa et al., 2018). Lycorine is used as a pesticide against fungi formed in tomato (Sener and Orhan, 2005).

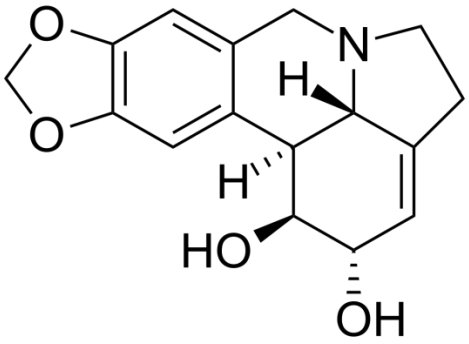

Figure 1. 4 Structural formula of lycorine

1.1.2.3 Acetylcholinesterase (AChE) Inhibitory Activity

The acetylcholine (ACh) is neurotransmitter at many synapses in the central nervous system (CNS), at many autonomically innervated organs, at all autonomic ganglia, and at the neuromuscular junction. In the autonomic nervous system (ANS), ACh which serves as the neurotransmitter in all the parasympathetic innervated organs is the neurotransmitter in the parasympathetic and preganglionic sympathetic neurons, as at the adrenal medulla. ACh is also the neurotransmitter at the piloerector muscle of the sympathetic ANS and at the sudoriparous glands (sweat glands). ACh is the neurotransmitter in the neuromuscular junction between the skeletal muscle and motor nerve in the peripheral nervous system (PNS). ACh which is found mainly in interneurons and a few important long-axon cholinergic pathways have also been identified in the central nervous system (CNS). One of these pathologies associated with AD is the degeneration of this pathway (Colovic et al., 2013). The enzyme AChE plays an important role in the hydrolysis of the neurotransmitter ACh into acetic acid and choline, this effect allows a cholinergic neuron to return to its resting state after activation (Marco and Carreiras, 2006).

AD which is memory loss is decribed as a progressive neurological disorder and selective neuronal cell death, the most known form of dementia. It is characterized serious enough to interfere with daily life the presence of extra cellular amyloid deposits in the core of neuritic plaques and the formation of intra neuronal neurofibrillary tangles in the brain of afflicted individuals. The disease depends on loss of cholinergic neurons in the brain and the decreased level of ACh. ACh is the

most abundant neurotransmitter in the body and the primary neurotransmitter in the brain which is responsible for cholinergic neurons (Colovic et al., 2013; Marco and Carreiras, 2006).

In the AD treatment strategies, the main therapeutic target is the inhibition of brain Acetylcholinesterase (AChE). There is no treatment for AD, and reversible AChE inhibitors, used in the cure, treat symptoms concerned to judgment, memory, language, thinking, and other thought processes. Actually, several physiological processes interested to AD destroy or damage cells that use and produce ACh, as a result of reducing the amount available to give messages to other cells (Colovic et al., 2013).

The cholinesterase enzyme from destroying ACh is inhibited by AChE inhibitors which rise both the duration and level of the neurotransmitter action. Tacrine which the oldest known AChE inhibitors was the first of the AChE inhibitors confirmed for the AD cure in 1993, but its usage has been abandoned for high rate of side effects including hepatotoxicity. Right now, medications approved by organizing agencies like the European Medicines Agency (EMA) and the U.S. Food and Drug Administration (FDA) to treat the cognitive manifestations of AD and develop life standart of the patients are: donepezil, galanthamine and rivastigmine as reversible AChE inhibitors, and memantine which is newer treatment of AD as an NMDA receptor antagonist. 19 new Alzheimer drugs are currently in clinical development. Based on the mode of action, there are two groups of AChE inhibitors which are reversible and irreversible. Reversible inhibitors, noncompetitive or competitive, mostly have therapeutic implementation, while toxic effects are relevant with irreversible AChE activity modulators (Colovic et al., 2013; Marco and Carreiras, 2006).

1.2 Plant Stress

Any increase or decrease in optimum environmental conditions restricts the growth of the plant. These unwanted unfavorable conditions are called stress. The environmental stresses in plants can be categorized as biotic stress and abiotic stress (Varma et al., 2013). Abiotic stresses (environmental stress) include temperature,

salinity, flood, drought, radiation, heavy metals etc. Biotic stresses (biological stress) include an attack by various pathogens like bacteria, fungi, herbivores etc.

Stress may be injured some plants, for this reason, they occur any metabolic dysfunctions. When the stress is taken away, the dysfunction (injury) can be temporary and the plant may recover if the stress is moderate and short term. Severe enough stress is caused disadvantages like seed formation, preventing flowering, and inducing age that lead to plant death. These plants are argued to be sensitive. Some plants eliminate the stress completely, like desert plants which are ephemeral, or short-lived.

Therefore many plants are considered to be stress resistant, they have the power to tolerate stress (Figure 1.5). The organism which requires stress resistance shows the capacity to acclimate or to adjust to the stress.

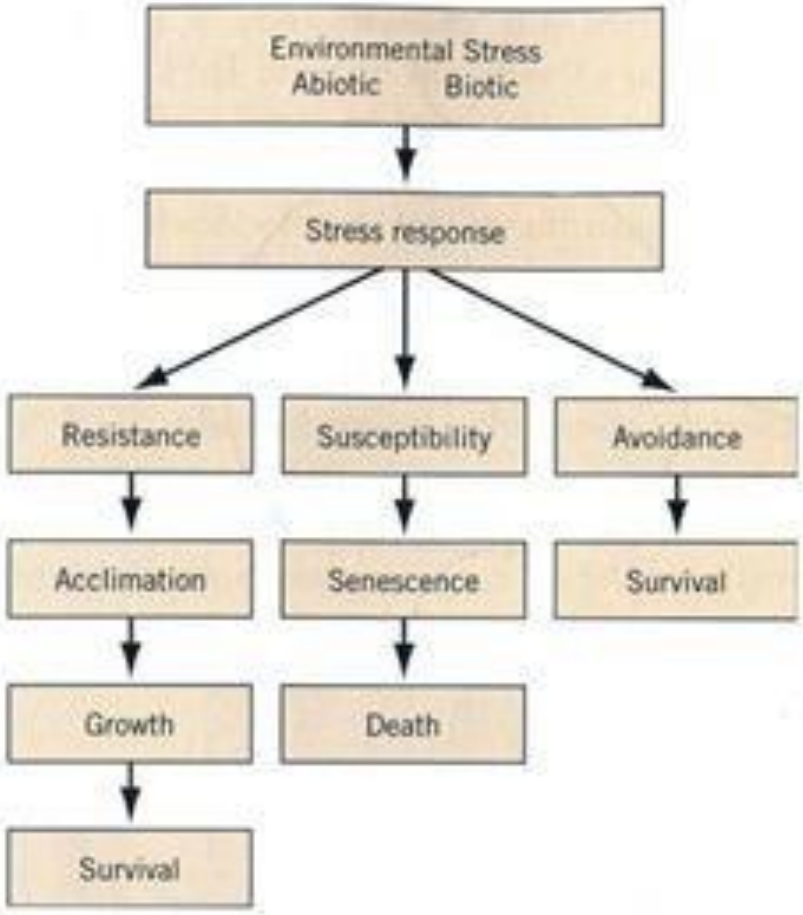

Figure 1. 5 The effect of environmental stress on plant survival

1.2.1 Biotic Stress

Biotic stress factors are plant pathogens (fungi, bacteria, viruses) and herbivorous animals

Living organisms which are weeds, viruses, insects, fungi, bacteria, arachnids and nematodes cause biotic stress. Unlike abiotic stress induced by environmental ingredients, biotic stress factors directly deny their host of its food, leading to decreased plant stamina and host plant's death. Biotic stress is one of the most important causes of pre-harvest and post-harvest harm in agriculture (Singla and Krattinger, 2016).

1.2.2 Abiotic Stress

Abiotic stress factors are excessively low and high temperature, excesses or deficiencies of water (flooding and drought), high salinity, lack of some nutrients, man-made chemicals and toxicity, extremes of soil pH, extremes of irradiance, xenobiotics (herbicides) and mechanical stress (wind, hail).

1.2.2.1 Salt Stress

Mineral stress from abiotic stresses is the stress factor that affects the usable areas after drought. Most of the mineral stress is salt stress. The salt stress surrounding the fertile lands in the world affects the development of plants by causing changes in their physiological, biochemical and molecular mechanisms (Culha and Cakırlar, 2011).

Salt stress prevents the development and growth of plants from causing osmotic and ion stress (Parida and Das, 2005). When salt amount increased in the root rhizosphere, firstly osmotic stress formed. The resulting osmotic stress causes a decrease in the amount of water available. This situation is called “physiological drought” (Tuteja, 2007). When the amount of water available decreases, cell expansion decreases and shoot development slows down. In the ion stress phase

following osmotic stress, the increasing Na and Cl ions in the environment compete with necessary nutrients such as K^{+}, Ca^{+2}, and NO^{-3}, nutrient deficiency occurs in the plant (Hu and Schmidhalter, 2005).

While salinity shows the direct effect on plants by forming osmotic and ion stresses, it shows the indirect effect (secondary effect) of these stress factors because of structural degradation of plants and the synthesis of toxic compounds. Major secondary effects of NaCl; synthesis of active oxygen species that damage DNA, chlorophyll, protein and membrane function; inhibition of photosynthesis; metabolic toxicity; inhibition of K^{+} intake; cell death (Botella et al., 2005; Hong et al., 2009).

The effects of salt stress on plants vary depending on the type of plant, the amount of salt applied and the amount of exposure. In salty environments, plants give very different responses due to genotypic differences. These different growth responses to salinity are valid not only for different plant species but also for different varieties of the same species (Munns, 2002).

1.3 Antioxidant Activities in Plants

Plants are an important part of the earth. Human beings have been using plants as medicine since old times. These medicinal properties of plants have bioactive constituents in them and plants represent the main source of biologically active molecules. Bioactive constituents reproduced from plant extracts are important scientifically for biological activities. Plants produce chemicals for protecting themselves, but new studies show that most of them can also be used against various human diseases. Flavonoids, alkaloids, steroids, terpenes, glycosides, and tannins are important biological active ingredients. In the preparation of drugs, these components can be used and extracted. The importance of biological, chemical and pharmacological evaluation of plant-sourced bioactive compounds used to cure human disease has been progressively recognized in the last years, but still there are countless useful medicinal plants waiting to be evaluated and exploited for their effective therapeutic application (Tabassum et al., 2016).

Plants include phytochemicals commonly known as antioxidants like phenolic acids, flavonols, polyphenols, isoflavones, tannins, and curcuminoids.

Antioxidants play a key role in the defensing mechanism of the organism counter pathologies related to the assault of free radicals. Glutathione peroxidase, catalase, superoxide dismutase or nonenzymatic compounds which are albumin, uric acid, metallothioneins, and bilirubin are substances that called endogenous antioxidants. Exogenous antioxidants requirement become as pharmaceutical products or nutritional supplements that contain as active part an antioxidant compound, if endogenous factors do not protect the organism against the reactive oxygen species. Vitamin E, vitamin D, vitamin C, beta-carotene, vitamin K3, flavonoids, and mineral Se are the most important exogenous antioxidants (Pisoschi and Negulescu, 2011).

Exogenous antioxidants are obtained from natural sources for example; some mineral compounds, flavonoids, vitamins, anthocyanins. Also, they could be synthetic compounds, such as gallates, butylhydroxytoluene, butylhydroxyanisole etc. (Pisoschi and Negulescu, 2011).

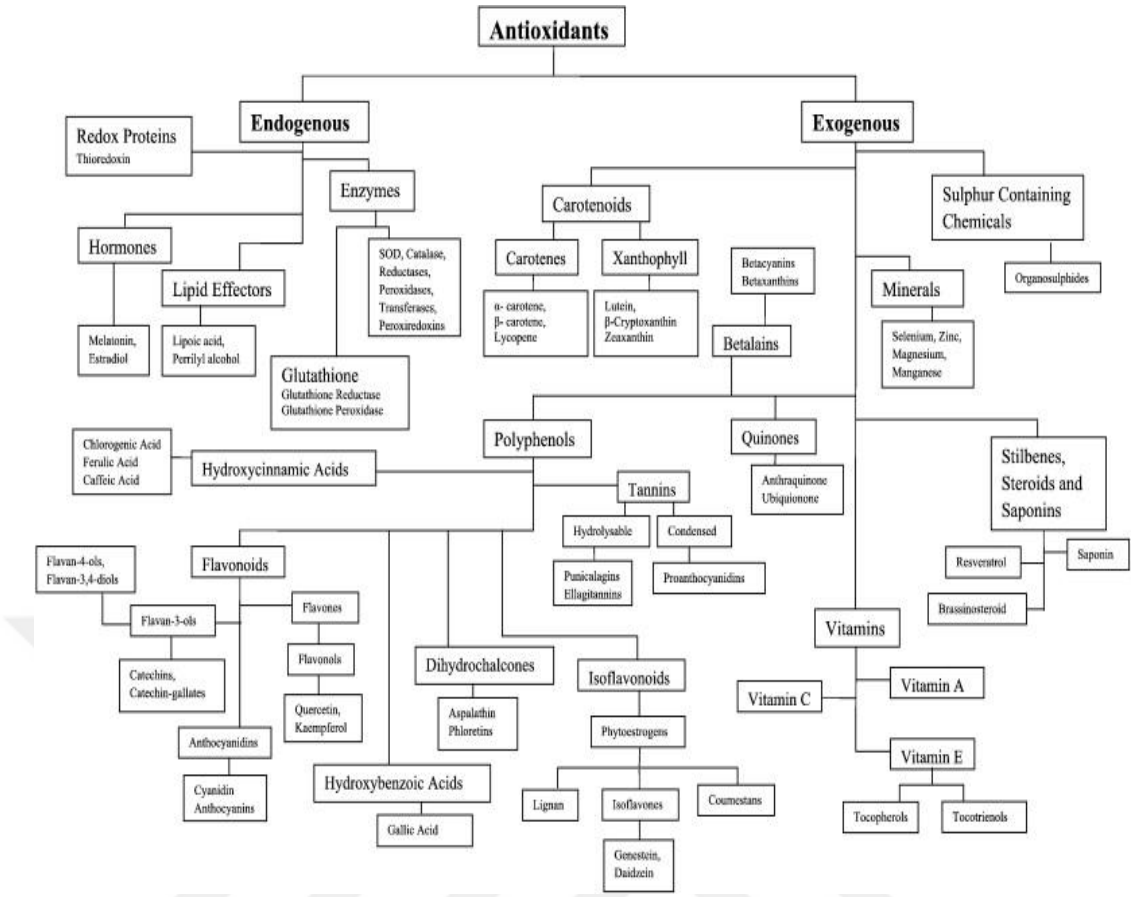

Figure 1. 6 Classes of antioxidant compounds (Wootton-Beard and Ryan, 2011)

1.3.1 Non-Enzymatic Antioxidant Activity

One of the most important health protective factor is antioxidants. Some scientific studies show that antioxidants decrease the risk of chronic diseases like cancer and heart disease. Main sources of natural antioxidants are vegetables, fruits, and grains. Plant-source antioxidants like vitamin C, vitamin E, carotenes, phenolic acids have been recognized as reducing disease risk (Shekhar and Anju, 2014). Antioxidant compounds generally belong to various classes of compounds with physical and chemical properties and are derived from plant sources (Sravani and Paarakh, 2012).

Antioxidant helps preserve cells from the damage caused by free radicals which are unstable molecules that are made during normal cell metabolism. They can

increase in cells and cause damage to other molecules. This damage may raise the risk of cancer and some diseases (National Cancer Institute, Free Radical Scavenger).

The use of the DPPH (free radical 2,2-Diphenyl-1-picrylhydrazyl) is a simple, rapid, and inexpensive method to measure the antioxidant capacity of the plant. Also, DPPH is widely used method to find out capacity of compounds to act as free radical scavengers and to assess antioxidant properties (Kirtikar and Basu, 2006).

The DPPH method is based on the reduction of free DPPH radical and it gives maximum absorption at 517 nm (Warrier et al., 1994).

1.3.2 Enzymatic Antioxidant Activity

Antioxidants prevent oxidative harm in biological macromolecules draw on reactive oxygen species. All aerobic organisms which preserve against ROS have an antioxidant defense system. Antioxidant defense system is form of both non-enzymatic and enzymatic systems. The enzymatic system has enzymes like catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSHPx). SOD catalyzes the dismutation of O_2 at a rate 10 times higher than natural dismutation at pH 7.4 (Panda, 2012).

SOD : $2\ O_2^- + 2\ H^+ \rightarrow H_2O_2 + O_2$

CAT : $2\ H_2O_2 \rightarrow 2\ H_2O + O_2$

GSHPx : $2\ GSH + 2\ H_2O_2 \rightarrow GSSG + 2\ H_2O$

SOD which was first published in 1969 is required to catalyze the transformation of O_2^- to H_2O_2 because of less harmful and reactive of hydrogen peroxide. Superoxide dismutase reacts with superoxide radicals in the presence of transition metals, and then resulting in more reactive OH radicals. SODs are metalloproteins active with Fe, Mn, Cu, or Zn as cofactors and occur in the mitochondria, chloroplasts, apoplast, cytosol, and peroxisomes (Szollosi, 2014).

