

**ABANT İZZET BAYSAL UNIVERSITY
THE GRADUATE SCHOOL OF NATURAL AND APPLIED
SCIENCES**



**DETERMINATION OF BIOLOGICAL ACTIVITIES
(ANTIBACTERIAL, ANTIOXIDANT AND
ANTIPROLIFERATIVE) OF SOME LICHEN SPECIES
COLLECTED FROM BOLU AND HPLC ANALYSIS OF SOME
LICHEN METABOLITES**

MASTER OF SCIENCE

İSA TAŞ

BOLU, NOVEMBER 2015

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THE GRADUATE SCHOOL OF NATURAL AND APPLIED
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DEPARTMENT OF BIOLOGY



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APPROVAL OF THE THESIS

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To my lovely family

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.

İsa TAŞ

ABSTRACT

DETERMINATION OF BIOLOGICAL ACTIVITIES (ANTIBACTERIAL, ANTIOXIDANT AND ANTIPROLIFERATIVE) OF SOME LICHEN SPECIES COLLECTED FROM BOLU AND HPLC ANALYSIS OF SOME LICHEN METABOLITES

MSC THESIS

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ABANT İZZET BAYSAL UNIVERSITY GRADUATE SCHOOL OF
NATURAL AND APPLIED SCIENCES

DEPARTMENT OF BIOLOGY

(SUPERVISOR: PROF. DR. ARZU UÇAR TURKER)

BOLU, NOVEMBER 2015

Lichens are symbiotic organisms which are composed of fungi and algae. Various species have been used for treatment of stomach diseases, diabetes, bronchitis and skin diseases throughout the ages. The aim of present thesis was to investigate the biological activities (antibacterial, antioxidant and antiproliferation) and chemical contents of thirteen different lichen species (*Ramalina fraxinea*, *Ramalina farinacea*, *Bryoria capillaris*, *Evernia prunastri*, *Ramalina fastigiata*, *Evernia divaricata*, *Anaphthycia ciliaris*, *Pseudoevernia furfuracea*, *Lobaria pulmonaria*, *Platismatia glauca*, *Cetraria islandica*, *Letharia vulpina* and *Usnea florida*). Antibacterial activity of lichens was evaluated by disc diffusion method (Kirby-Bauer). Methanol extract of *R. fraxinea* was the most active antibacterial agent, inhibiting growth of 8 tested species of bacteria with the diameter of inhibition zone ranging from 9.5 mm to 30.5 mm. Antioxidant activities were determined by free radical scavenging (DPPH), total phenol and total flavonoid assays. *P.furfuracea* showed the largest DPPH radical scavenging activity (81.1%). Highest total phenol and flavonoid compounds were identified in *B. capillaris* (respectively 163.3 and 154.5 mg CE/g dried mass). Antiproliferative activities on the human breast cancer (MCF-7) and human hepatocellular carcinoma (HepG2/C3A) cell lines of six lichen species were determined by MTT assay. Only acetone extracts of samples showed antiproliferative activity against human hepatocellular carcinoma (HepG2/C3A) cell line. *L. pulmonaria* exhibited the best antiproliferative activity with IC₅₀ value of 90.2±1.2 µg/mL. Lastly, Quantification of chosen five standards (usnic acid, atranorin, stictic acid, evernic acid and fumarprotocetraric acid) in thirteen lichen species was performed by HPLC. Fumarprotocetraric acid was only found in *C. islandica*, while stictic acid was mostly detected in *L. pulmonaria*. *U. florida* had the greatest amount of usnic acid. *E. prunastri* was the main source of evernic acid. Atranorin was mostly detected in *P. furfuracea*. Fourteen different lichen compounds in the lichen species were also identified qualitatively by comparing of the absorbance spectral data in the literatures.

KEYWORDS: Lichen, Antiproliferative, Antibacterial, Antioxidant, TLC, HPLC

ÖZET

BOLU'DAN TOPLANAN BAZI LİKENLERİN BİYOLOJİK AKTİVİTELERİNİN (ANTİBAKTERİYEL, ANTİOKSİDANT VE ANTİPROLİFERATİF) BELİRLENMESİ VE BAZI LİKEN METABOLİTLERİNİN HPLC ANALİZİ

YÜKSEK LİSANS TEZİ

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ABANT İZZET BAYSAL ÜNİVERSİTESİ FEN BİLİMLERİ ENSTİTÜSÜ

BİYOLOJİ ANABİLİM DALI

(TEZ DANIŞMANI: PROF. DR. ARZU UÇAR TURKER)

BOLU, KASIM - 2015

Likenler, alg ve mantarların birleşimiyle oluşan simbiyotik organizmalardır. Çeşitli türleri, çağlar boyunca mide hastalıkları, şeker hastalığı, bronşit ve cilt hastalıkları tedavisinde kullanılmıştır. Mevcut tezin amacı, on üç farklı liken türünün (*Ramalina fraxinea*, *Ramalina farinacea*, *Bryoria capillaris*, *Evernia prunastri*, *Ramalina fastigiata*, *Evernia divaricata*, *Anapthygia ciliaris*, *Pseudoevernia furfuracea*, *Lobaria pulmonaria*, *Platismatia glauca*, *Cetraria islandica*, *Letharia vulpine* ve *Usnea florida*) biyolojik aktivitelerini (antibakteriyel, antioksidant ve antiprolifereatif) ve kimyasal içeriklerini araştırmaktır. Likenlerin antibakteriyel etkileri disk difüzyon yöntemi (Kirby-Bauer) ile değerlendirilmiştir. *R. fraxinea*'nın metanol özütü, 8 farklı bakteri üzerinde gösterdiği 9.5-30.5 mm aralığındaki inhibisyon çapıyla en yüksek etkiyi sergilemiştir. Antioxidant aktivite değerlendirmesi için DPPH serbest radikali giderme, toplam fenol ve toplam flavonoit testleri kullanılmıştır. *P.furfuracea*, en yüksek DPPH aktivitesini göstermiştir (81.1%). En yüksek toplam fenol ve flavonoid içeriğine *B. capillaris*'in sahip olduğu belirlenmiştir (sırasıyla 163.3 ve 154.5 mg CE/g kuru ağırlık). Altı farklı liken türünün MCF-7 (insan meme kanseri) ve HepG2/C3A (karaciğer kanseri) üzerindeki antiproliferatif aktiviteleri MTT assay ile belirlenmiştir. Örneklerin sadece aseton özütleri HepG2/C3A hücrelerine karşı etki göstermiştir. *L.pulmonaria*, 90.2 ± 1.2 µg/mL IC₅₀ değeriyle en yüksek antiproliferatif etkiyi sergilemiştir. Son olarak, likenlerin bazı sekonder metabolit içerikleri, kullanılan standartlarla (usnik asit, stiktik asit, evernik asit, fumarprotosetrarik asit ve atranorin) HPLC aracılığıyla karşılaştırılmıştır. Stiktik asit çoğunlukla *L. pulmonaria* da görülürken, fumarprotosetrarik asit sadece *C. islandica*'da bulunmaktadır. *U. florida* en yüksek usnik asit içeriğine, *E. prunastri* ise en yüksek evernik asit içeriğine sahiptir. En fazla Atranorin *P. furfuracea*'da tespit edilmiştir. Örneklerde bulunan 14 farklı lichen maddesi de, literatürde belirtilen UV spectrumları karşılaştırılarak tanımlanmıştır.