CAT is an antioxidant enzyme existing in every aerobic organism. CAT is known to catalyze hydrogen peroxide (H_2O_2) into H_2O and O_2 in a productive energy way in the cells exposed to environmental stress. It is located in all important sites of hydrogen peroxide generation in the cellular environment of higher plants. CAT which protects the cell from oxidative damage by reactive oxygen species (ROS) is a very significant enzyme. CAT, each of 500 amino acids in length, is a tetramer of four polypeptide chains. CAT includes four iron-containing heme groups which let the enzyme to react with the H_2O_2 (Chelikani et al., 2004; PDB-101, Catalases).

2. AIM AND SCOPE OF THE STUDY

- To cultivate *L. aestivum* for 3.5 months under salt stress treatments (6 treatment groups and control): Irrigation with 2 different salt types (NACl and $CaCl_2$) having 3 different concentrations (2, 4 and 8 g/L) and only water (control).
- To collect leaves and bulbs grown under salt stress treatments.
- To prepare methanol extracts for leaves and bulbs grown under salt stress treatments.
- To determine alkaloid (galanthamine and lycorine) content of methanolic extract s of bulbs and leaves grown under salt stress treatments by HPLC-DAD system.
- To determine shoot length, bulb size and water capacity of *L. aestivum* grown under salt stress treatments.
- To determine non-enzymatic antioxidant activities of *L. aestivum* bulbs and leaves grown under salt stress treatments.
    - Free Radical Scavenging Activity (DPPH Method)
    - Total Phenolic Content (Folin-Ciocaltaeu Method)
    - Total Flavonoid Content ($AlCl_3$ Colorometric Method)
- To determine enzymatic antioxidant activities of *L. aestivum* bulbs and leaves grown under salt stress treatments.
    - Superoxide Dismutase (SOD) (Lowry Method)
    - Catalases (CAT) (Lartillot Method)

3. MATERIALS AND METHODS

3.1 *L. aestivum* Cultivation Under Salt Stress

The bulbs of *L. aestivum* were provided by Floramarla Company, Yalova, Turkey. Similar size of *L. aestivum* bulbs were grown directly in a mixture of peat, sand, soil and perlit in pots (sizes 17x20 cm) in the greenhouse. The ratio of soil mixture in pots was given in the table (Table 3.1). Salt stress was applied by supplementing with NaCl and $CaCl_2$ at three different concentrations (2 g/L, 4 g/L and 8 g/L in distilled water for each salt). There were 7 different groups for the experiment (3 NaCl and 3 $CaCl_2$ stress treatments and control). There were 3 pots for each treatment and the experiment was repeated twice. The plants were irrigated by two day intervals in all treatments. When necessary all groups including control were irrigated once a week with pure water (according to the physiological appearance of the plant). The volume of irrigation for each group was varied between 100 and 150 mL per pot. The experiment was carried out in a greenhouse at 24 ºC under a 16 h photoperiod and relative humidity of 60% and 70% for four months at the Art and Science Faculty, Department of Biology. In this experiment, the bulbs were irrigated using distilled water until leaves appeared on the bulbs and then the bulbs were developed in the same soil mixture in the pots by irrigating with distilled water and salt water until they were harvesting.

At the end of the salt stress treatments, *L. aestivum* bulbs and leaves were seperately harvested and stored in a deep-freezer at -20 ºC until extraction and biological activity studies.

Furthermore, leaves and bulbs from each group were taken separately and shoot length and bulb size and their fresh weights (FW) were determined and also their dry weights (DW) were calculated by drying the bulbs and leaves in freeze-dryer at -65 °C (low pressure drying). Thus, the proportional water content (WC) in the bulbs and leaves were determined according to following equation:

WC (%) in plant sample: [[FWs - DWs)/FWs] x 100

Table 3. 1 The ratio of soil mixture in pots.

Peat	Sand	Soil	Perlite	Total
6 units	1 unit	1 unit	1 unit	9 units
66.66 %	11.11 %	11.11 %	11.11 %	100 %

3.2 Preparation of *L. aestivum* Leaf and Bulbs Extracts

Bulbs and leaves of *L. aestivum* grown in pots were harvested in batches at the end of the vegetative development stage (before flowering). Methanol extracts were prepared from bulbs and leaves of plant. Collected bulbs and leaves from seven different treatments were freeze-dried and then lyophilized by using freeze-dryer at -65 ºC in order to save their biological contents and activities and then ground into a powder. Powdered leaves and bulbs have been extracted with methanol at 40 ºC in a water bath for 18-24 hours and then filtered. Methanol was evaporated under vacuum using rotary evaporator at 50 ºC to get crude methanol extracts. Yields which were obtain after extraction were determined according to following equation:

Yield (%) = Obtained extract weight (g) / Initial plant material weight (g) X100.

When extracts were used for biological studies and HPLC analyses, each residue was dissolved in methanol to produce a known final concentration.

3.3 Determination of the Alkaloid Content of Bulb and Leaf Methanol Extracts by HPLC

Methanolic extracts of bulb and leaf were analyzed for two standard alkaloids using a HPLC-DAD system (VWR-Hitachi LaChrom Elite®). Galanthamine and lycorin were used as alkaloid standards (Sigma®). Standard stock solutions were separately prepared in %1 TFA and different concentrations (10, 20, 40, 60, 80, 100 and 200 mg/L) of standards were prepared for obtaining standard curve. Analysis was performed using HPLC system (VWR-Hitachi LaChro Elite) equipped with L-2455 Diode-Array Detector (DAD), Hitachi L-2130 Pump, Hitachi L-2200

autosampler. Chromatographic separation was performed using Hitachi column oven L-2300 and Venusil XBP C18 column (Bonna-Agela Technologies, 5 μm particle size, 4.6 x 250 mm inner diameter). HPLC grade (Merck) solvents were used and eluent was composed of trifluoroacetic acid, water, acetonitrile (0.01:90:10, v/v/v) (Kaya et al., 2014). An isocratic elution was applied at flow rate of 1 ml/min for 10 min. 20 μL of each extract was injected for HPLC-DAD and separations were done at 25 ºC oven. The chromatograms were recorded at 290 nm for galanthamine and lycorine standards.

Mobil phases were filtered through 0.45 μm hydrophilic polypropylene membrane filter (Pall Corporation) prior to HPLC injection. All extracts and standards were filtered through a 0.2 μm GHP Acrodisc (Pall Corporation) intro HPLC vials. All procedures have been repeated 3 times for each sample tested.

3.4 Non-Enzymatic Antioxidant Activity Studies

Antioxidant activities of methanolic *L. aestivum* bulbs and leaves were evaluated by using DPPH (Sigma-Aldrich Chemie, Steinheim, Germany) radical photometric assay according to Blois (1958)' s method modified by Coruh et al. (2007). At the same time, to determine total phenolic and total flavonoid contents of these methanolic extracts, Folin-Ciocalteu (Slinkard and Singleton, 1977) and aluminium colorimetric assay (Marinova et al., 2005; Chang et al., 2002) were used, respectively.

3.4.1 Free Radical Scavenging Activity (DPPH Method)

Free radical scavenging activity of the methanolic extracts of *L. aestivum* was determined spectrophotometrically by monitoring the disappearance of DPPH at 517 nm (Angayarkanni et al., 2010).

All methanolic extracts of *L. aestivum* were weighted as 0.050 g and they were dissolved 5 ml of methanol. Finally, this stock solution was prepared as 25000 μg/ml concentration. The extracts were diluted with methanol (1000, 2000, 4000, 8000, 10000, 15000, 18000, 20000 μg/ml) from the stock extract solutions.

Quercetin as an antioxidant was used and a standard curve was prepared in methanol by using various concentrations of quercetin (12.5, 25, 50, 100, 200 and 400 μg/ml). 0.13 mM solution of DPPH• was prepared daily in methanol to obtain approximately 1.4 absorbance unit at 517 nm before UV measurements. To determine antioxidant potential of the extracts, 0.1 ml of the diluted samples or quercetin was mixed vigorously with 1.4 ml of DPPH solution. These solutions were shaken vigorously and incubated in the dark for 30 minutes at room temperature. After 30 minutes, the decrease in the absorbance of these solutions was measured at 517 nm with Hitachi U-1900, UV-Vis Spectrophotometer 200V against blank samples (positive control). The blank samples includes same amount of methanol and DPPH• solution (0.1 ml methanol and 1.4 ml DPPH•) was used as a positive control. All analyses were made in triplicate. The DPPH radical scavenging activity of *L. aestivum* methanolic extracts was calculated according to following formula:

DPPH radical was dissolved in methanol to obtain approximately 1.4 absorbance unit (0.13 mM DPPH solution) at 517 nm.

DPPH· Scavenging Effect (% inhibition) = $[(A_0-A_1/A_0) \times 100]$ (Turker et al., 2018) where A_0 is the absorbance of the control reaction and A_1 is the absorbance of *L. aestivum* extracts.

3.4.2 Total Phenolic Content

Total phenolic contents in the methanolic extracts were determined with Folin Ciocaltaeu method using gallic acid as a standard phenolic compound (Slinkard and Singleton, 1977). Gallic acid stock solution was prepared and diluted with methanol to get a standard calibration curve. To prepare gallic acid stock solution, 0.5 mg of dry gallic acid was dissolved in 5 ml methanol (1 mg/ml) and diluted as 0, 12.5, 25, 50, 100, 200 and 400 μg/ml gallic acid. Then, a calibration curve was obtained by using these gallic acid solutions.

To prepare extract solution, 0.01 g each methanolic extract of *L. aestivum* was weighted and 2 ml of methanol was added to extracts (5 mg/ml). Later, these extract solutions were diluted as 1 mg/ml. To measure total phenolic contents of methanolic

extracts, sodium carbonate (Na_2CO_3) solution and Folin-Ciocalteu reagent were used. 20 g of anhydrous Na_2CO_3 was dissolved in 100 ml of water.

Twenty µl gallic acid solution of various concentrations, *L. aestivum* methanol extracts or methanol as a blank was placed into separate glass tubes containing 1.58 ml distilled water, and 100 µl of the Folin-Ciocalteu reagent was added into each and then mixed well. Two minutes later, 300 µl sodium carbonate solution was added to each test tube, and was shaken vigorously. Each solution was incubated at 25 ºC fo 2 hours and the absorbance of each solution was measured at 765 nm against blank (all reagents without sample) using the spectrophotometer (Hitachi U-1900, UV-Vis Spectrophotometer 200V, Japan). The absorbance vs. concentration was plotted. After the created calibration curve with standard gallic acid, the total phenolic content in the extracts were determined using an formula that was gotten from standard gallic acid graph. The results were expressed as mg gallic acid equivalent / g crude extracts and all analysis was performed in triplicate.

3.4.3 Total Flavonoid Content

The total flavonoid contents in methanolic extracts were determined by aluminium chloride colorimetric method with some modifications. Quercetin was used as a reference flavonoid. Quercetin stock solution was prepared and diluted with methanol to obtain standard calibration curve. For this, 0.5 mg of quercetin was weighted and then dissolved in 5 ml methanol (1 mg/ml). Then, to obtain a standard calibration curve, different concentrations of quercetin (25, 50, 100, 200, 400 and 800 µg/ml) were prepared using this stock quercetin solution.

To prepare extract solution, 0.01 g each methanolic extract of *L. aestivum* was weighed and added into test tube containing 2 ml of methanol (5 mg/ml). Later, these extract solutions were diluted as 1 mg/ml. To determine total flavonoid contents in methanolic extracts, sodium nitrate ($NaNO_3$) solution, aluminum chloride ($AlCl_3$) and sodium hydroxide (NaOH) were also used. For this, 5 g of $NaNO_3$, 10 g of $AlCl_3$ and 4 g of NaOH was dissolved separately in 100 ml of water to obtain %5 $NaNO_3$, %10 $AlCl_3$ and 1M NaOH.

In measurement process, 500 µl quercetin solution of various concentrations, *L. aestivum* methanol extracts or methanol as a blank was added to glass tubes containing 2 ml distilled water. At zero time, 150 µl $NaNO_2$ (5%) was added to each vial and mixed well. After 5 minutes, 150 µl $AlCl_3$ (10%) was added and at 6th minutes, 1000 µl NaOH (1M) was added to each mixture. Instantly, the reaction tube was made up to 5 ml by adding distilled water and shaken thoroughly. Each solution was incubated in dark at 25 ºC for 10 minutes and its absorbance was measured at 415 nm against the blank using the spectrophotometer (Hitachi U-1900, UV-Vis Spectrophotometer 200V, Japan). The standard qurcetin curve was prepared by plotting absorbance value versus known concentrations of standard and then total flavonoid contents were estimated by using the equation obtained from the standard calibration graph. The results were expressed as mg quercetin equivalent / g crude extracts and all analysis was performed in triplicate.

3.5 Enzymatic Antioxidant Activity Studies

Protein contents in bulbs and leaves was determined prior to SOD and CAT enzyme activity assays. For the determination of protein content, the Lowry method was used (Lowry et al., 1951). Bovine serum albumine was used as a standard protein and various concentrations of protein (12.5, 25, 50, 100, 200, 400 and 1000 µg/ml) were prepared using this standard protein.

The extractions of SOD and CAT enzyme from fresh bulbs and leaves were made using extraction buffer. 0.1 g each plant sample of *L. aestivum* was weighed and thoroughly ground with a liquid nitrogen on an ice bath. The sample powders were separatelly homogenized in 50 mM phosphate buffer and centrifuged for 15 minutes at 12000 Hg at 4 ºC. The supernatants were used for determination of total protein content, SOD enzyme and CAT enzyme activities.

To measure the total protein level in bulb and leaf extract solutions, 0.2 ml of prepared each extract solution was taken and placed in the test tube and then alkali copper sulfate reagent was added into the plant extract solution. These solutions were left for 10 minutes at room temperature and their absorbances were measured at 660

nm against the blank using the spectrophotometer (Hitachi U-1900, UV-Vis Spectrophotometer 200V, Japan).

3.5.1 Superoxide Dismutase (SOD)

To determine SOD activity in *L. aestivum* bulbs and leaves, two test tubes were used for each enzyme extract solution. One tube was used as blank and the other tube was used as the sample. 1.425 ml of reagent mixture was added into both tubes and then 0.025 ml of xanthine oxidase solution was added into the blank tube and left for 20 minutes at room temperature. At the end of time, the reaction in the blank tube was stopped adding 0.05 ml of copper chloride. Finally, 0.05 ml of enzyme extract solution was added and absorbance of the blank was recorded at 560 nm against distilled water using Hitachi U-1900, UV-Vis Spectrophotometer 200V.

To measure SOD enzyme activity, 0.05 ml of enzyme extract solution and 0.25 ml xanthine oxidase were added to the sample tube containing 1.425 ml of reagent mixture and left for 20 minutes at room temperature. Then, the reaction was stopped adding 0.05 ml of copper chloride and absorbance of the samples were recorded at 560 nm against distilled water using Hitachi U-1900, UV-Vis Spectrophotometer 200V.

3.5.2 Catalases (CAT)

Catalase activity in *L. aestivum* bulbs and leaves was determined by Lartillot method (Lartillot et al., 1988). To measure CAT activiy, three test tubes were used for each enzyme solution. One tube was used as the sample, the other was used as a blank and the final tube was used as protein absorbance in each enzyme solution.

To prepare the sample tube: 3 ml of the reaction mixture containing 50 mM phosphate buffer and 10 mM H_2O_2 was added to the sample tube and then 20 µL of enzyme extract was added to each tube to initiate the reaction. Two min. later, reaction was terminated by adding 0.5 ml of 1M HCl solution. Thus, CAT activity in each sample was determined by the consumption of H_2O_2 in two minutes at 240 nm.

To determine the initial absorbance value of H_2O_2 (the blank tube), 2.5 ml of reaction mixture and 0.5 ml of 1 M HCl were mixed vigorously and then its absorbance was measured at 240 nm spectrophotometer. The third tube was used to measure protein absorbance in each enzyme solution. For this, 20 µL enzyme extract, 2.5 ml of 50 mM phosphate buffer solution and 0.5 ml of 1M HCl were added in the test tube and mixed well and then its absorbance was measured at 240 nm spectropotometer. Finally, CAT enzyme activity in each bulb and leaf was calculated by using changes in absorbance values.

3.6 Data Analysis

Analysis of variance (ANOVA) and Duncan's Multiple Range Tests using SPSS vers. 15 (SPSS Inc, Chicago, IL, USA) were performed for data analysis.

4. RESULTS AND DISCUSSION

4.1 Cultivation of *L. aestivum*

This study was carried out to determine the Effect of salt water on bulb and leaves of *L. aestivum* in terms of alkaloid content (galanthamine and lycorine), growth, total phenol and flavonoid content, antioxidant activity and antioxidant enzymes (SOD and CAT). Two different salt types (NACl and $CaCl_2$) were used in three different concentrations (2, 4 and 8 g/L) and distilled water was used in the control group.

At the end of the salt application, leaves and bulbs were taken separately from each group (Figures 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7 and 4.8). And then, shoot length, bulb diameter and water capacity (%) were determined (Table 4.1 and 4.2).

Figure 4. 1 *L. aestivum* in pots.

Figure 4. 2 Bulbs of control group.

Figure 4. 3 Bulbs and leaves of 2 g/L $CaCl_2$ group.

Figure 4. 4 Bulbs and leaves of 4 g/L $CaCl_2$ group.

Figure 4. 5 Bulbs of 8 g/L $CaCl_2$ group

Figure 4. 6 Bulbs and leaves of 2 g/L NaCl group

Figure 4. 7 Bulbs of 4 g/L NaCl group

Figure 4. 8 Bulbs of 8 g/L NaCl group

Different salt type and concentrations did not show an effect on shoot length and bulb size. There is no statistical difference among them. Furthermore, there is no big difference in water content under salt stress (Table 4.1 and 4.2).

Table 4. 1 Shoot length and bulb size of *L. aestivum* under salt stress.

Treatments	Concentration (g/L)	Shoot length (cm)	Bulb size (cm)
Control	-	9.0 ± 1.0^{a}	9.1 ± 1.2^{ab}
$CaCl_2$	2	9.5 ± 0.8^{a}	7.5 ± 0.8^{b}
	4	9.6 ± 0.7^{a}	8.6 ± 1.0^{ab}
	8	10.7 ± 0.9^{a}	11.0 ± 0.5^{a}
NaCl	2	10.0 ± 0.8^{a}	9.3 ± 0.9^{ab}
	4	9.3 ± 0.7^{a}	9.3 ± 0.9^{ab}
	8	10.3 ± 0.7^{a}	8.0 ± 1.0^{ab}

Table 4. 2 Water content (%) of *L. aestivum* under salt stress.

Treatments	Concentration (g/L)	Water content (%)	
		Bulb	Leaf
Control	-	75	83
$CaCl_2$	2	76	87
	4	75	87
	8	75	87
NaCl	2	76	87
	4	77	84
	8	75	86

4.2 HPLC Analyses of the Methanol Extracts for Galanthamine and Lycorine

Leaves and bulbs of *L. aestivum* unders salt stress were extracted with methanol. Yield for each extract was calculated and listed in Table 4.3. Quantification of two alkaloids (galanthamine and lycorine) in fourteen different extracts was conducted with HPLC-DAD analysis and results were summarized in Table 4.4. The chromatogram of the used standards was shown in Figure 4.9. Galanthamine and lycorine in the extracts were compared with the standard chromatogram and the concentrations (ppm) of each alkaloids were found. Finally, the results were converted into mg alkaloid/g dry extract.

According to the results of HPLC analyses in bulb extracts, the highest amount of lycorine and galanthamine was found in extract treated with 4 g/L $CaCl_2$. When compared with galanthamine content in control (6.9 mg/g), 4 or 8 g/L $CaCl_2$ treatment increased the galanthamine content approximately 4 (28.5 mg/g) or 3 (17 mg/g)-fold, respectively. When the amount of lycorine was compared, it was determined that all concentrations of NaCl and $CaCl_2$ at high concentration (8 g/L) caused a decrease in lycorine amount compared to the control. Similar to lycorin

results, it was found that all concentrations of NaCl caused a decrease in the amount of galantamine when compared with control. In addition, a decrease in the amount of galanthamine was observed when low concentrationof $CaCl_2$ (2 g/L) was used (Table 4.4; Fig. 4.10).

In leaf extracs, the amount of studied lycorine was found higher in control extract than other extracts. Among the plants grown with salt stress, high amount of lycorine was found in the plant treated with 4 g/L $CaCl_2$. In other words, the lowest amount of lycorine was found in the plants treated with the lowest concentration of $CaCl_2$ (2 g/L) and the highest concentration of NaCl (8 g/L). However the highest amount of galanthamine was found in the plant treated with the highest concentration of NaCl (8 g/L). The amount of galanthamine was higher in the plants treated with salt stress except 2 g/L $CaCl_2$ and 4 g/L NaCl (Table 4.4; Fig. 4.11).

Table 4. 3 Extraction yield (%) of methanolic *L. aestivum* extracts under salt stress.

Treatments	**Concentration (g/L)**	**Extraction yield (%)**	
		Bulb	**Leaf**
Control	-	8	36
$CaCl_2$	2	7	35
	4	9	45
	8	8	29
NaCl	2	8	35
	4	9	31
	8	8	21

Table 4. 4 Alkaloid quantities (galanthamine and lycorine) (mg/g) in the bulb and leaf extract of *L. aestivum* under salt stress with HPLC-DAD analysis. Data were presented as a mean number of galanthamine or lycorine content ± standard error (SE). Means with the same letter within columns are not significantly different at $P>0.05$.

Treatments	Concentration (g/L)	*L. aestivum* extracts (mg/g dry extract)			
		Bulb		Leaf	
		Galanthamine	Lycorine	Galanthamine	Lycorine
Control	-	6.9 ± 0.4 ^{c}	50.1 ± 0.7 ^{b}	11.7 ± 0.4 ^{d}	36.5 ± 0.6 ^{a}
$CaCl_2$	2	0.7 ± 0.0 ^{e}	52.9 ± 0.1 ^{b}	8.7 ± 0.2 ^{e}	23.7 ± 0.5 ^{d}
	4	28.5 ± 2.8 ^{a}	69.5 ± 5.1 ^{a}	16.4 ± 0.2 ^{b}	32.1 ± 0.3 ^{b}
	8	17.0 ± 0.2 ^{b}	8.5 ± 0.2 ^{f}	13.4 ± 0.6 ^{c}	26.0 ± 0.8 ^{c}
NaCl	2	4.4 ± 0.1 ^{cd}	31.7 ± 0.9 ^{c}	13.1 ± 0.2 ^{c}	26.3 ± 0.3 ^{c}
	4	0.8 ± 0.1 ^{e}	15.4 ± 0.4 ^{e}	11.3 ± 0.1 ^{d}	27.0 ± 0.9 ^{c}
	8	1.5 ± 0.0 ^{de}	23.3 ± 0.4 ^{d}	23.5 ± 0.2 ^{a}	23.9 ± 0.0 ^{d}

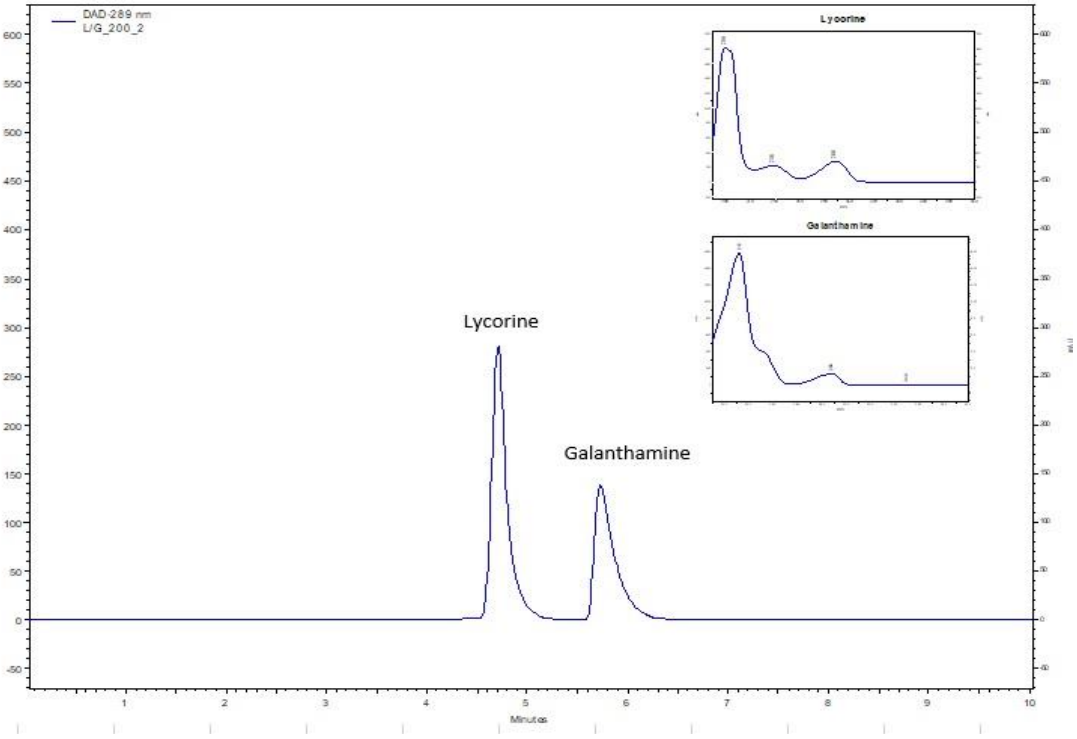

Figure 4. 9 HPLC chromatogram of the standards. Retention times: 1. Lycorine-4.82 min, 2. Galanthamine-5.95 min.

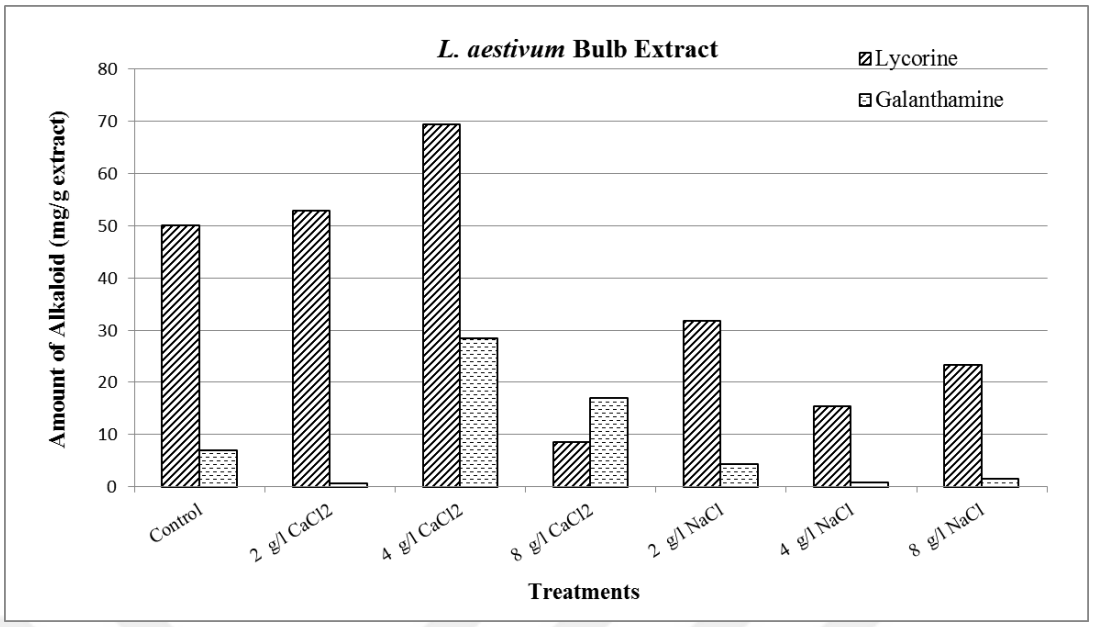

Figure 4. 10 Galanthamine and lycorine content in the bulb extract of *L. aestivum* under salt stress.

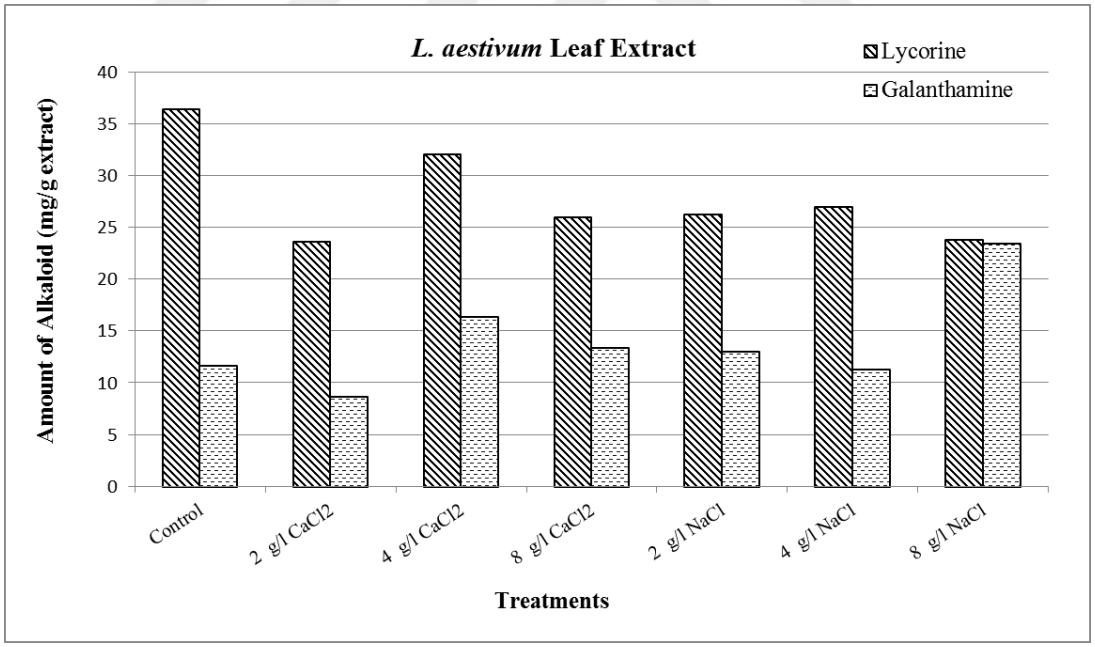

Figure 4. 11 Galanthamine and lycorine content in the leaf extract of *L. aestivum* under salt stress.

4.3 Free Radical Scavenging Activity–DPPH

The DPPH radical scavenging activity was determined by measuring the decrease in its absorbance at 517 nm. Therefore, DPPH is often used as the substrate to assess the activity of cleaning the antioxidant compounds.

In the present study, methanolic extracts of *L. aestivum* bulb and leaves grown under different salt stress conditions was used to evaluate the antioxidant activity. The results were given as an IC_{50} which is the concentration of any sample at which 50% inhibition. Quercetin was used as standard antioxidant in the experiment. In order to compare IC_{50} values of these extracts, DPPH inhibition curve were plotted using standard quercetin (Figure 4.5). The results of the antioxidant assay (DPPH IC_{50} inhibition) of the tested extracts were shown in Table 4.5.

In antioxidant assay for snowflake leaves extract, leaves grown under 4 g/L $CaCl_2$ salt stress showed the best antioxidant activity with IC_{50} value of 14.4 mg/ml. In the other words, this extract scavenged 50% of DPPH radical at 14.4 mg/ml concentration. Also leaves grown under 8 g/L NaCl salt stress showed higher antioxidant activity than rest of the NaCl salt stress and control. The IC_{50} value of this extract for DPPH inhibition was 17.52 mg/ml. Among snowflake bulb extracts, control and all bulb samples grown under both stress showed antioxidant activity with above 20 mg/ml IC_{50} value.

On the other hand, the results indicated that leaf extracts under grown 4 g/L $CaCl_2$ and 8 g/L NaCl salt stress showed higher antioxidant activity than the rest of the leaf extracts and all bulb extracts. However, this better antioxidant activity was not considered strong or good activity. According to Phongpaichit et al. (2007) plant extracts ranged from 0.05 to 0.1 mg/ml IC_{50} values are considered as intermediate antioxidant activity. Meanwhile, plant extracts ranged from 0.01 to 0.05 mg/ml IC_{50} values are considered as strong antioxidant activity.

Nikolova et al. (2011) studied the antioxidant capacity of *L. aestivum* folia methanolic extracts using DPPH radical scavenging assay. Similar to our result (control) (Table 4.5), *L. aestivum* leaves had IC_{50} value of greater than 20 mg/ml. Furthermore, Hundur et al. (2018) determined the IC_{50} values of methanolic extract of *L. aestivum* bulb and leaves were 317 µg/ml and 345 µg/ml, respectively (>200 µg/ml).