ANAHTAR KELİMELEER: Liken, Antiproliferatif, Antibakteriyel, Antioksidant, TLC, HPLC.

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LIST OF ABBREVIATIONS AND SYMBOLS

AChE	: Acetylcholinesterase
MAO	: Monoamine oxidase
TSB	: Tryptic Soy Broth
TSA	: Tryptic Soy Agar
AlCl₃	: Aluminium chloride
Na₂CO₃	: Sodium carbonate
ANOVA	: Analysis of Variance
DPPH	: 2,2-diphenyl-1-picrylhydrazil
EtOH	: Ethanol
MeOH	: Methanol
GAE	: Gallic acid equivalent
CE	: Catechol equivalent
MCF-7	: Human breast adenocarcinoma
HepG2/C3A	: Human hepatocellular carcinoma
MTT	: 3-[4-5 dimethylthiozol-2-yl] 2-5 diphenyl-tetrazolium bromide
HPLC	: High Performance Liquid Chromatograph
DAD	: Diode-Array Detector
RT	: Retention time
Nm	: Nanometer

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1. INTRODUCTION

1.1 Lichens

Lichens are a symbiotic life form between a fungus (mycobiont) and an alga or a cyanobacterium (photobiont) with unique characteristics in plant kingdom (Molnar and Farkas, 2010). Since the mycobiont is unique in the symbiotic association and usually dominated the association, lichens are traditionally classified as a life form of fungi (Boustie and Grube, 2005). It is estimated that there are approximately 25.000 species of lichens (Mitrović et al., 2011). They are proven as the earliest colonizers of terrestrial habitats on the earth with a worldwide distribution from arctic to tropical regions and from the plains to the highest mountains (Müller, 2001). They cover about 8 % of the earth's land surface (Ahmadjian, 1995).

The fungal partners are mostly (98%) Ascomycota and the others belong to the Basidiomycota and anamorphic fungi. Approximately 21% of all fungi are able to act as a mycobiont, thus lichens form the largest mutualistic group among fungi (Molnar and Farkas, 2010). Only 40 genera are involved as photosynthetic partners in lichen formation: 25 algae and 15 cyanobacteria (Kirk et al., 2008). The most common photobionts are the genera *Trebouxia*, *Trentepohlia* and *Nostoc*. The genera *Trebouxia* and *Trentepohlia* are of eucaryotic structure and belong to the green algae, while the genus *Nostoc* belongs to the prokaryotic cyanobacteria. Eucaryotic photobionts are sometimes called phycobionts (90 % of lichens), while cyanobacterial photobionts are termed cyanobionts (10 % of lichens) (Nash, 2008).

In lichen associations, both partners have benefit. The mycobiont has two principal roles in the lichen symbiosis: to protect the photobiont from exposure to intense sunlight and desiccation and to absorb mineral nutrients from the underlying surface or from minute traces of atmospheric contaminants. The photobiont also has two roles: to synthesize organic nutrients from carbon dioxide and, in the case of cyanobacteria, to produce ammonium (and then organic nitrogen compounds) from

N₂ gas, by nitrogen fixation. In some ecosystems such as desert soils, tundra heaths and Douglas-fir forests of the Pacific Northwest of the United States, lichens can provide the major input of nitrogen which supports other forms of life (Hale, 1973; Nash, 2008).

A lichen thallus usually consists of layers such as an upper and lower cortex, algal layer and medulla (Fig. 1.1). The layers differ in thickness and are better developed in some species than in others. Fungal hyphae make up most of the thallus; photobionts are cells of only a small percentage (about 7 %) of the total volume (Ahmadjian, 1995). There are three main types of thalli: crustose, foliose and fruticose. A crustose thallus lacks a lower cortex and is generally considered to be the most primitive type. Many crustose lichens stick tightly to the substratum and appear to be painted on it. Squamules are a specialised type of crustose thallus and are attached at only one end to the substratum. A foliose thallus has an upper and lower cortex, an algal layer and medulla and is usually loosely attached to the substrate by hair-like structures called rhizines. Some foliose lichens have thalli that are attached to the substrate by only one central point. Fruticose thalli are upright or hanging, round or flat and often highly branched. The layers of a fruticose thallus may surround a central thick cord, as in *Usnea*, or a hollow space as in some *Cladonia* species (Nash, 2008).

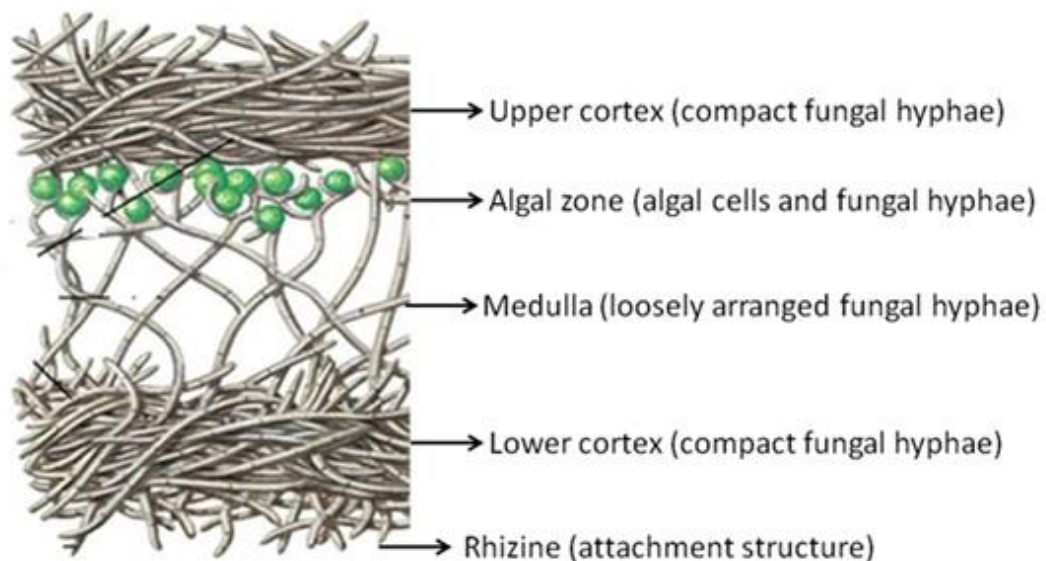


Figure 1.1. Layers of lichen thallus (www.plantscience4u.com)