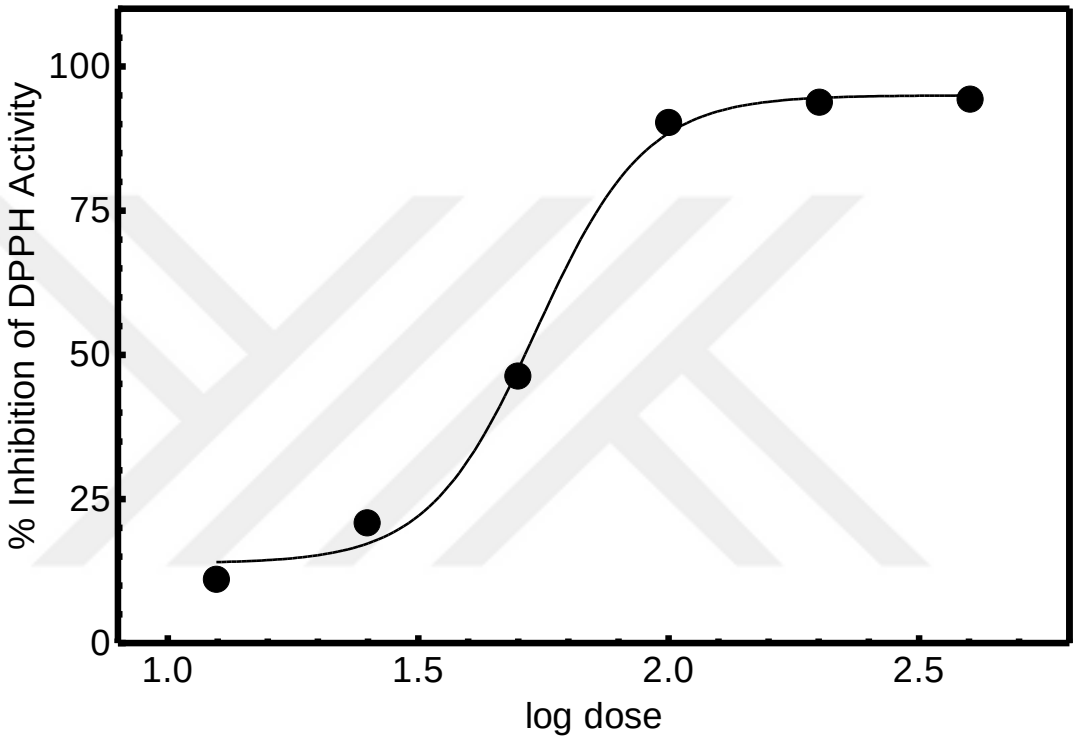

Figure 4. 12 IC_{50} value of quercetin standard for DPPH free radical scavenging effect.

Table 4. 5 DPPH radical scavenging activity of *L. aestivum* methanol extracts under salt stress.

Treatments	Concentration (g/L)	*L. aestivum* extracts	
		DPPH IC_{50} (mg/ml)	
		Bulb	Leaf
Control	-	> 20.0	> 20.0
$CaCl_2$	2	> 20.0	> 20.0
	4	> 20.0	14.4 ± 0.01
	8	> 20.0	> 20.0
NaCl	2	> 20.0	> 20.0
	4	> 20.0	> 20.0
	8	> 20.0	17.5 ± 0.01
Quercetin		0.05 ± 0.0	0.05 ± 0.0

4.4 Total Phenolic Assay (Folin–Ciocalteau Method)

Phenolic compounds are very important plant constituents because of their hydroxyl groups on phenolic compounds having scavenging ability. Total phenolic contents of *L. aestivum* extracts were detected using the Folin Ciocalteau reagent. Total phenolic content of *L. aestivum* extracts was calculated from the gallic acid calibration curve (R^2=0.9993) and expressed as milligrams of gallic acid equivalents (GAE) (Figure 4.13). Total phenolic contents of all methanol extracts were indicated in Table 4.6. Generally, some salt stress treatments increased the total phenolic content in both bulb and leaves.

When total phenolic content of control group (19.02 mg GAE/g dry extract) for bulb extracts were compared with total phenolic content of salt stress groups, the highest phenolic content was obtained with the plant grown at 4 g/L concentration of

$CaCl_2$ (27.67 mg GAE/g extract) and 8 g/L concentration of NaCl (23.94 GAE/g extract) (Table 4.6).

When leaf extracts were compared with respect to the their total phenol content, plants grown in all salt stress contained higher phenolic content than control except plant grown at 4 g/L NaCl. The highest phenolic content was found in plant grown at 2 g/L NaCl (36.5 ± 0.06 mg gallic acid/g extract) and followed by the plant grown at 2 g/L $CaCl_2$ (31.0 ± 0.06 mg gallic acid/g extract). In other words, the highest phenolic content in extracts was obtained with plants grown at low concentration of salt stresses (2 g/L $CaCl_2$ or NaCl) (Table 4.6).

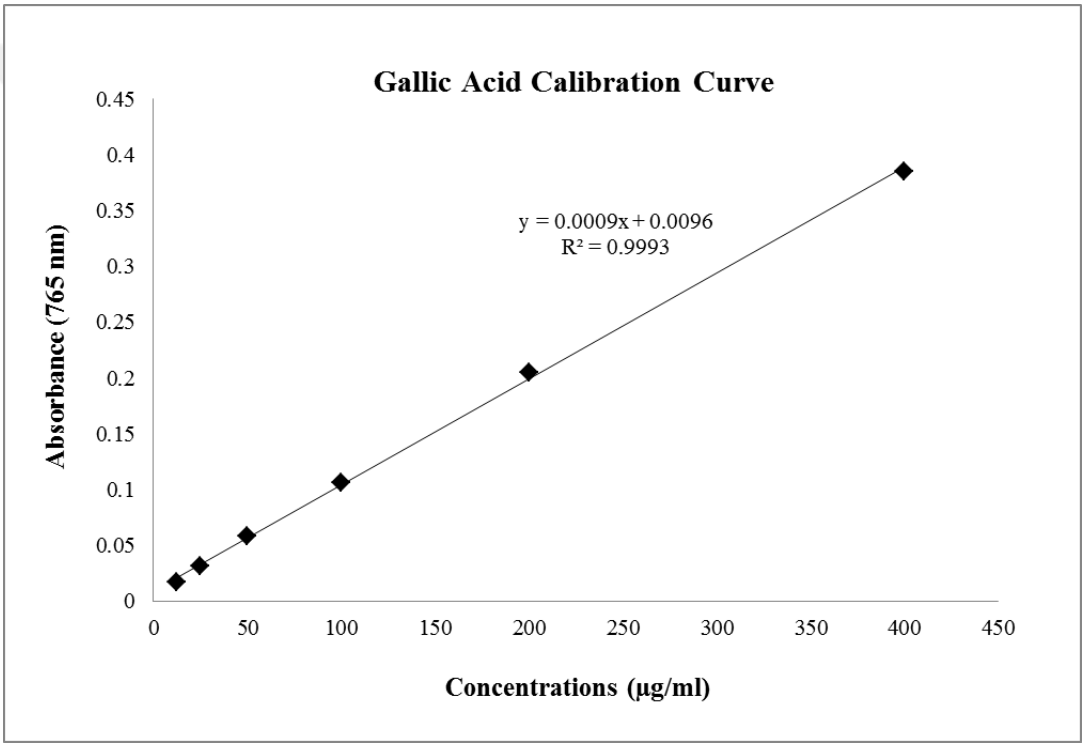

Figure 4. 13 Calibration curve for gallic acid standard.

4.5 Total Flavonoid Assay (Aluminium Chloride Colorimetric Assay)

Aluminium chloride was used for the colorimetric determination of total flavonoid content in *L. aestivum* extracts. Total flavonoid content of *L. aestivum* extracts was calculated from the quercetin calibration curve (R^2=0.9996) and expressed as milligrams of quercetin equivalents (QE) (Figure 4.14). Total flavonoid

contents of all *L. aestivum* extracts were shown in Table 4.6. Generally, some salt stress treatments increased the total flavonoid content in both bulb and leaves.

When bulb extracts were compared with respect to the their total flavonoid contents, only plants grown at 4 g/L $CaCl_2$ contained the higher total flavonoid content (10.95 mg QE/g dried extract) than control group (8.22 mg QE/g dried extract). Moreover, the extracts grown in high concentrations of NaCl and $CaCl_2$ contained the lowest flavonoid contents (1.37 and 1.39 mg QE/g dried extract, respectively) (Table 4.6).

The results for total flavonoid content in the leaf extracts indicated that plants grown in high concentration of NaCl (8 g/L) contained the highest flavonoid content as 126.23 mg QE/g dried extract. In addition, it was found that $CaCl_2$ salt stress treatments at all concentrations caused a decrease in total flavonoid contents. Among all leaf extracts, it was found that control plant contained higher flavonoid content than plants grown salt stresses except 2 and 8 g/L concentrations of NaCl.

Hundur et al. (2018) reported higher total phenol and flavonoid content of *L. aestivum* bulbs and leaves than our study (control) (Table 4.6). They determined total phenolic content of bulbs and leaves as 58.92 GAE mg/g and 53.93 GAE mg/g, respectively. Total flavonoid content was also determined as 85 QE mg/g and 68.33 QE mg/g, respectively (Hundur et al. 2018).

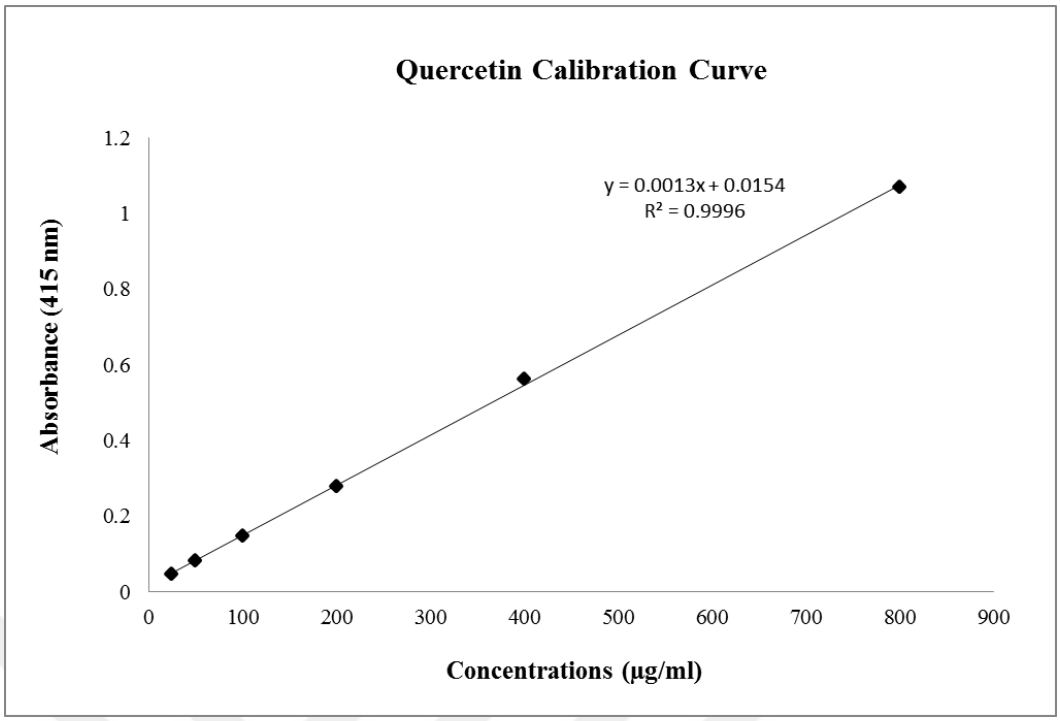

Figure 4. 14 Calibration curve for quercetin standard

Table 4. 6 Total phenol and flavonoid contents of methanolic extracts of the *L. aestivum* under salt stress. Data were presented as a mean number of phenolic/flavonoid content ± standard error (SE). Means with the same letter within columns are not significantly different at $P>0.05$. GAE: Gallic acid equivalent, QE: Quercetin equivalent.

Treatments	Concentration (g/L)	*L. aestivum* extracts			
		Total Phenol (mg GAE/g extract)		Total Flavonoid (mg QE/g extract)	
		Bulb	Leaf	Bulb	Leaf
Control	-	19.02 ± 0.08 [c]	11.46 ± 0.03 [f]	8.22 ± 0.02 [b]	41.50 ± 0.04 [c]
$CaCl_2$	2	14.24 ± 0.03 [e]	31.00 ± 0.06 [b]	2.04 ± 0.04 [d]	14.91 ± 0.02 [f]
	4	27.67 ± 0.00 [a]	12.28 ± 0.11 [e]	10.95 ± 0.02 [a]	39.73 ± 0.04 [d]
	8	8.43 ± 0.06 [f]	14.35 ± 0.14 [d]	1.39 ± 0.02 [f]	41.50 ± 0.00 [c]
NaCl	2	18.00 ± 0.06 [d]	36.5 ± 0.06 [a]	4.56 ± 0.04 [c]	66.46 ± 0.04 [b]
	4	19.00 ± 0.00 [c]	7.15 ± 0.03 [g]	1.78 ± 0.04 [e]	31.38 ± 0.00 [e]
	8	23.94 ± 0.00 [b]	26.56 ± 0.06 [c]	1.37 ± 0.12 [f]	126.23 ± 0.00 [a]

4.6 Superoxide Dismutase (SOD)

The changes in superoxide dismutase (SOD) activity of *L. aestivum* bulb and leaf were given in Table 4.7. According to the SOD results of *L. eastivum* bulb, the effect of all salt stress on the SOD activity was found to be significant when compared with control (Table 4.7 and Figure 4.15). On the other hand, it was found that the highest amount of SOD activity (mg/g) was observed when $CaCl_2$ was used at high concentrations (8 g/L). The second high SOD activity (mg/g) was obtained when $CaCl_2$ was used at low concentration (2 g/L). Moreover, among all salt stress treatments, the lowest SOD activity was found in bulb grown in 4 g/L concentration of $CaCl_2$.

According to the SOD results of L. eastivum leaf, the effect of all treatments to SOD activity of the plant extracts was found to be significant. Accordingly, there were differences in their SOD activities depending on the salt treatments. SOD activity was significantly increased when salt stress was used at the concentrations of 2 g/L $CaCl_2$ or 2 g/L NaCl (0.11 mg/g for both concentrations) when compared to the control leaf. When the concentrations were used higher than 2 g/L in both salt treatments, a decrease was observed in SOD activity when compared to control (Table 4.7 and Figure 4.15).

Table 4. 7 SOD and CAT activity of methanolic extracts of the *L. aestivum* under salt stress. Data were presented as a mean number of SOD/CAT activity ± standard error (SE). Means with the same letter within columns are not significantly different at $P>0.05$.

Treatments	Concentration (g/L)	*L. aestivum* extracts			
		SOD Activity (mg/g)		CAT Activity (mg/g)	
		Bulb	Leaf	Bulb	Leaf
Control	-	0.03 ± 0.00 [f]	0.10 ± 0.00 [b]	73.0 ± 3.9 [c]	55.1 ± 7.3 [d]
$CaCl_2$	2	0.13 ± 0.02 [b]	0.11 ± 0.00 [a]	50.1 ± 2.3 [d]	27.6 ± 3.2 [e]
	4	0.07 ±0.00 [e]	0.10 ± 0.00 [b]	85.9 ± 1.2 [b]	100.4 ± 4.8 [a]
	8	0.15 ± 0.3 [a]	0.09 ± 0.00 [c]	44.7 ± 0.8 [e]	59.4 ± 3.9 [c]
NaCl	2	0.11 ± 0.01 [c]	0.11 ± 0.00 [a]	16.2 ± 0.8 [g]	59.0 ± 2.9 [c]
	4	0.09 ± 0.02 [d]	0.09 ± 0.00 [c]	110.9 ± 1.6 [a]	23.1 ± 1.8 [f]
	8	0.11 ± 0.02 [c]	0.08 ± 0.00 [d]	42.5 ± 0.9 [f]	80.4 ± 3.4 [b]

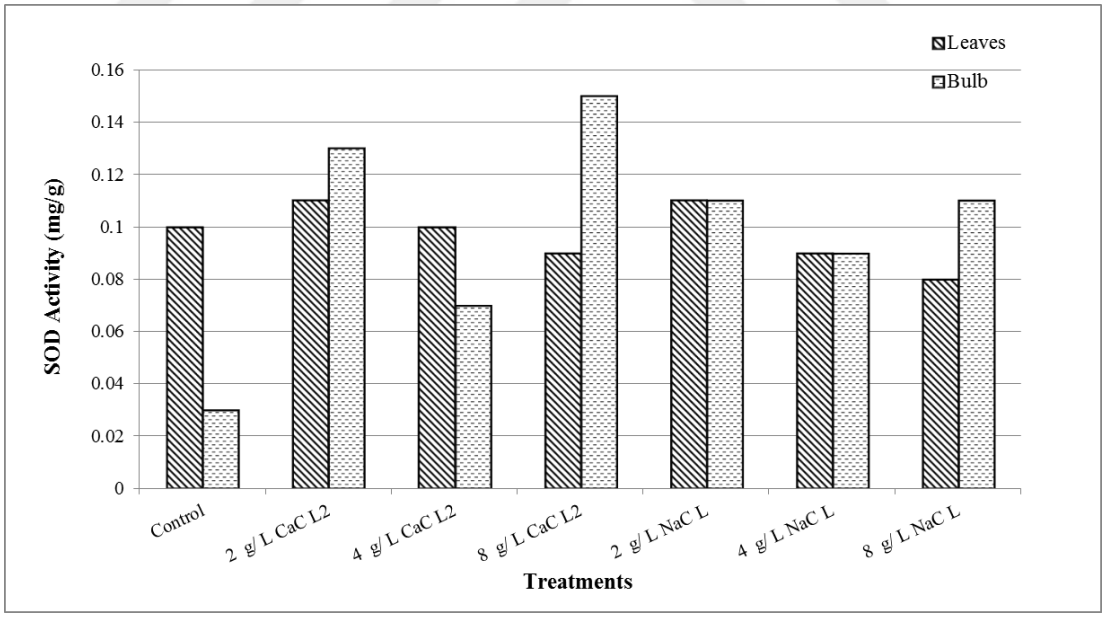

Figure 4. 15 SOD activity of methanolic extracts of the *L. aestivum* under salt stress.

4.7 Catalases (CAT)

The changes in Catalase (CAT) activity of *L. aestivum* leaf and bulb were given in Table 4.7. According to the CAT results of *L. eastivum* bulbs, the effect of salt stress on the CAT activity was found to be significant when 4 g/L NaCl and $CaCl_2$ concentrations were applied (Table 4.7 and Figure 4.16). On the other hand, the highest amount of CAT activity (110.9 mg/g) was obtained when 4 g/L $CaCl_2$ concentration was applied. Besides, the second highest CAT activity (85.9 mg/g) was obtained when $CaCl_2$ was 4 g/L in comparison to the control bulb. Lastly, the lowest CAT activity (16.2 mg/g) was observed in bulb grown at 2 g/L NaCl concentration (Table 4.7 and Figure 4.16).

When leaf extracts were compared with respect to their CAT activities, the CAT activity significantly increased when *L. aestivum* bulbs were grown in salt stress treatments except 2 g/L $CaCl_2$ and 4 g/L NaCl. When 4 g/L $CaCl_2$ salt stress treatment was used, approximately 2-fold higher CAT activity was observed with respect to the control leaf. However, when NaCl salt stress was used as 4 g/L concentration, CAT activity decreased approximately 2-fold with respect to the control leaf (Table 4.7 and Figure 4.16).

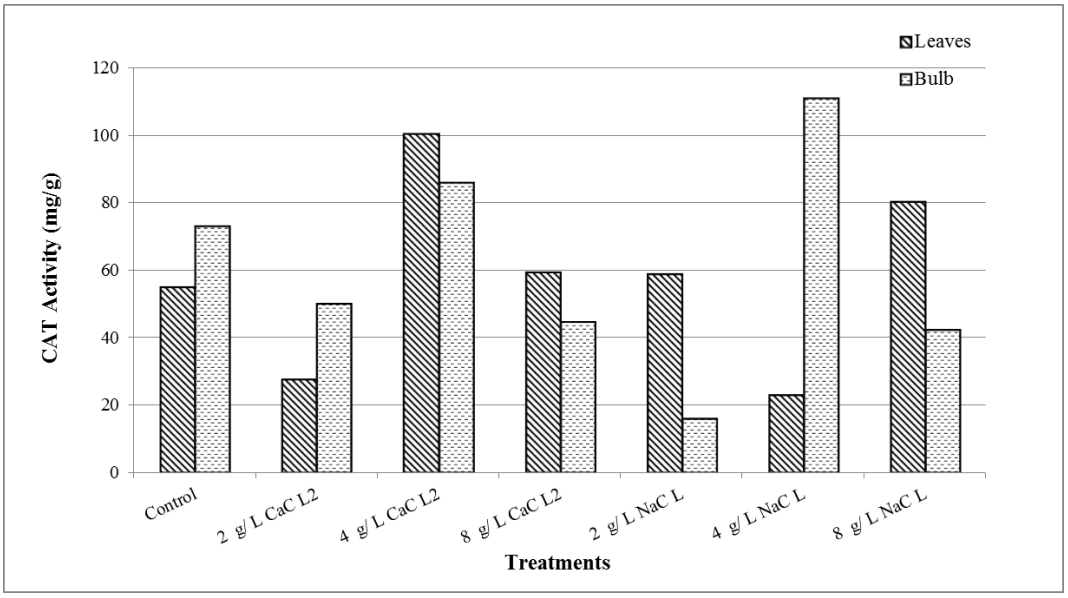

Figure 4. 16 CAT activity of methanolic extracts of the *L. aestivum* under salt stress.

Growing conditions like light regime, temperature and nutrient supply powerfully affects the accumulation of natural products. Vigorous environmental factors like different stress conditions also influence the metabolic pathways responsible for the accumulation of secondary plant products (Bohnert et al., 1995; Selmar and Kleinwächter, 2013). For example, many studies were reported about drought stress related increase in the concentrations of alkaloids such as trigonelline, pyrrolizidine alkaloids, quinolizidine alkaloids, steroid alkaloids, morphine alkaloids, indole alkaloids, nicotiana alkaloids and benzylisoquinolines (Selmar and Kleinwächter, 2013). There is a common tendency for alkaloid concentrations to be higher during dry periods than in wet periods as in the case of wild hemlock (*Conium maculatum* L.), cinchona (*Cinchona calisaya* Wedd.) and opium poppy (*Papaver somniferum* L.). During dry and hot season, wild hemlocks are most toxic. Cinchona does not produce quinine during the rainy season and field-grown opium poppy shows a keen decrease in morphine and codeine content in a wet period (Timmermann et al. 1984).

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.] and indicated that highest amount of total alkaloids and phenols were observed in summer, while the lowest in rainy season. Guo et al. (2007) concluded that high temperature could enhance the accumulation of some alkaloids in *Catharanthus roseus* (L.) G.Don. related with the treatment time. Zu et al. (2003) reported that alkaloid camptothecin and hydroxycamptothecin were involved in *Camptotheca acuminata* Decne. Heat shock resistance from its environment and under heat-shock condition, alkaloids changed sensitively in young leaves and buds.

Pavlov et al. (2007) displayed that light raised the galanthamine production in the shoot-clump of *L. aestivum*. Concentrations of nutrients in the medium affected the galanthamine biosynthesis in *in vitro* cultured *L. aestivum* (Georgiev et al. 2009). Diop et al. (2006) determined the galanthamine content in both *in vivo* and *in vitro* extracts of *L. aestivum* at different stages of morphogenesis. They found a correlation between the galanthamine content and the state of differentiation. 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 beneficial practice to enhance bulb yield in the cultivation of *L. aestivum* under field conditions.

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. 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.

Ptak et al. (2019) investigated the effect of NaCl (50, 100, 150 and 200 mM), melatonin (1, 5, 10 μM) and NaCl (200 mM) in combination with melatonin (1, 5 or 10 μM) in *in vitro*-cultured *L. aestivum* in regard to pigment contents, antioxidant enzyme activities and alkaloids. Addition of 100 mM NaCl to the medium resulted in a 2.6-fold increase in the galanthamine content comparing with the control. The highest quantity of galanthamine was observed with 5 μM melatonin (58.6-fold increase). Enrichment of the medium with 200 mM NaCl with 5 μM of melatonin induced a 6.4-fold increase in galanthamine. Furthermore, it was observed that NaCl increased CAT, POD and SOD activities in *in vitro*-grown *L. aestivum* plants. However, supplementation of melatonin into medium containing NaCl decreased the activity of antioxidant enzymes conspicuously and it was proved that melatonin enhanced salt stress tolerance in *L. aestivum* plant cultures (Ptak et al. 2019).

Salt stress decreases plant growth and development and change a number physiological and metabolic process. It induces the formation of different kinds of secondary metabolites such as phenols, terpenes, and alkaloids. Salt stress causes cellular dehydration and osmotic stress, resulting in a reduction of the cytosolic and vacuolar volumes. It often leads to both ionic and osmotic stress in plants that brings about increase or decrease in specific secondary metabolites in plants (Thakur et al. 2019). Stress conditions may increase secondary metabolites in some plants or decrease in the salt-sensitive species. Under stress conditions, hugely supply of reduction equivalents is produced. To avoid damage by oxygen radicals, NADPH + H^+ is reoxidized by photorespiration or violaxanthine cycle. Excessive amount of

reduction equivalents also causes an excessive ratio of synthesis of highly reduced compounds such as isoprenoids, phenols or alkaloids (Selmar 2008). Increased concentrations of NADPH in stressed plants cause increased rate of biosynthesis and thereby alkaloid concentration in the plant enhances under various stress conditions (passive shift) (Yahyazadeh et al. 2018) (Figure 4. 17). Yahyazadeh et al. (2018) also reported that stress-related rises in alkaloid content may not only be brought about by the well-known stress-related passive shift, but may also be because of an improvement of enzymatic capacity.

Although salt stress generally inhibits plant growth and development as well as leads to cellular dehydration and osmotic stress (Yahyazadeh et al. 2018; Thakur et al. 2019), a decrease in shoot length, bulb size and water content was not observed with salt stress applications in our study (Table 4.1 and 4.2). Also, noticeable increase was observed in galanthamine content in the bulbs and leaves with some salt stress treatments (Table 4.4). Similar to our result, salt stress caused strong increase on the production of tropane alkaloids in *Datura innoxia*, trigonelline in *Glycine max*, benzylisoquinoline alkaloids in *Chelidonium majus*, ergonovine and ergine in *Achnatherum inebrians*, and vincristine and vinblastine in *Catharanthus roseus* (Selmar 2008; Zhang et al. 2011; Yahyazadeh et al., 2017; Thakur et al. 2019).

Plants have antioxidant systems to increase the endurance for oxidative damage under different stress conditions both enzymatically like SOD, CAT, GSHPx etc. and non-enzymatically in the form of metabolites like phenols, flavonoids, ascorbic acid, glutathione, α-tocopherol, carotenoid etc. Furthermore, higher antioxidant enzyme activity and metabolites was observed in tolerant cultivars than the susceptible ones (Sairam et al. 2002). Enhanced total phenol and flavonoid content and also SOD and CAT activity were observed with some salt stress treatments in our study.

It is obvious that *L. aestivum* is a salt tolerant plant and pharmaceutically valuable alkaloid galanthamine can be enhanced with the exposure of salt stress conditions.

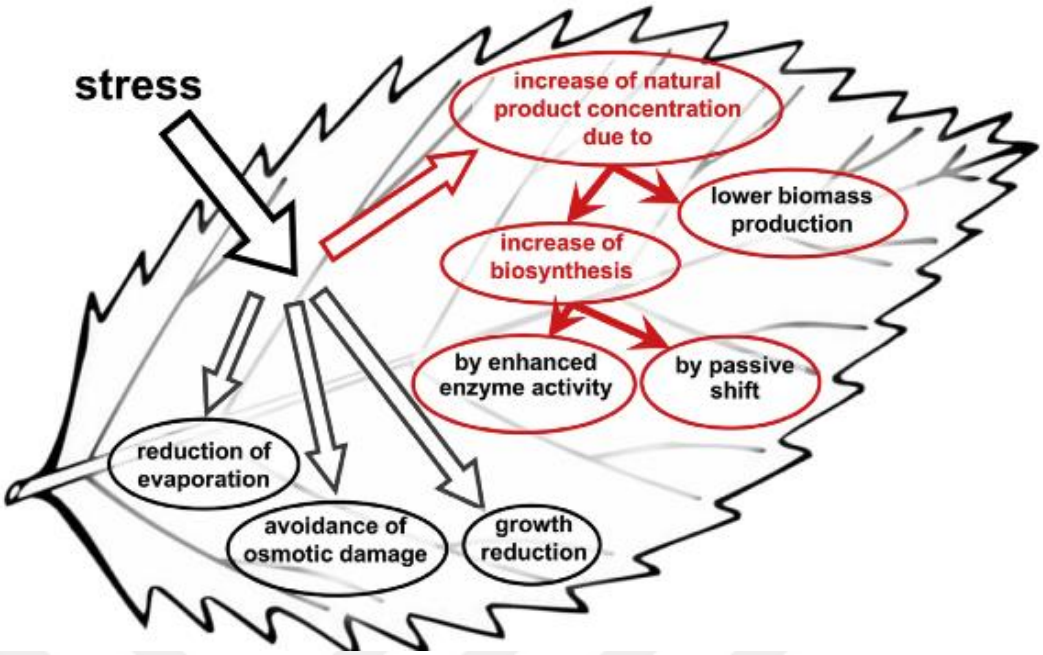

Figure 4. 17 Influences of salt stress and drought on concentration and content of natural product formation (Yahyazadeh et al. 2018).

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5. CONCLUSIONS AND RECOMMENDATIONS

Three different concentrations (2, 4 and 8 g/L) of two different salts ($CaCl_2$ and NaCl) were used to reveal the effect of salt stress on galanthamine and lycorine content, growth and development, non-enzymatic and enzymatic antioxidant activities in *L. aestivum* bulb and leaves.

Different salt stress applications did not change the shoot length, bulb size and water content percentage in *L. aestivum* significantly.

Among applications of 6 different salt stress treatments, 4 g/L $CaCl_2$ enhanced galanthamine and lycorine amounts in the bulbs. Especially, galanthamine in the bulb increased 4 times with this salt stress treatment. Two-fold increase of galanthamine was observed with 8 g/L NaCl treatment in the leaves. Salt stress treatments did not change the lycorine content in the leaves.

Non-enzymatic antioxidant activity was determined with free radical scavenging activity (DPPH) and total phenol-flavonoid content. Some salt stress treatment increased the antioxidant activity only in the leaves. Leaves grown under 4 g/L $CaCl_2$ salt stress showed better antioxidant activity with IC_{50} value of 14.4 mg/ml. All salt stress treated bulbs showed IC_{50} value of >20 mg/ml. Total Phenolic and flavonoid content were enhanced with 4 g/L $CaCl_2$ in the bulbs. Some concentrations of NaCl were more effective in the enhancement of total phenol and flavonoid content in the leaves. Total phenolic and flavonoid content were increased significantly in the leaves with 2 and 8 g/L NaCl, respectively.

Enzymatic antioxidant activity of *L. aestivum* was determined by SOD and CAT. In bulb extracts, althoug the highest SOD activity was observed with 8 g/L $CaCl_2$, the highest CAT activity was obtained with 4 g/L NaCl. In leaf extracts, the highest SOD activity was observed with 4 g/L $CaCl_2$ or 2 g/L NaCl, and the highest CAT activity was obtained with 4 g/L $CaCl_2$.

We can conclude that *L. aestivum* can be cultivated easily in soils with high salt content increasing its production of galanthamine.

Galanthamine is a medicinally important alkaloid and enhancement of this alkaloid was observed with salt stress treatments with this study for the first time under pot culture conditons. Improvement of this alkaloid should be studied with different stress applications for future studies.

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APPENDICES

7. APPENDICES

Appendix A. Bulb HPLC Chromatograms of *Leucojum aestivum* L.