Lichen thalli are poikilohydrous, which means that their water status passively follows the atmospheric humidity (Kappen, 2000). The presence of water rapidly activates lichen metabolism. Recovery of the photosynthetic apparatus after the dark winter takes only minutes in Antarctic lichens, whereas in mosses it is a longer process (Schlensog et al., 2004). The right choice of the photobiont, the water holding structures, and a tolerance to osmotic stress are some of the survival strategies of lichens. While green algae in lichens are able to activate their photosynthesis with water vapor, cyanobacteria in lichens need liquid water. This explains why green algal lichens survive in dryer habitats than cyanobacterial lichens, which in humid tropics represent nearly half of the known lichen species (Richardson, 2002).

In nature, lichens grow very slowly. Their radial growth is measured in millimeters per year. Hale (1973) made the following generalization about growth rates of lichens: most foliose species grow 0.5–4 mm/year, fruticose species 1.5–5 mm/year and crustose species 0.5–2 mm/year.

1.1.1 Lichen Metabolites

Lichens produce a great variety of secondary metabolites, and most of them are unique to lichen-forming fungi (Molnar and Farkas, 2010). They are mostly produced by the mycobiont, and accumulate in the cortex or in the medullary layer as extracellular tiny crystals on the outer surfaces of the hyphae (Brunauer et al., 2007). Although lichen secondary metabolites are exclusively of fungal origin, the metabolic interaction between the mycobiont and photobiont is essential to the production of these secondary chemicals (Shrestha and Clair, 2013). This has been documented by studies where the mycobiont grown without the photobiont does not produce the same metabolites as the intact lichen or produces a completely different suite of chemical products (Molina et al., 2003; Fazio et al., 2009).

They apparently involved diverse biosynthetic pathways to produce the variety of compounds mainly polymalonate, shikimic acid and mevalonic acid pathways (Boustie and Grube, 2005). Lichen secondary metabolites synthesized via

polymalonyl pathway belong to unique class of chemical compounds mainly, depsides, depsidones, dibenzofurans, anthraquinones, xanthenes and naphthaquinones. Lichen substances synthesized via shikimate pathway are mainly pulvinic acid and terphenylquinone. Mevalonic acid pathway mainly results in the synthesis of terpenes (Stocker-Wörgötter, 2005).

Secondary metabolites from lichens, especially polymalonyl derived polyketides, have been found to exhibit manifold biological activities in various screening (Müller, 2001). Past and current studies show that lichen's secondary metabolites exert a wide variety of biological activities that include antibiotic, antimycobacterial, antiviral, antiinflammatory, analgesic, antipyretic, plant growth inhibitory, enzyme inhibitory, antiproliferative and cytotoxic effects (Gayathri and Swamy, 2012)

The main natural roles of these compounds include: protection against a large spectrum of microbes, animal predators and plant competitors; defense against environmental stress like UV radiation and desiccation; physiological regulation of metabolism, such as the ability to increase algal cell membrane permeability in order to increase the flow of nutrients to the fungal component (Huneck and Yoshimura, 1996).

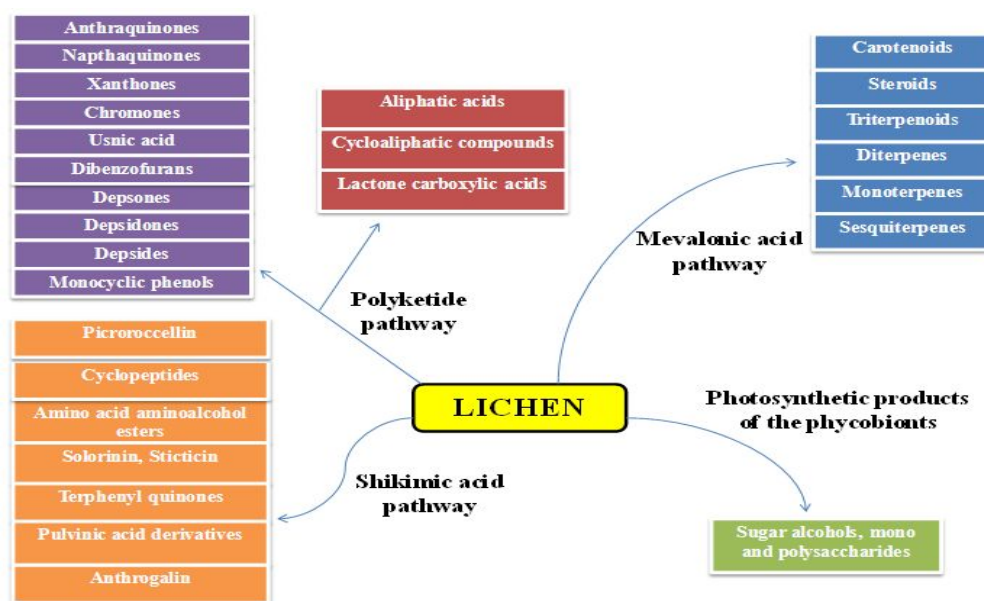


Figure 1.2. Biosynthetic pathways of lichens (wgbis.ces.iisc.ernet.in).

1.2 Ecological Role of Lichens

1.2.1 Biomonitoring

Air pollution is an essential problem in many urban, industrialized and densely populated areas. Pollution is increasing as a consequence of combustion for transport power generation and other human activities. Combustion process produce a mixture of pollutants including gaseous sulphur and nitrogen compounds in association commonly have high concentrations of heavy metals, organic compounds and others. Air pollution is a major environmental-related health treat. In order to protect living organisms from exposure to environmental hazards, better understanding of the relationship between environmental conditions and response of organisms are required (Upreti et al., 2015).