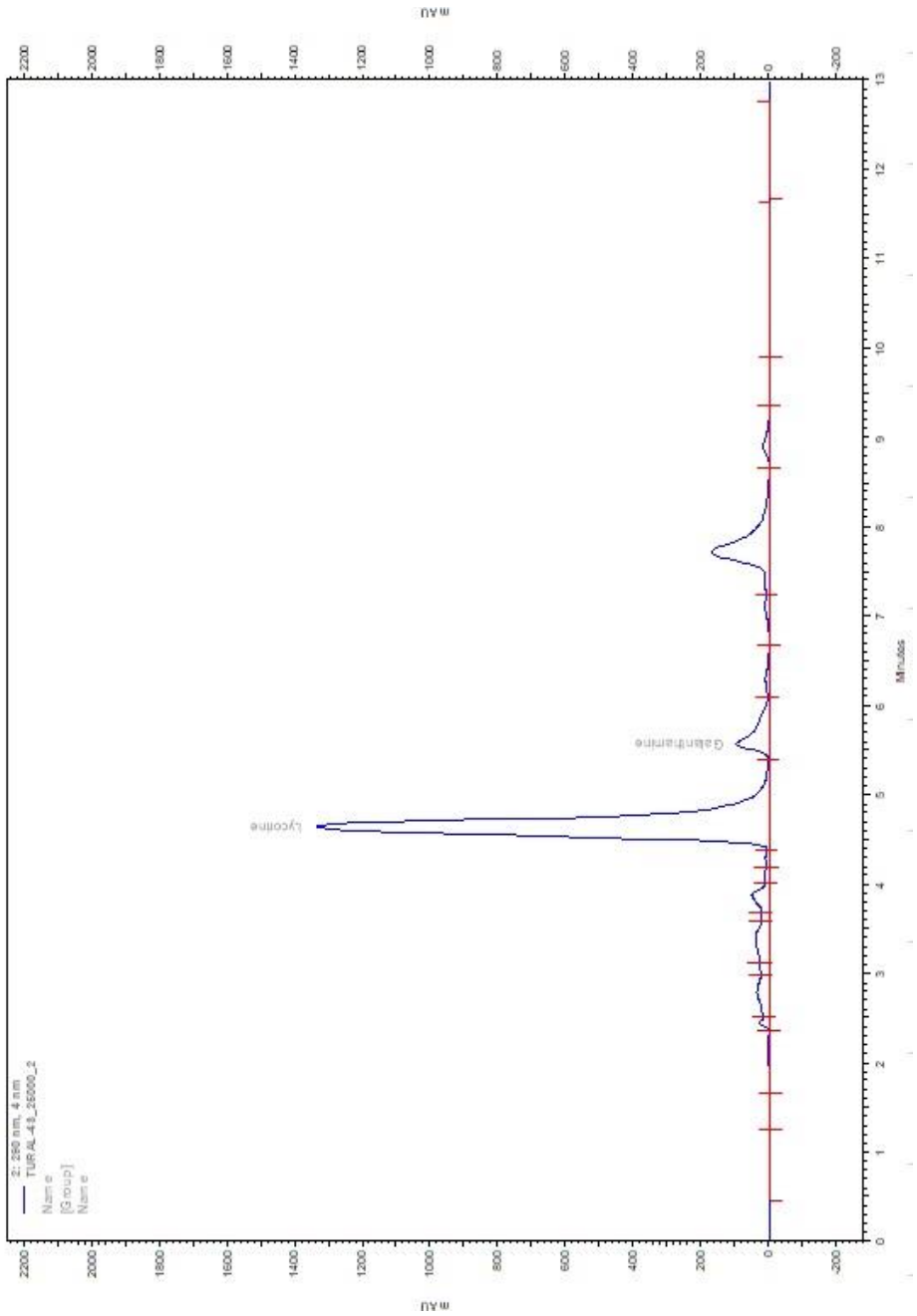

Figure A. 1 Control group of *L. aestivum* bulbs

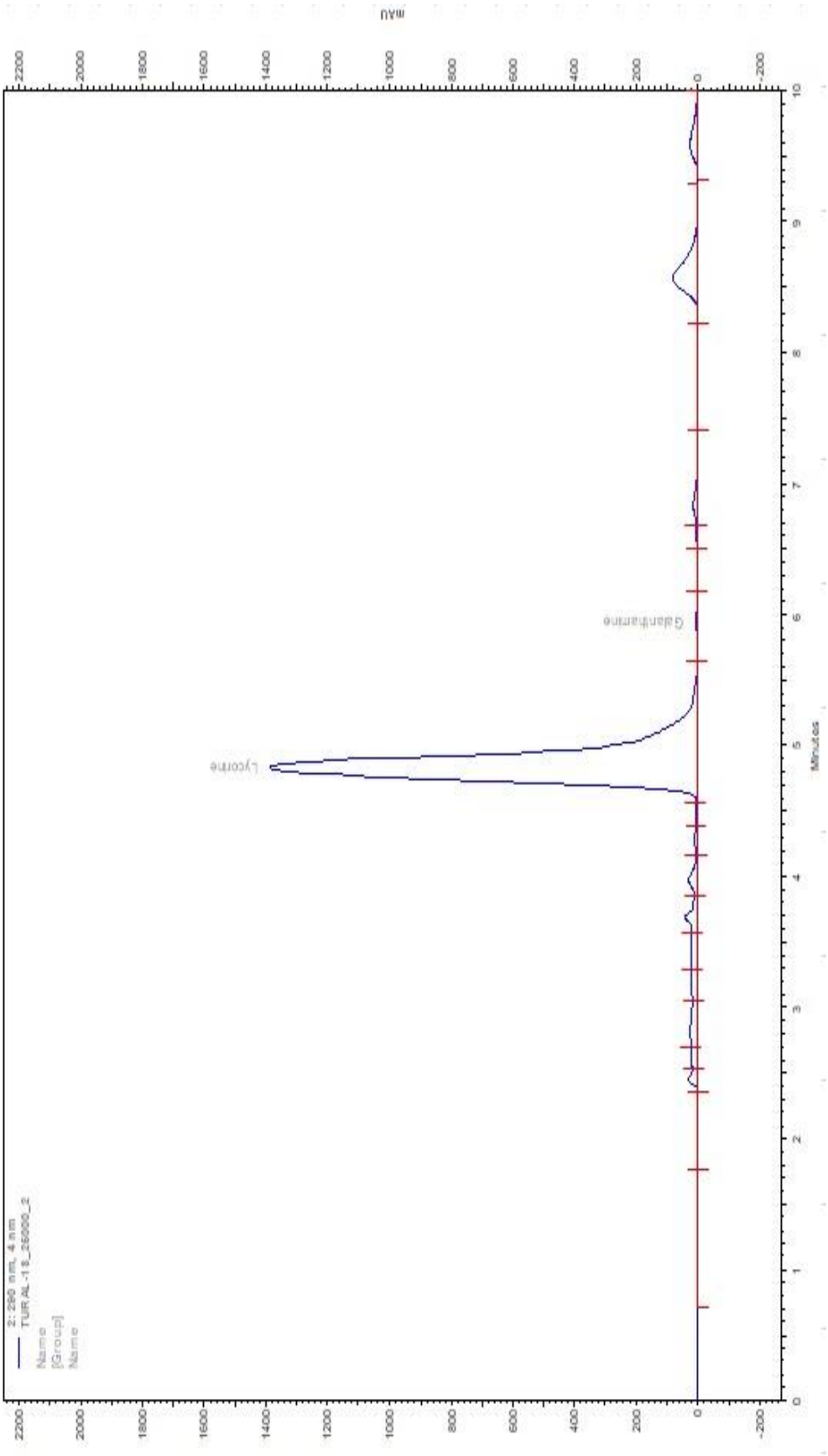

Figure A. 2 2 g/L $CaCl_2$ group of *L. aestivum* bulbs

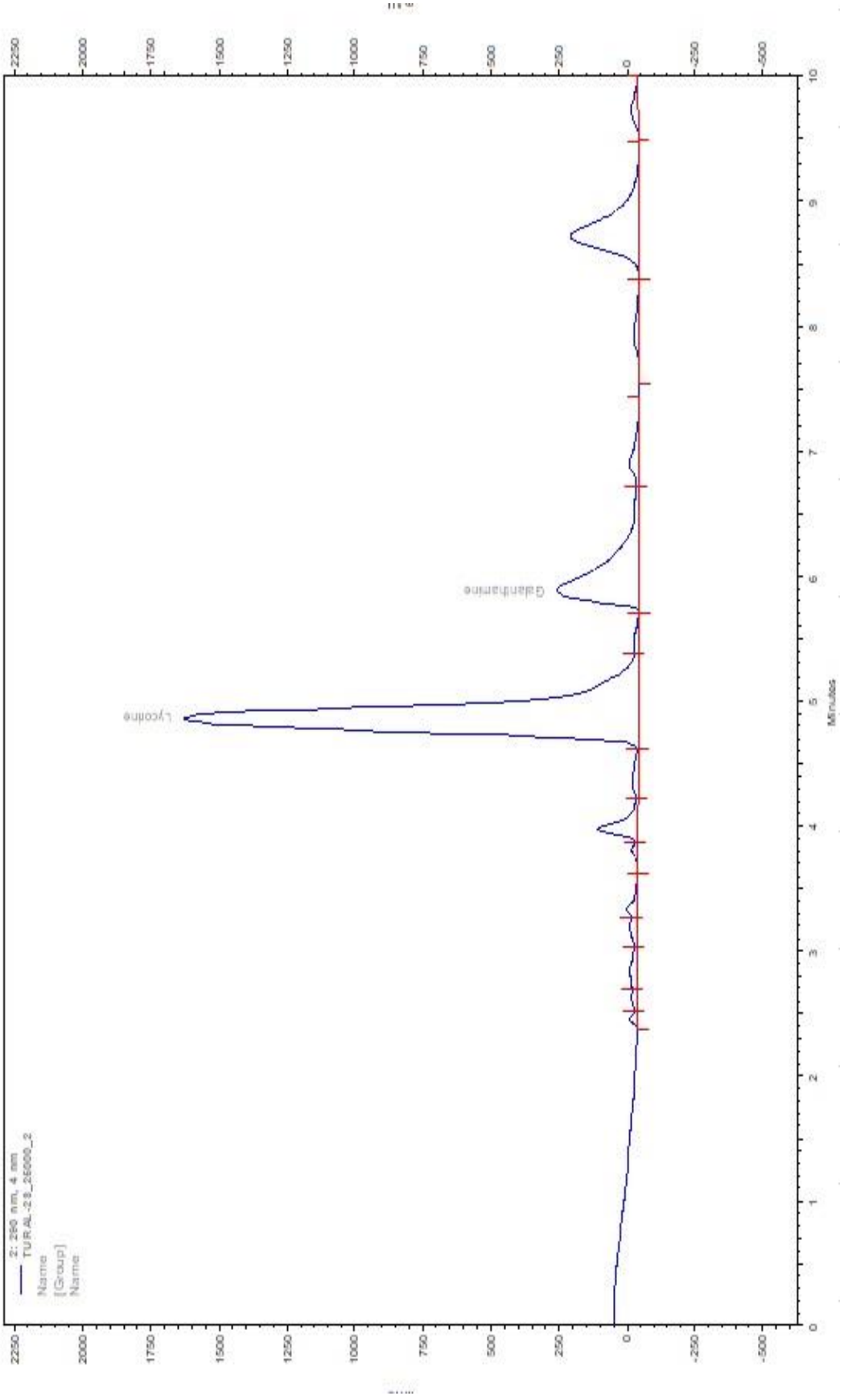

Figure A. 3 4 g/L $CaCl_2$ group of *L. aestivum* bulbs

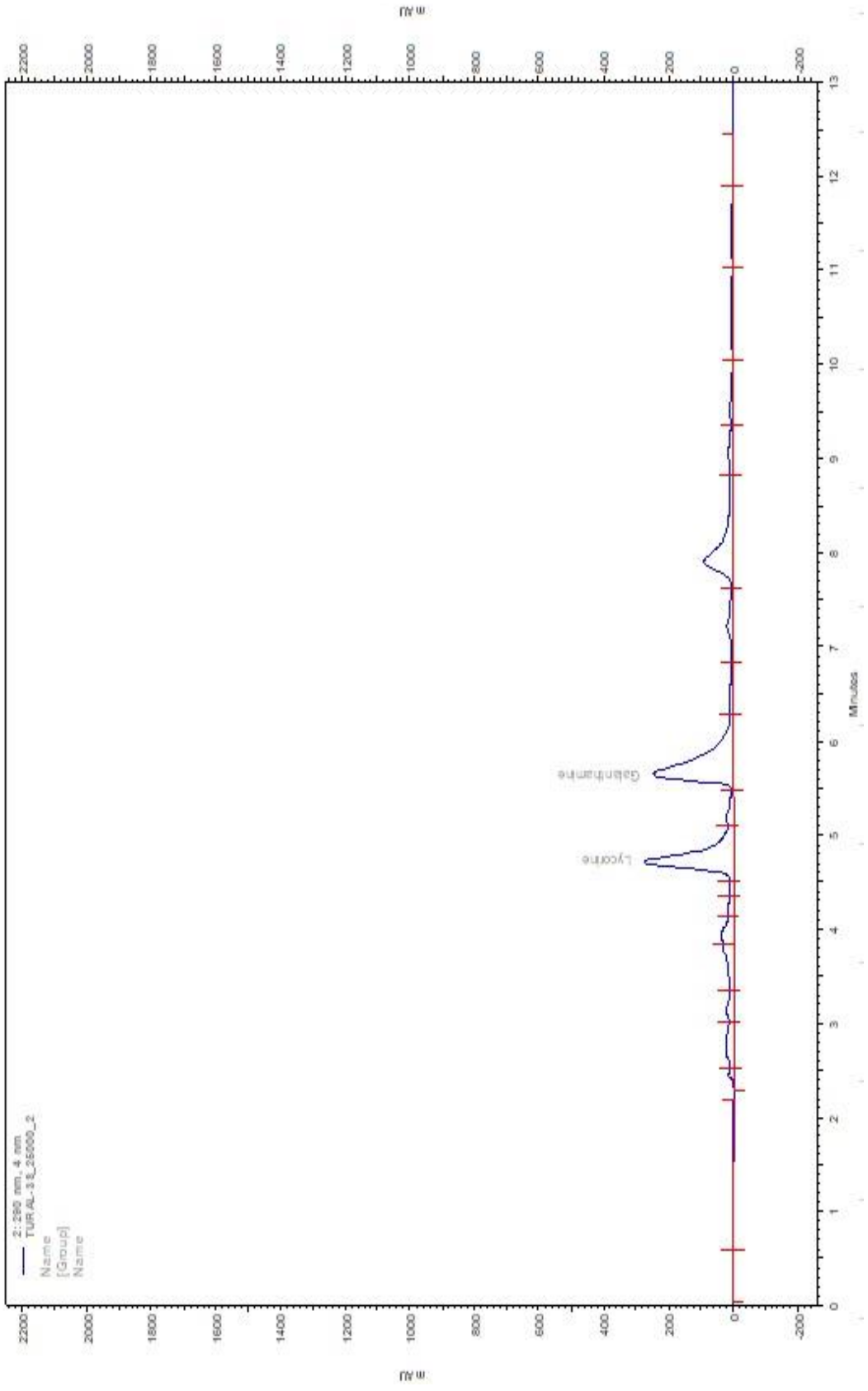

Figure A. 4 8 g/L CaCl2 group of L. aestivum bulbs

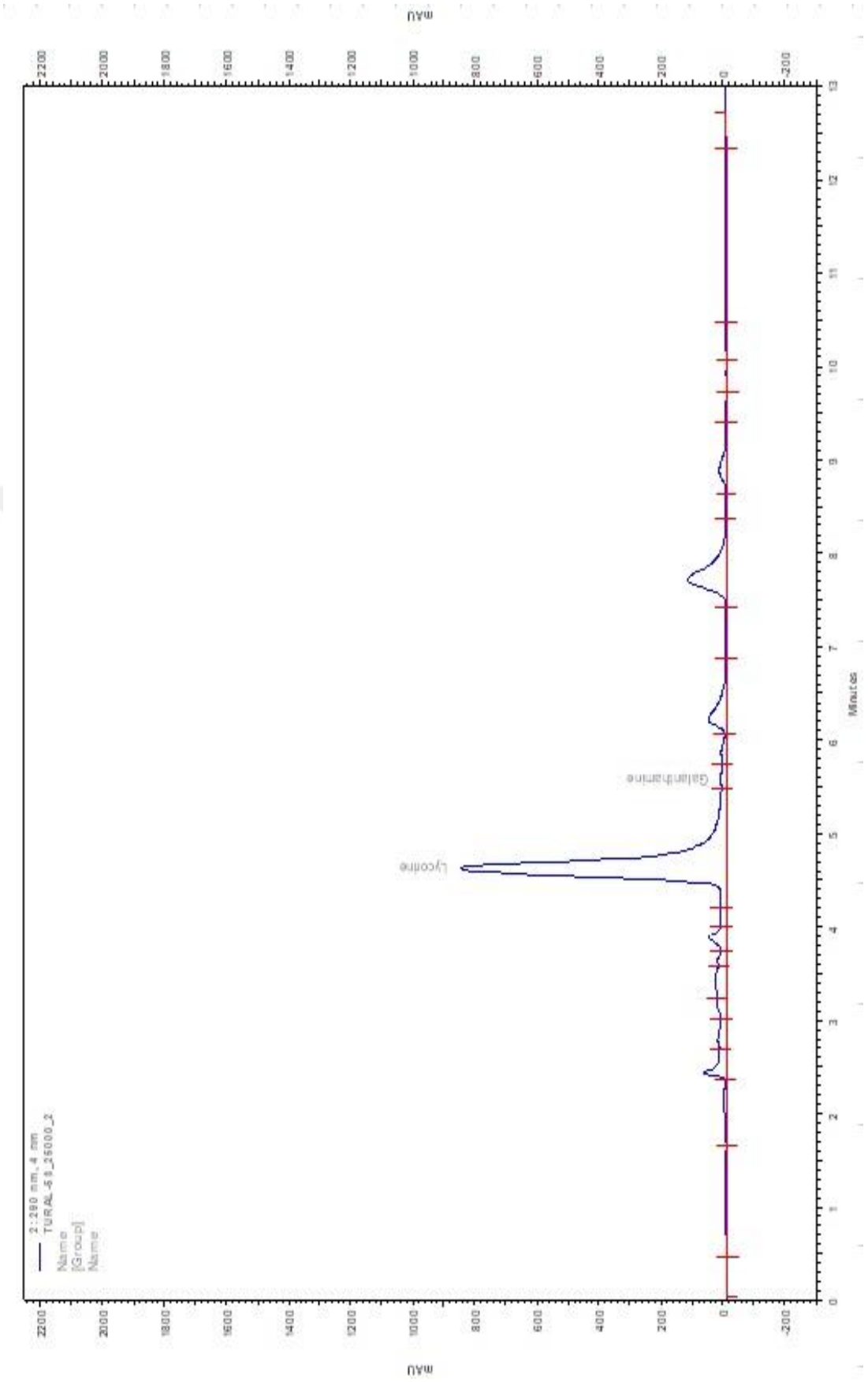

Figure A. 5 2 g/L NaCl group of *L. aestivum* bulbs

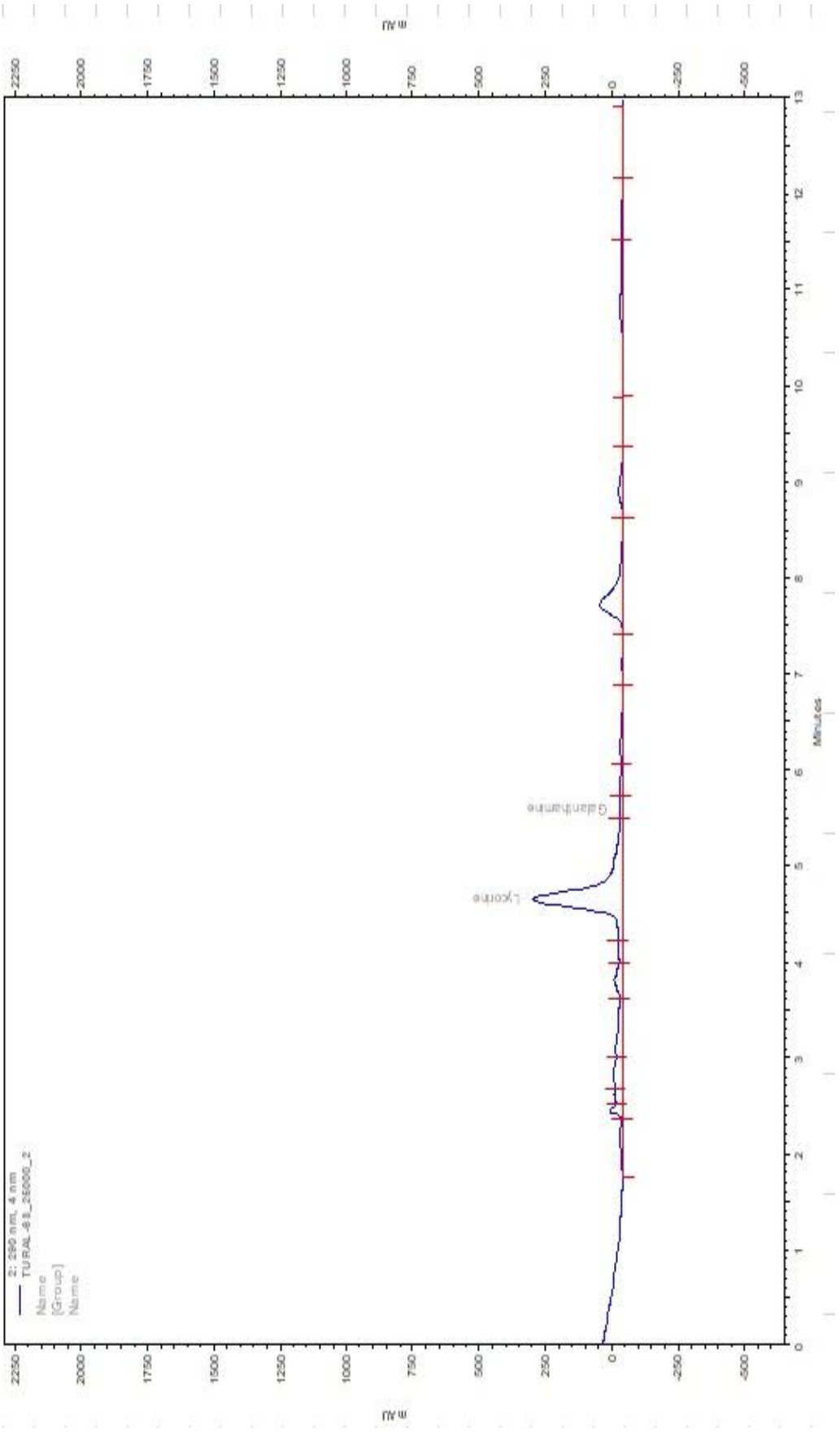

Figure A. 6 4 g/L NaCl group of *L. aestivum* bulbs

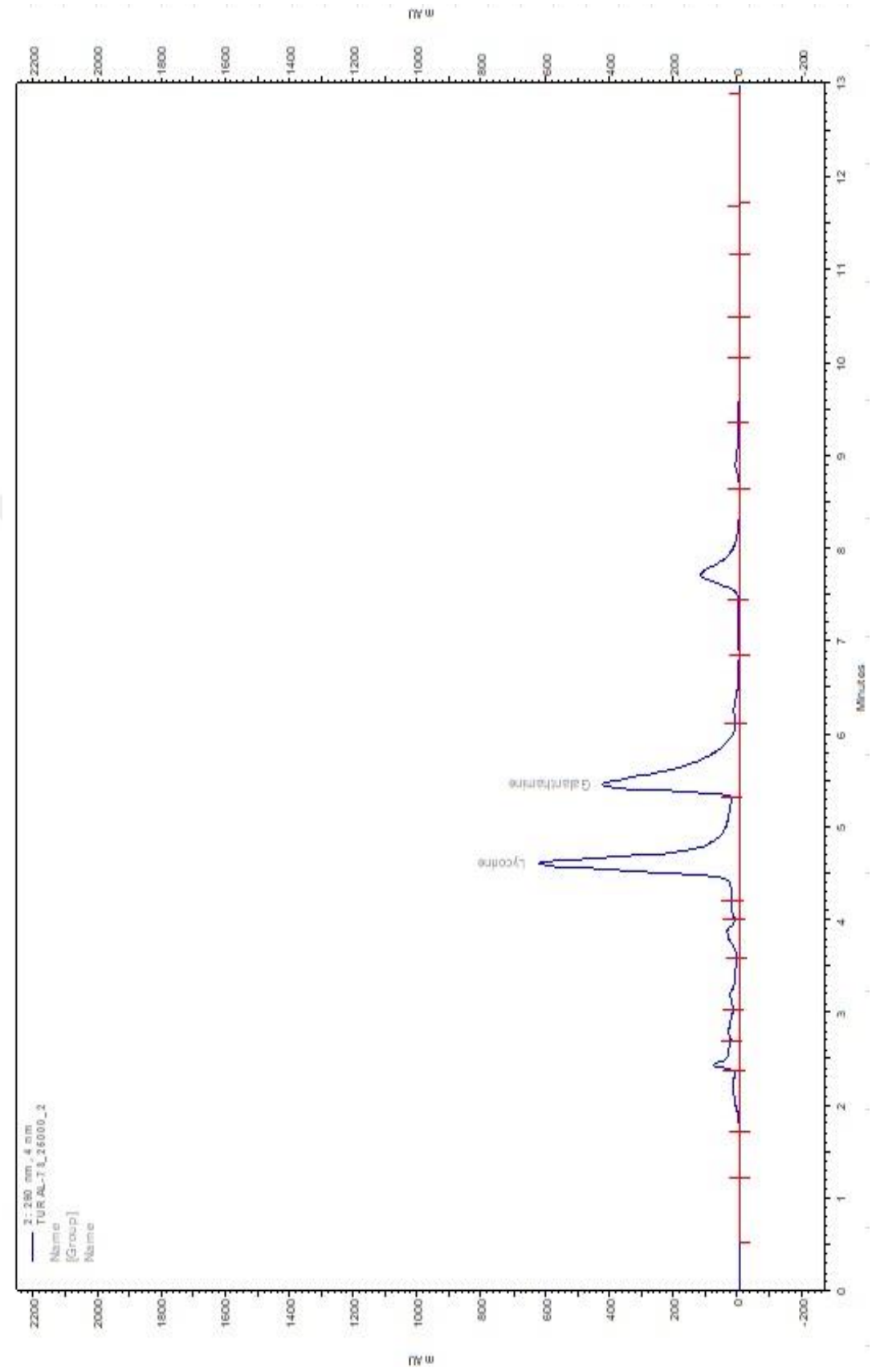

Figure A. 7 8 g/L NaCl group of *L. aestivum* bulbs

Appendix B. Leaf HPLC Chromatograms of *Leucojum aestivum* L.