As environmental contamination becomes actual problem, scientists have to find methods of detecting and quantifying atmospheric pollution. An automated monitoring system is an useful tool for the determination of levels of atmospheric contamination. One of the limitations of such system is large number of sampling sites that leads to high cost. Alternatively, many recent studies on atmospheric pollution have used biological samples such as lichens or mosses (Conti and Cecchetti, 2001; Wolterbeek et al., 2003; Seaward, 2004). Biological monitoring is based on the assumption that changes in environment affect the biota and these changes can be effectively used as an early warning signal in order to detect environmental changes (Garty, 2001). The advantage of the biomonitors usage is the monitoring of several sites at low cost. In addition, their reaction reflects the effects of integrated pollutants impact over long period (Upreti et al., 2015).

Lichens are one of the most sensitive and valuable biomonitors of atmospheric pollution and can be used as indicators of air pollution at community-level mapping lichen species present in an area, transplanting healthy lichens and measuring deterioration in thallus, assessing the contaminants accumulated in lichen thallus. Lichens have been effective biomonitoring tools for decades because they absorb substances directly form the air and are sensitive to them (Upreti et al., 2015).



Figure 1.3. Lichens are used for biomonitoring. *Xanthoria parietina* can be used as a monitor for nitrogen in the atmosphere. It is a pollution-tolerant species (by İsa Taş).

1.2.2 Nitrogen Fixing

Nitrogen is a macronutrient essential for life in the formation of proteins and nucleic acids. Its limited availability frequently constrains growth and productivity, both of individual organisms, such as lichens (Crittenden et al., 1994), and of ecosystems as a whole. On the other hand, nitrogen may also be present in excessive amounts in some regions, such as those affected by high deposition of ammonia associated with fertilizer use and/ or animal husbandry. Because nitrogen is not part of the Earth's crust's material, rock weathering and subsequent soil formation do not provide a nitrogen source, as they do for many other elements. Rather the principal pool of nitrogen is atmospheric nitrogen N_2 , which is not readily utilized by most organisms. Nitrate (NO_3^-) and ammonia (NH_3), or ammonium ions, NH_4^+ are the inorganic forms of nitrogen that are universally processed by organisms, and their availability is critical to growth and survival of green-algal lichens (Nash, 2008).

With chlorolichens, nitrogen concentrations can often be related to atmospheric deposition of these ions (Hyvarinen and Crittenden, 1998). At least some cyanolichens are part of the small group of organisms, including some bacteria

and actinomycetes, capable of utilizing atmospheric N₂ directly. As a result cyanolichens have higher total N concentrations (2.2–4.7%) than chlorolichens (0.4–0.85%) (Rai, 1988). Because cyanolichens are abundant in a number of ecosystems, their potential contribution to nitrogen fixation in these ecosystems has led to attempts to quantify the rate of nitrogen fixation by these lichens and to understand the environmental and physiological factors limiting their fixation rates (Nash, 2008).

1.2.3 Rock weathering

The major long-term role has been as biological weathering agents, with a pedogenic action which is both physical and chemical in nature (Syers and Iskander, 1973; Jones, 1988). Despite early controversy concerning the pedogenic significance of lichens, their effectiveness in the biodeterioration of rocks has been clearly demonstrated by recent research, which has revealed that substantial quantities of the substrate can be degraded even over relatively short periods of time. Furthermore, lichens have the capacity to accumulate elements such as nitrogen, phosphorus, and sulfur, thereby increasing the potential bioavailability of these elements to successive life forms which may replace lichens during soil development. Organic material derived from lichen decomposition, together with detached particles of the substrate and atmospherically derived dusts trapped by thalli, all contribute to the development of soils. However, in a few cases, lichens have been considered as being bioprotective, the thallus acting as an umbrella to protect the underlying rock from rain, pollutants, salt deposition, and insolation (Carter and Viles, 2003, 2005).

The weathering action of saxicolous lichens can be physical, due to penetration by rhizinae and expansion and contraction of thalli, and/or chemical, due to carbon dioxide, oxalic acid, and the complexing action of lichen substances. The latter have a low but significant solubility in water, forming soluble metal complexes under laboratory conditions when they react with minerals and rocks, particularly limestone (Syers and Iskander, 1973). Oxalic acid, formerly considered of minor importance in the biodeterioration process, has been proved otherwise. The widespread occurrence of metal oxalates, particularly calcium oxalate, in lichens

(and in nature generally), the nature of the thallus substrate interface, and the chemical disruption of the substrate are significant components of the weathering process (Nash, 2008).



Figure 1.4. Lichens play an important role in breaking down rock minerals and contribute the soil formation. (www.buffelskloof.info/images/Lichen%208.JPG)

1.2.4 Food and nesting material

Many bird species use lichens as nesting material, some showing a definite preference for certain types of lichen not only for nest construction and camouflage, but also, as in the case of bower birds, for purely decorative purposes. The disappearance of epiphytic lichens, mainly due to deforestation and air pollution, has depleted this source of nest-building material and, more importantly, the consequent loss of the associated lichenophagous insect fauna. The loss of this important food source as well as egg-laying sites for invertebrates affects the complex food webs upon which bird populations depend (Nash, 2008).

Lichens are also used as nesting material by flying squirrels (Hayward and Rosentreter, 1994), which were highly selective in their choice of lichens, as 96% by

volume of their nests were constructed of three species of Bryoria. Interestingly, these three species contain little or no lichen substances, making them more palatable to these squirrels, which are known to feed of lichens.

A large number of mammal species are known to feed on lichens, although their importance in the animal's diet varies considerably. Deer, elk, ibex, gazelle, musk ox, mountain goat, polar bear, lemming, vole, tree mouse, marmot, squirrel, monkeys, and some domestic animals may include lichens in their diets, perhaps fortuitously, but more likely as a means of supplementing their normal diet or as winter feed (Nash, 2008).



Figure 1.5. Birds use lichens for nesting material (www.ecology.com).

1.3 Biological Activity of Lichens

1.3.1 Antimicrobial Activity

The drug resistance of human and animal pathogens is one of the best documented in biological evolution and a serious problem in both developed and developing countries. The consumption of more than one ton daily antibiotics in some European countries has resulted in resistance to bacterial populations, thus causing a serious public health problem. In view of this scenario, the search for new antimicrobial substances from natural sources, including lichens, has gained importance in pharmaceutical companies (Rankovic et al., 2015)

Since lichens produce a variety of substances with antimicrobial properties, it is expected that screening programs discover candidate compounds for the development of new antibiotics. However, scientific research to determine the therapeutic potential of lichens is limited, and there is a lack of scientific studies that confirm the possible experimental antibiotic properties of a large number of lichens. It is expected that compounds that reach targets different from those used by known antibiotics may be active against resistant pathogens (Rankovic et al., 2015).