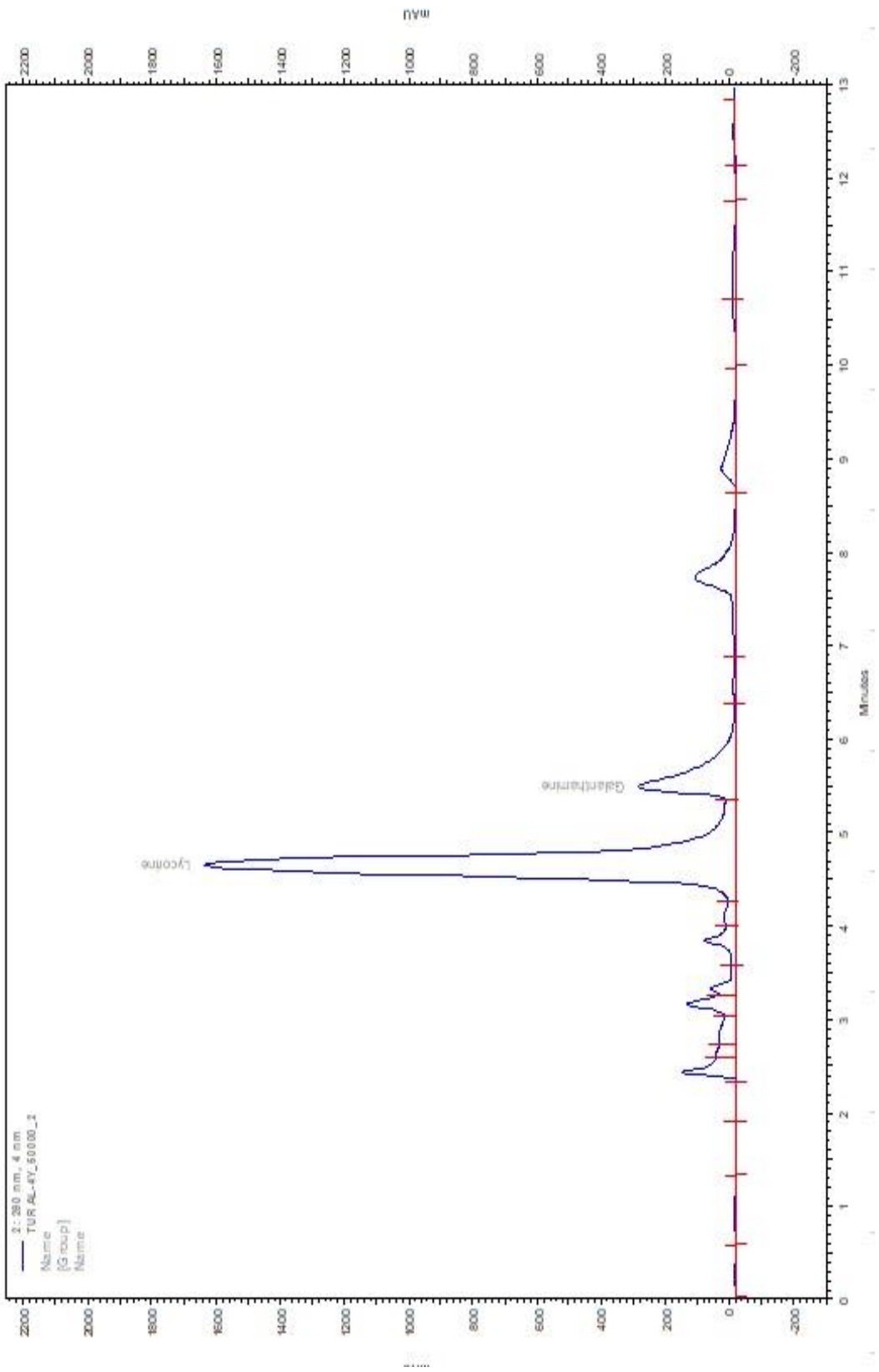

Figure B. 1 Control group of *L. aestivum* leaves

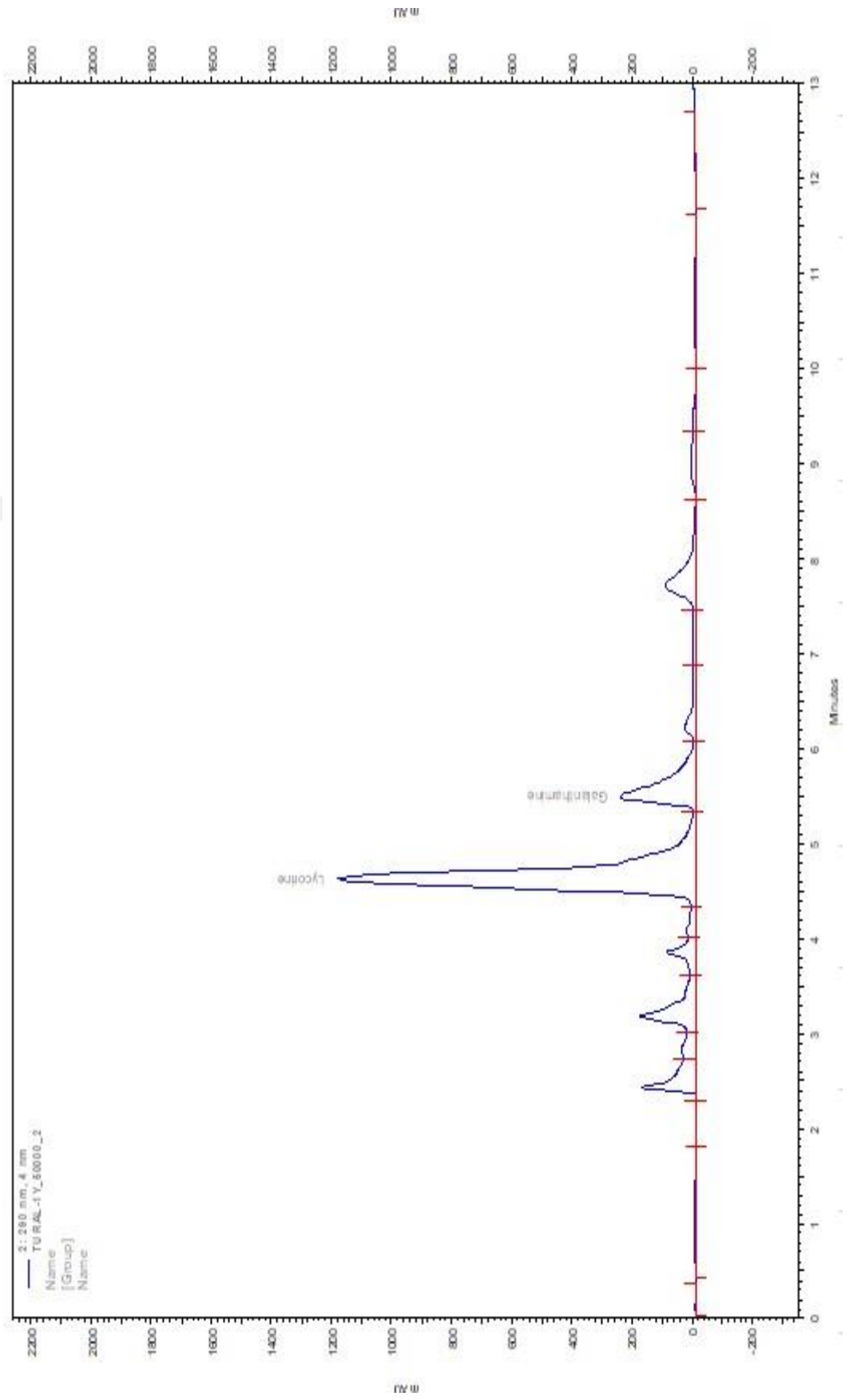

Figure B. 2 2 g/L $CaCl_2$ group of *L. aestivum* leaves

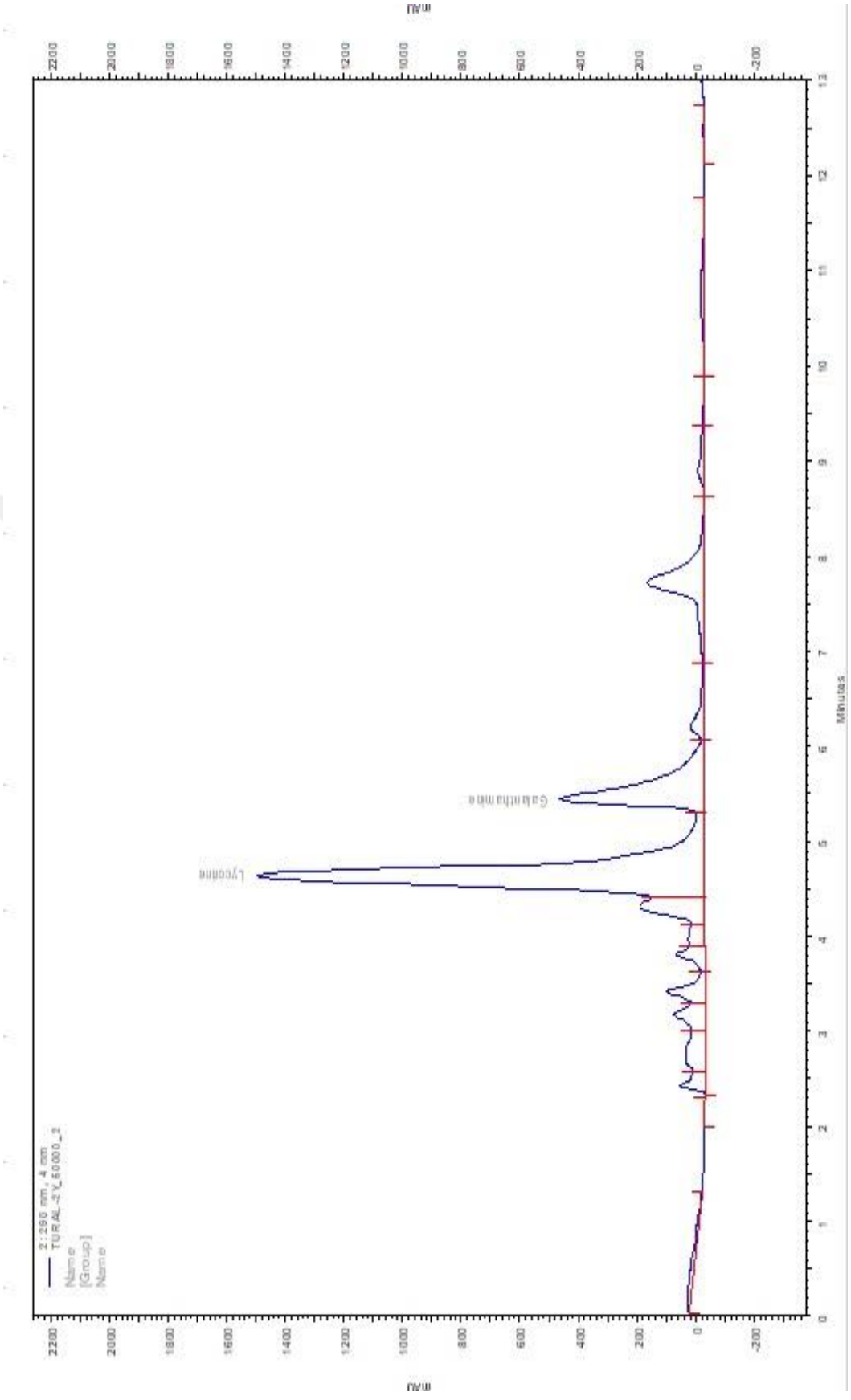

Figure B. 3 4 g/L $CaCl_2$ group of *L. aestivum* leaves

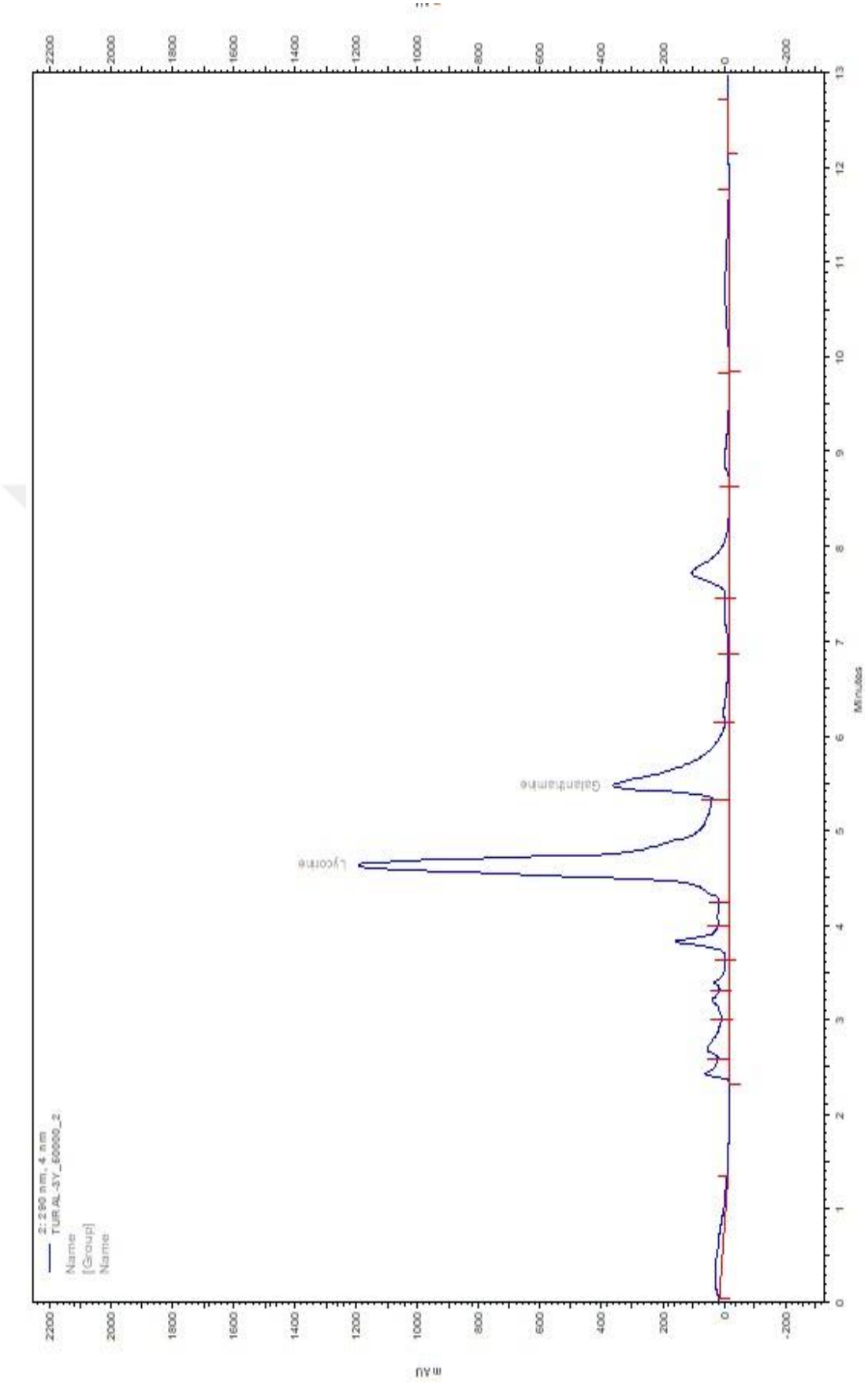

Figure B. 4 8 g/L $CaCl_2$ group of *L. aestivum* leaves

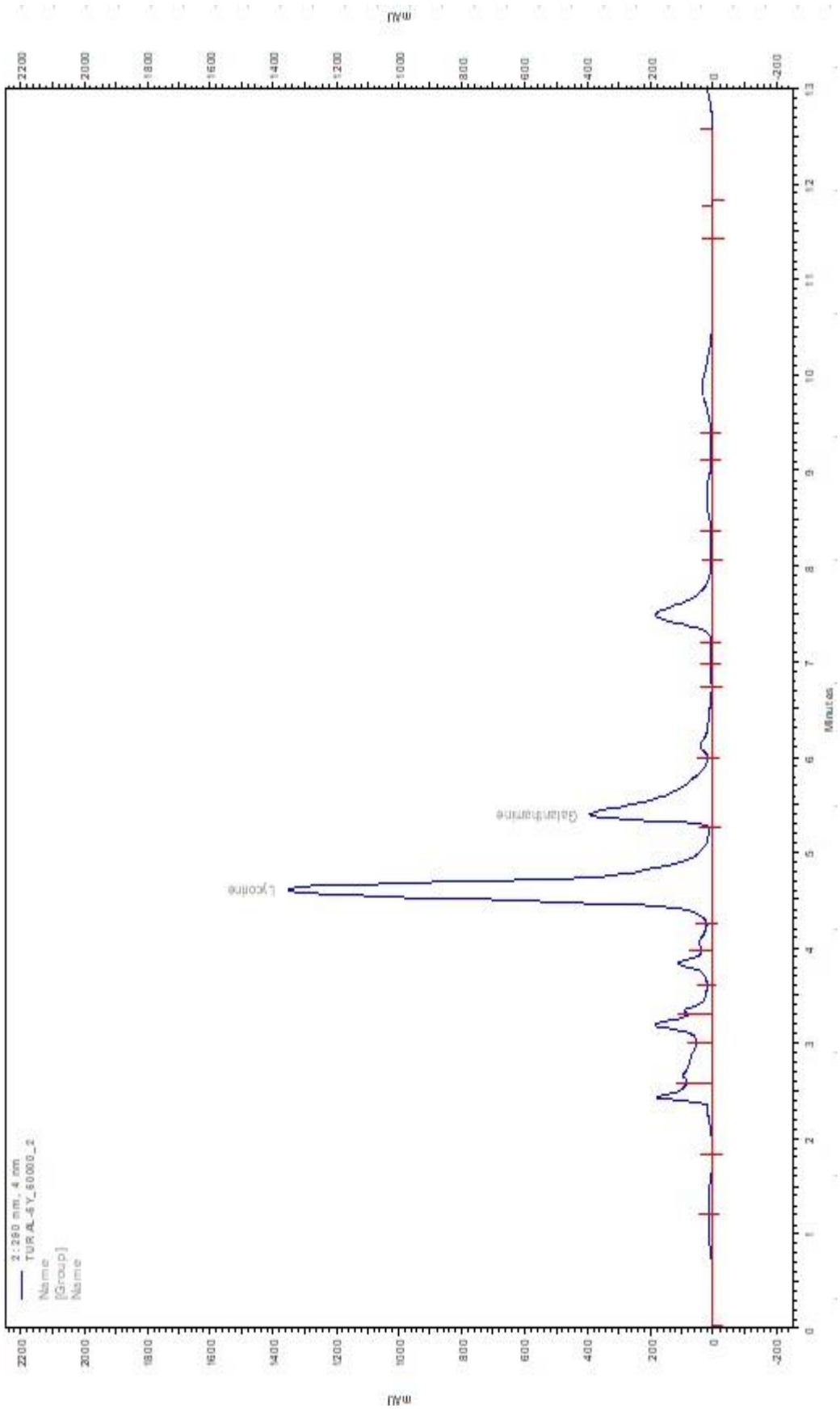

Figure B. 5 2 g/L NaCl group of *L. aestivum* leaves

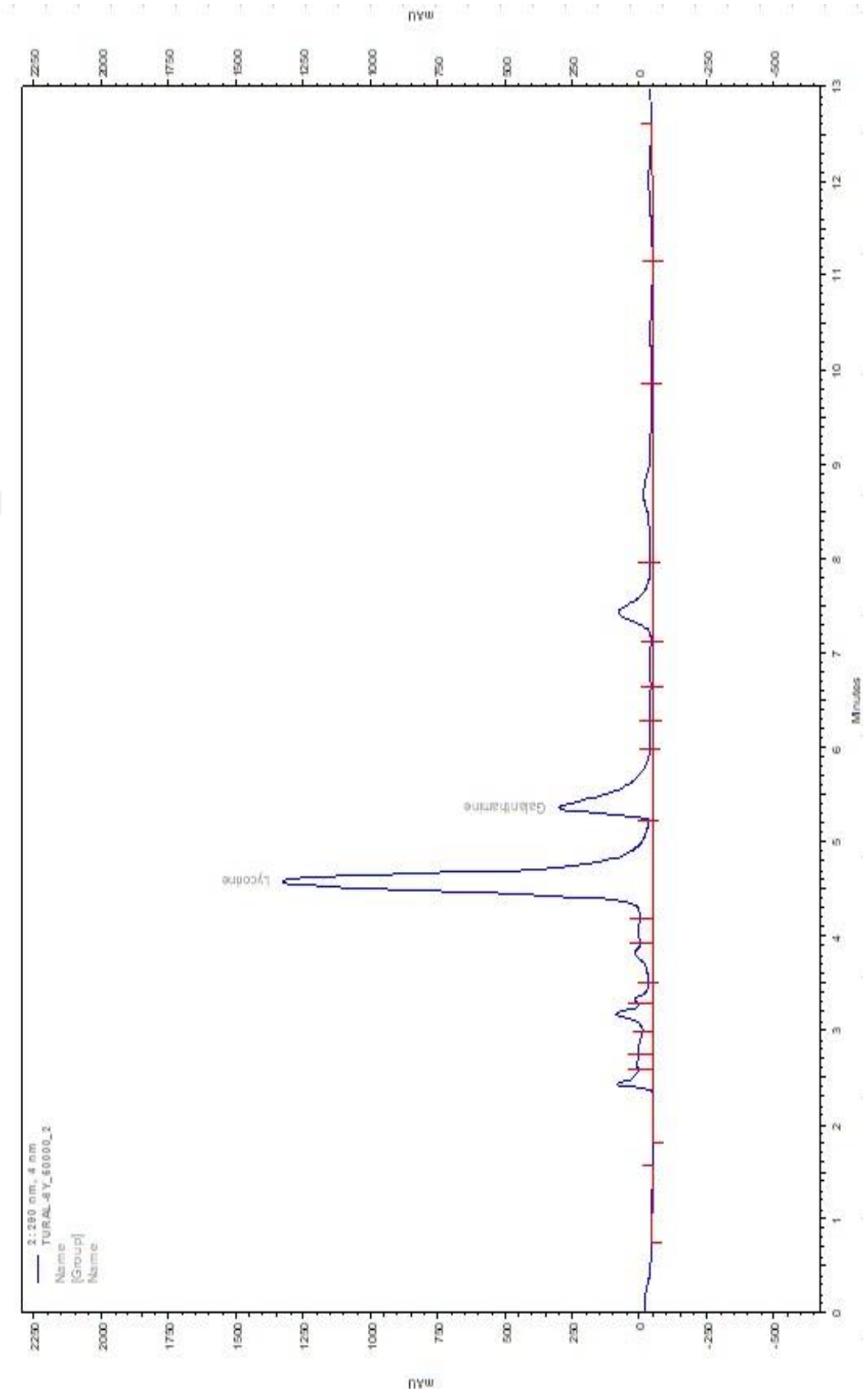

Figure B. 6 4 g/L NaCl group of *L. aestivum* leaves

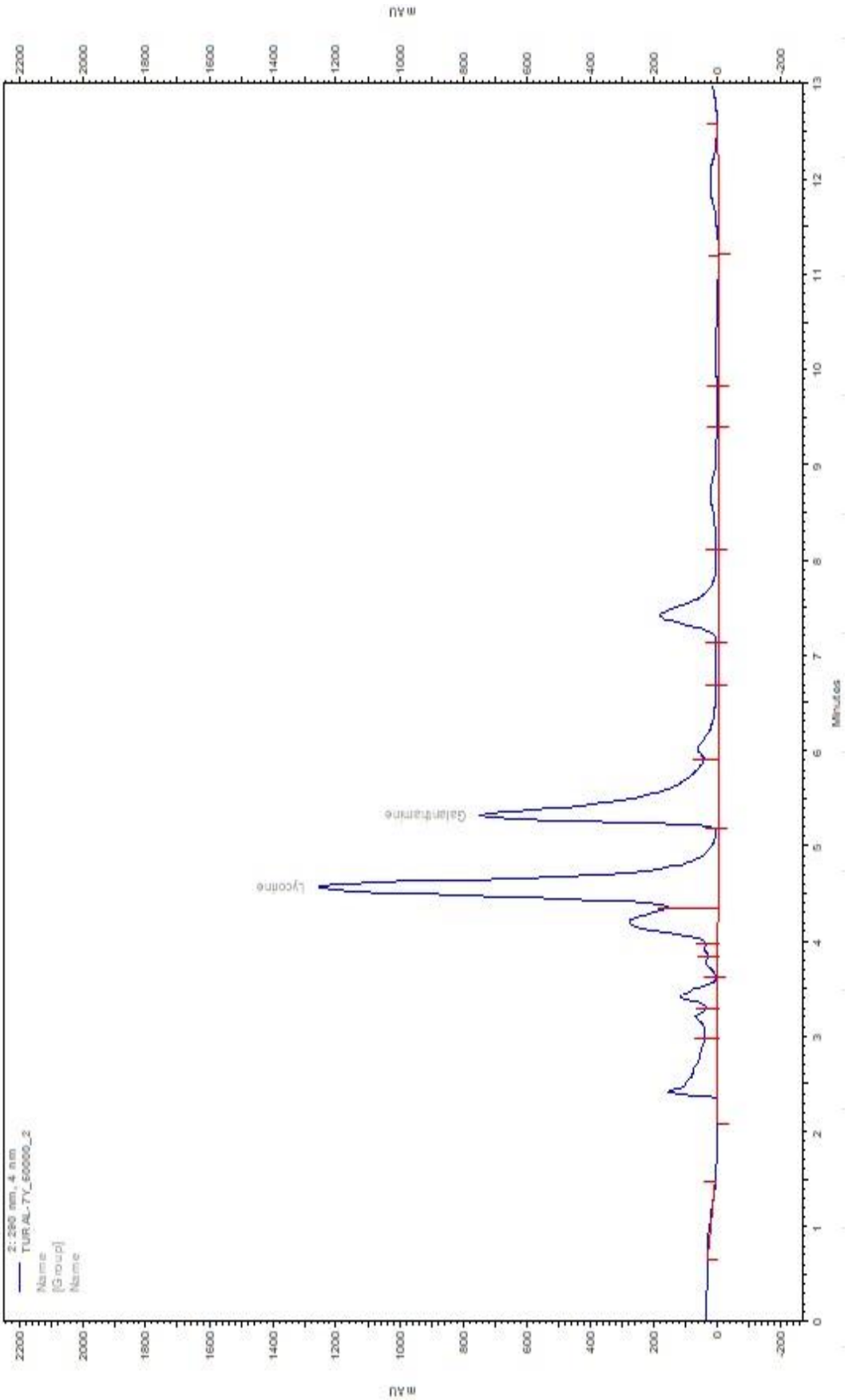

Figure B. 7 8 g/L NaCl group of *L. aestivum* leaves

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