The probable mechanisms of antimicrobial action of lichens are: inhibition of cell wall synthesis, inhibition of protein synthesis (translation), alteration of cell membranes, inhibition of nucleic acid synthesis and antimetabolite activity.

The screening of lichen extracts has been of great interest to scientists for the discovery of new compound effective in the treatment of microbial infection. There are various reports on the antimicrobial activity of crude lichen extracts. Rankovic et al. (2007a,b) tested water, acetone, and methanol extracts of *Cladonia furcata*, *Parmelia caperata*, *Parmelia pertusa*, *Hypogymnia physodes*, *Umbilicaria polyphylla*, *Lasallia pustulata*, *Parmelia sulcata*, *Umbilicaria crustulosa*, and *Umbilicaria cylindrical* from Serbia on six species of bacteria and ten species of fungi. The strongest activity was exhibited with methanol extracts of *Parmelia pertusa* and *Parmelia sulcata*, and the weakest activity was manifested by *Parmelia caperata* and *Umbilicaria cylindrica*. Aqueous extracts of all tested lichen species

were inactive. *Bacillus mycoides* was the most sensitive bacterial species tested, whereas *Candida albicans* was the most sensitive fungal species examined.

Karagöz et al. (2009) evaluated the aqueous and ethanol extracts of 11 different species from Turkey and determined potent antibacterial activity of aqueous extract of *Peltigera polydactyla* and ethanol extract of *Ramalina farinacea*. According to Schmeda-Hirschmann et al. (2008), dichloromethane and methanol extracts of *Protosnea poeppigii* had strong antifungal effects against the fungal pathogens *Microsporum gypseum*, *Trichophyton mentagrophytes*, and *T. rubrum*.

1.3.2 Antioxidant Activity

Free radicals (reactive oxygen species, such as the hydroxyl radical, superoxide anion and hydrogen peroxide, and reactive nitrogen species, such as nitric oxide and peroxynitrite) play an important role in many chemical processes in the cells. At low or moderate concentrations, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are necessary for the maturation process of cellular structures and can act as weapons for the host defence system (Sangameswaran et al., 2009; Huda-Faujan et al., 2009). Under normal conditions, the balance between the generation and diminution of ROS is controlled by the antioxidant defence system.

When produced in excess, free radicals and oxidants generate a phenomenon called oxidative stress, a deleterious process that can seriously alter the cell membranes and other structures such as proteins, lipids, lipoproteins and deoxyribonucleic acid (Betteridge, 2000). If not regulated properly, oxidative stress can induce a variety of chronic and degenerative diseases such as Alzheimer's disease, atherosclerosis, emphysema, hemochromatosis, many forms of cancer (e.g. melanoma, Parkinson's disease and schizophrenia) as well as the ageing process (Fig. 4.2) (Sangameswaran et al., 2008; Sachindra et al., 2010; Rankovic et al., 2015).

In addition, in order to protect biomolecules against the attack of ROS and/or to suppress the resultant damage, synthetic antioxidants have been used for industrial processing in recent years. However, since synthetic antioxidants are often

carcinogenic, finding natural substitutes is of great interest (Zhang et al. 2009). Concerns regarding toxicological effects and carcinogenic potential of synthetic antioxidants have prompted the need for natural alternatives in the last few decades (Es-Safi et al. 2006; Rankovic et al., 2015).

Lichens are proven to be good source of antioxidants, and a plenty of literatures have supported the antioxidant action of these organisms. Few reports concerning the antioxidative nature of pure lichen metabolites are available in the literature most of the publications describe the antioxidant activities of crude lichen extract (Rankovic et al., 2015).

Acetone, methanol and aqueous extracts of the lichens *Cetraria islandica*, *Lecanora atra*, *Parmelia pertusa*, *Pseudevernia furfuracea* and *Umbilicaria cylindrica* were found to possess effective antioxidant activities (Kosanic' and Rankovic', 2011). Antioxidant activities of the tested extracts were studied by DPPH radical scavenging, superoxide anion radical scavenging and reducing power. The DPPH radical scavenging activity for studied species ranged from 32.68 to 94.70 %. For reducing power, measured values of absorbance varied from 0.016 to 0.109. The superoxide anion scavenging activity for different extracts was 7.31– 84.51 %. In addition, the high contents of total phenolic compounds suggest that phenols might be the major antioxidant compounds in tested extracts. Sisodia et al. (2013) evaluated antioxidant activity for different extract of *Ramalina roesleri* species and found that the DPPH radical scavenging activity of extracts ranged from 29.42 to 87.90 %. Manojlovic et al. (2010) explored antioxidant properties of *Laurera benguelensis* by scavenging of DPPH radical and found that this lichen provided a weak radical scavenging activity.

1.3.3 Anticancer Activity

Cancer is the life threatening and dreadful disease characterized by the abnormal proliferation of cells that invade the adjacent tissues and causes the destruction of these tissues (Gennari et al., 2007). Cancer spreads to distant organs through blood stream and lymphatic vessels. It results when disturbance occurs in

two types of genes i.e., oncogenes responsible for the growth of cancer cells, and tumor suppressor genes which prevent cancer from developing (Knudson et al., 2001). It is often impossible to evaluate the specific cause for specific cancer. However there are certain factors known to increase the risk of cancer such as tobacco use, alcohol, environmental pollutant, infectious agents, custom habits and life styles. Approximately 10% to 15% cancer is entirely hereditary (Anand et al., 2008).

Cancers are named according to the type of cell the tumor resembles, for example carcinoma if they arise from the epithelial cell lining, sarcoma if they arise from mesodermal cell lining mean cancer of muscles, bones, cartilage and connective tissue, lymphoma if they arise from cells of immune system, leukemia if they arise from cells of bone marrow (Sultana et al., 2014).

Cancer is the serious health issue of both developed and developing countries. In 2012, there were an estimated 14.1 million cases around the world, of these 7.4 million cases were in men and 6.4 million women. This number is expected to increase to 24 million by 2035 (Sultana et al., 2014). Chemotherapy as well as conventional treatment for the cure of cancer causes the adverse and toxic side effects therefore fails to control the cancer disease. The need for novel chemotherapeutics has promoted a search for alternative cancer treatments which are more effective but have fewer side effects. Natural products, derived from plants, microbes, fungi, and marine have proven to be promising sources of anticancer drugs (Mondal et al., 2012). Over 60% of the current anticancer drugs have been developed from natural sources (Cragg and Newman, 2009). However, there are still many unexamined natural sources that merit further investigation. Among them are lichens.

The methanolic extract of *H. physodes* reduced cell viability in dose dependent manner. The IC_{50} values for MCF-7 (Human breast cancer cell lines) and MDA-MB-231 (Human breast cancer cell lines) were 50 and 441 μ g/ml, respectively. Apoptosis was observed only in MCF-7 but not in MDA-MB-231 as it was found M30-antigen level increased in MCF-7 but remained unchanged in MDA-MB-231. Nuclear fragmentation was also observed in MCF-7 further confirming apoptosis. It

was further found that *H. physodes* was genotoxic at higher concentrations (250 and 500 µg/ml) (Ari et al., 2012).

1.3.4 Allelopathic Activity

Lichen secondary metabolites can function as allelopathic agents (called allelochemicals), i.e., they may affect the development and growth of neighboring lichens, mosses and vascular plants, as well as microorganisms (Macías et al., 2007; Romagni et al., 2004; Rundel, 1978). Allelopathic compounds are released into the environment and might influence other organisms' photosynthesis, respiration, transpiration, protein and nucleic acid synthesis, ion membrane transport, and permeability (Chou, 2006; Macías et al., 2007; Molnar and Farkas, 2010).

Whiton and Lawrey (1984) found that vulpinic and evernic acids severely inhibited ascospore germination of the crustose lichens *Graphis scripta* and *Caloplaca citrina*. Atranorin had an inhibitory effect only on *C. citrina*, completely eliminating its spore germination. Neither species was affected by stictic acid.

Populations of mosses and lichens frequently occur together on rocks, soil, and trees and they compete for light, substrate, nutrients, and water (Lawrey, 1977). Lichen substances also have inhibitory effects against other cryptogams in overlapping niches, such as mosses, and might significantly influence the competitive interactions in cryptogam communities. In the Great Smoky Mountains of the eastern United States, Heilman and Sharp (1963) observed that the lichen *Thelotrema petractoides* (as *Ocellularia subtilis*) was inhibiting and overgrowing a colony of *Frullania eboraensis* on the bark of *Aesculus octandra*.

Lichens have also long been known to inhibit or greatly retard the growth of higher plants. *Cladonia stellaris* (as *C. alpestris*) and *C. rangiferina*, two common species in boreal forests, have been shown to have allelopathic effects on jack pine (*Pinus banksiana*) and white spruce (*Picea glauca*) (Fisher, 1979; Molnar and Farkas, 2010).

1.3.5 Enzyme Inhibitory Activity

1.3.5.1 Inhibition of Tyrosinase and Melanin Biosynthesis

Tyrosinase, a kind of phenol oxidases, acts as a catalyst from tyrosine to dopaquinone via dopa in the biosynthetic pathway of melanin; therefore, tyrosinase inhibitors such as ascorbate, arbutin, and kojic acid have been used as whitening agents in cosmetics (Yamamoto et al., 2015). Yamamoto et al. (1998) reviewed tyrosinase inhibitory activity of tissue cultures of lichens and tyrosinase inhibitors by screening the inhibitory activity of tyrosinase obtained from mushroom in ethanol extracts of 108 natural thalli of 91 species. Extracts of *Baeomyces placophyllus* and *Stereocaulon intermedium* showed the strong activity among them.

1.3.5.2 Inhibition of Monoamine Oxidase

Monoamine oxidase (MAO) is a key enzyme which plays an essential role in the turnover of biogenic amines. MAO inhibitors have been used for the treatment of depression, hypertension, etc. The norsolorinic acid, solorinic acid, and averantin 6-O-methyl ether from *Solorina crocea* also possess MAO inhibition properties (Okuyama et al., 1991). Screening test on MAO inhibition was performed for the methanol extracts of 30 species of natural thalli and 26 species of cultured mycobionts of lichens by Kinoshita et al. (2006). The extracts of natural thalli of *Parmeliella mariana*, *Lobaria subexornata*, and *Peltigera horizontalis* showed strong MAO inhibitory activity, while the extract of a cultured mycobiont of *Graphis scripta* showed the strong inhibition (Yamamoto et al., 2015).

1.3.5.3 Inhibition of Acetylcholinesterase (AChE)

In order to develop new and effective agents for anti-AD (Alzheimer's disease) from lichen products, both the AChE inhibitory and the neuroprotective effects were evaluated by Luo et al. (2013). The AChE inhibitory assay was

performed based on Ellman's reaction, and the neuroprotective effect was evaluated by using the MTT method on injured PC12 cells. Biruloquinone as an AChE inhibitor (IC₅₀ 27.1 µg/ml) was isolated from the extract of lichen-forming fungus *Cladonia macilenta*, which showed the most potent AChE inhibitory activity (Yamamoto et al., 2015).

1.3.6 Antiviral Activity

Antiviral agents refer to chemicals, moiety, and therapeutic substances employed for the treatment of virus infections and diseases. Therefore, viruses which cause a number of infections and diseases in plant, animals, and humans are targeted by these agents. Viruses are very tiny parasite that cannot reproduce by their own. Following infection of a host (e.g., a cell), it can direct the cell machinery to produce more viruses (Rankovic, 2015).

Antiviral agents target different antigen of the respective virus (virion) or other host cell factor to terminate or control the putative viral infection/disease. Antiviral agents have been shown to act by one or more of the following processes: (a) Inactivating extracellular virus particles (b) Prevent viral attachment and/or entry (c) Prevent replication of viral genome (d) Prevent synthesis of specific viral protein (e) Prevent assembly or release of new infectious virions (Rankovic, 2015).

The bioactive molecules occur in plants as secondary metabolites and as defense mechanisms against predation, herbivores, fungal attack, microbial invasion, and viral infection. During the past decade, potent agents have become available against viral infections, and extensive studies have shown that medicinal plants of several parts of the world contain compounds active against viruses that cause human diseases (Kott et al. 1999; Semple et al. 1998; Sindambiwe et al., 1999). Not left out of these are lichens which are known as promising sources of diverse secondary metabolites, the majority of which exhibit various biological properties including extensive antiviral properties (Molnar and Farkas, 2010; Stocker-Worgotter, 2008; Pengsuparp et al., 1995; Cohen et al., 1996; Neamati et al., 1997).

Lichens have proven a useful source of antiviral agents. Established antiviral properties have been attributed to some specific lichen secondary metabolites including usnic acid, parietin, anthraquinones, hypericin, lichenan, etc. For instance, lichenan, which is widely distributed in lichens, is demonstrated to inhibit the tobacco mosaic virus (Stubler and Buchenauer, 1996). In a screening exercise involving a total of 69 lichen species from New Zealand by Perry et al. (1999), they identified the three species *Cladia retipora*, *Pseudocyphellaria glabra*, and *P. homoeophylla* with antiviral activity. These antiviral activities were later traced to the usnic acid constituent following a bioactivity guided isolation process. Viruses strongly inhibited are the herpes simplex virus type 1 (HSV-1) and the polio type 1 virus (Rankovic, 2015).

Esimone and coworkers, in their increasing study of West African lichen species, identified the utility of *R. farinacea* derivatives against lentiviruses and adenoviruses. Esimone et al. (2005) initially showed that the ethyl-acetate-soluble fraction (ET4) from the lichen *Ramalina farinacea* inhibited the infectivity of lentiviral and adenoviral vectors, as well as wild-type HIV-1. Esimone et al. (2009) determined the antiviral activity of ET4 against other wild-type viruses, including the herpes simplex virus type 1 (HSV-1) and the respiratory syncytial virus (RSV). They found ET4 to inhibit HSV-1 and RSV potently with IC₅₀ of 6.09 and 3.65 µg/ml, respectively (Rankovic, 2015).

1.4 Biological Activities and Constituents of Lichen Species in the Presented Research

Bryoria capillaris (Ach.) Brodo & D. Hawksw. belongs to Parmeliaceae family. It is known as horsehair lichen. It showed antibacterial and antifungal activity. Also, basic lichen metabolites are fumarprotosetraric acid, alectorialic acid and barbatolic (Çobanoğlu et al., 2006) In addition, Türkez et al., (2012) observed genoprotective activity of *Bryoria capillaris*.

Ramalina fraxinea (L.) Ach. is from Ramalinaceae family. It has antibacterial and antioxidant activity. As lichen metabolites it contains usnic acid (Cansaran et al., 2008; Yamamoto et al., 2010; Şahin et al., 2015).

Evernia prunastri (L.) Ach. is placed on Parmeliaceae family. It is known as “oak moss” in folk medicine. *Evernia prunastri* is a valuable product for perfumery and the pharmaceutical industry (Podterob, 2008). About 700 tons/year of *Evernia prunastri* (“oak moss”) are being processed in the perfume industry (mainly at Grasse, France) (Huneck, 1999). Mitrovic et al. (2011) determined strong antifungal effect of *Evernia prunastri*. The cytotoxic activity of *Evernia prunastri* on two murine and four human cancer cell lines was reported by Bézivin et al. (2003). Aslan et al. (2006) revealed the antioxidant and antimicrobial properties of the *Evernia prunastri*. It has some important substances including atranorin, usnic acid, chloroatranorin and evernic acid (Kakubin et al., 2007; Kosanic et al., 2012).

Cetraria islandica (L.) Ach. belongs to Parmeliaceae family and known as “iceland moss”. It is ancient cough remedy known as “tonicum amarum” accepted as a mucilage drug (Müller, 2001). Iceland moss has long been used in tuberculosis, chronic bronchitis, diarrhea, inflammation of the throat and oral cavity, stomach diseases, gastric and duodenal ulcers, reduced acidity, poor digestion and absorption, and as a nutrient and a tonic (Podterob, 2008). It contains some bioactive metabolites such as usnic acid, fumarprotocetraric acid and protocetraric acid (Ingólfssdóttir et al., 1997). Antioxidant (Gülçin et al., 2002; Cocchiello et al., 2002; Grujicic et al., 2014),

antimicrobial (Dulger et al., 1998; Grujicic et al., 2014), and anticancer (Grujicic et al., 2014; Müller, 2001; Mitrovic, 2011) activities of *Cetraria islandica* were reported.

Anaptychia ciliaris (L.) Korb. ex A. Massal. belongs to Physciaceae family. Antibacterial (Karagöz et al., 2009; Çobanoğlu et al., 2006; Rankovic et al., 2010) and antifungal (Çobanoğlu et al., 2006; Rankovic et al., 2010), antioxidant (Rankovic et al., 2010) activities were reported. Atranorin ve zeorin are the main lichen metabolites (Çobanoğlu et al., 2006).

Pseudevernia furfuracea (L.) Zopf. is from Parmeliaceae family. Antibacterial (Türk et al., 2006; Kırmızıgül et al., 2003), antifungal (Çobanoğlu et al., 2006), antioxidant (Rankovic and Kosanic, 2011), anti-inflammatory (Güvenç et al., 2012) and antimutagenic (Türkez et al., 2010) effects of *P. furfuracea* were determined. 1900 tons/year of *Pseudevernia furfuracea* are being processed in the perfume industry (mainly at Grasse, France) (Huneck, 1999). Main secondary metabolites are; usnic acid, atranorin, evernic acid and physodic acid (Türk et al., 2006, Koparal et al., 2010).

Ramalina fastigiata (Pers.) Ach. is from Ramalinaceae family. It includes usnic acid and evernic acid (Cansaran et al., 2008; Krog and Osthagen, 1980). It showed antibacterial activity against some Gram (-) negative and Gram (+) bacteria (Cansaran et al., 2007; Şahin et al., 2015). Also, it has antioxidant activity (Şahin et al., 2015).

Evernia divaricata (L.) Ach. is member of Parmeliaceae family. Antibacterial (Aslan et al., 2006; Çobanoğlu et al., 2010; Rankovic et al., 2010), antifungal and antioxidant (Aslan et al., 2006) activities were determined. As secondary metabolites; divaricatic acid, usnic acid, atranorin ve evernic acid can be isolated from *E. divaricata* (Çobanoğlu et al., 2010; Rankovic et al., 2010).

Letharia vulpina (L.) Hue. belongs to Parmeliaceae family. It is a poisonous lichen species. The main seconder metabolite of *L. vulpina* is vulpinic acid (Lawrey, 1983; Lauterwein et al., 1995). In folk medicine, it has been used as remedies in stomach disorders and skin infections for centuries (Malhotra et al., 2008).

Kırmızıgül et al. (2003) reported that it also exhibited antibacterial and antifungal activities. According to Khanuja et al. (2007) extracts from lichen *Letharia vulpina* showed potent insecticidal activities. Extract from *Letharia vulpina* was found to inhibit tyrosinase enzyme (Higuchi et al., 1993).

Platismatia glauca (L.) W.L.Culb. & C.F.Culb. belongs to Parmaliaceae family. Caperatic acid and atranorin are found in *Platismatia glauca* (Çobanoğlu et al., 2010). Antifungal and antibacterial effects were exhibited (Çobanoğlu et al., 2010; Güllüce et al., 2006). The cytotoxic activity of *Platismatia glauca* on two murine and four human cancer cell lines was reported by Bézivin et al. (2003).

Usnea florida (L.) Weber ex Wigg. is a member of Parmaliaceae family. It has several biological activities including antioxidant (Odabaşoğlu et al., 2004), antibacterial (Cansaran et al., 2006), antifungal (Molnar and Farkas, 2010). *Usnea* species have been used in Asia, Africa and Europe for pain relief and fever control (Mitrovic et al., 2011).

Lobaria pulmonaria L. (Hoffm.). places on Lobariaceae family and known as lung worth due to its shape (Boustie ve Grube, 2005). It has been used widely in folk medicine for treatment of various diseases, such as eczema, respiratory diseases, pulmonary diseases and arthritis, as well as being used as food and cosmetics (Zeybek and John, 1992). Anti-inflammatory, antiulcer (Süleyman et al., 2003), antioxidant (Odabaşoğlu et al., 2004) antibacterial, antifungal (Çobanoğlu et al., 2006) and gastroprotective (Odabaşoğlu et al., 2009) activities were revealed. Major components of this lichen are gyrophoric acid, stictic acid and norstictic acid (Huneck and Yoshimura, 1996; Çobanoğlu et al., 2006).

Ramalina farinacea (L.) Ach., is from Ramalinaceae family. Anticancer, anti-inflammatory, antibacterial and fungicidal activities were reported (Çobanoğlu et al., 2010; Ofokansi and Esimone, 2005; Karagöz et al., 2009). An ethyl acetate-soluble fraction (ET4) of the crude methanolic extract of *Ramalina farinacea* was found to be a broad-spectrum antiviral agent against RNA (respiratory syncytal virus and HIV- 1) and DNA (adenovirus and herpes simplex virüs type 1) viruses (Esimone et al., 2009). Usnic acid, norstictic acid and protosestric acid are the main lichen metabolites (Tay et al., 2004).



Figure 1.6. *Bryoria capillaris* (<http://www.rolv.no>)



Figure 1.7. *Evernia prunastri*, Oak moss (by İsa Taş)



Figure 1.8. *Cetraria islandica*, Iceland moss (by İsa Taş)



Figure 1.9. *Ramalina fraxinea* (by İsa Taş)



Figure 1.10. *Pseudoevernia furfuracea*
(by İsa Taş)



Figure 1.11. *Letharia vulpina*, wolf lichen
(by İsa Taş)



Figure 1.12. *Usnea florida* (by İsa Taş)



Figure 1.13. *Lobaria pulmonaria*, lungwort (by İsa Taş)



Figure 1.14. *Ramalina fastigiata* (by İsa Taş)



Figure 1.15. *Anaphthycia ciliaris* (by İsa Taş)



Figure 1.16. *Ramalina farinacea*
(www.waysofenlichenment.net)



Figure 1.17. *Evernia divaricata* (by İsa Taş)



Figure 1.18. *Platismatia glauca* (www.galeria.nagrzyby.pl)

2. AIM AND SCOPE OF THE STUDY

For hundreds of years, lichens have been used in many countries as a cure for stomach diseases, diabetes, coughs, pulmonary tuberculosis, wound curing, and dermatological disease. Lichens synthesize various bioactive secondary components which exert a large variety of biological actions including antibiotic, antimycotic, antiviral, anti-inflammatory, analgesic, antipyretic, antiproliferative and cytotoxic effect. However, therapeutic potential of lichens has not yet been fully explored.

In this study, main objectives were to evaluate the lichen extracts obtained from 13 different lichens as a possible antibacterial agent against human bacterial pathogens; to explore the new natural antioxidant sources to protect the body from oxidative stress; to screen antiproliferative activity against cytotoxic cell lines. Also, the aim of the present work was to identify and quantify secondary metabolite compositions of tested lichens by HPLC-DAD.

3. MATERIAL AND METHODS

3.1 Lichen Material and Extraction

Lichen materials (*Ramalina fraxinea*, *Ramalina farinacea*, *Bryoria capillaris*, *Evernia prunastri*, *Ramalina fastigiata*, *Evernia divaricata*, *Anapthygia ciliaris*, *Pseudoevernia furfuracea*, *Lobaria pulmonaria*, *Platismatia glauca*, *Cetraria islandica*, *Letharia vulpina* and *Usnea florida*) were collected from different provinces (Lake Abant Natural Park, Aladağlar province, Lake Seben province, Yedigöller National Park, Lake Gölcük Natural Park) of Bolu/Turkey between 2012-2014 years. All samples identified by Assoc. Prof. Dr. Gülşah Özyüğütoğlu and voucher specimens were deposited at the Abant İzzet Baysal University Herbarium, İstanbul/Turkey.

Collected lichens were dried in a room avoiding sun light and then ground into a powder. Each lichen was extracted by three different solvents (water, methanol and acetone). For water (W) extracts, about ten grams of each lichen species were extracted with 200 ml water at 40 °C in a water bath for 24 hours. For methanol (M) extracts, ten grams of each lichen species have been extracted with 200 ml methanol at 45 °C in a water bath for 12 hours. The extracts were evaporated under low pressure at a temperature not higher than 45 °C using rotary evaporator. In acetone (A) extractions, about 10 grams of each lichen sample were extracted by soxhlet with 200 mL of acetone at 50°C for 12 hours and then filtered. Filtrates were evaporated under vacuum using rotary evaporator to give the crude extracts. Lichen materials, their designations, locations and extraction yields are represented in Table 3.1.

Table 3.1. Tested lichen species, localities, extraction types, designation and yields.

Lichen Species	Location	Coordinate	Altitude	Collection number	Extract	Designation	Yield (%)
<i>Bryoria capillaris</i>	Aladağlar Province	40°36.014 N, 031°16.655 E	1353 m	isa1006	Methanol	1M	5.3
					Acetone	1A	2.3
					Water	1W	8.3
<i>Ramalina fraxinea</i>	Lake Abant Natural Park	40°36.745 N, 031°17.100 E	1330 m	isa1003	Methanol	2M	2.6
					Acetone	2A	1.6
					Water	2W	4.2
<i>Evernia prunastri</i>	Lake Golcuk Natural Park	40°39.215 N, 031°37.688 E	1233 m	isa1012	Methanol	3M	8.6
					Acetone	3A	7
					Water	3W	2.2
<i>Cetraria islandica</i>	Lake Seben Province	40°31.518 N, 031°36.880 E	1455 m	isa1009	Methanol	4M	6.7
					Acetone	4A	7.4
					Water	4W	5.9
<i>Anaptychia ciliaris</i>	Lake Abant Natural Park	40°36.589 N, 031°17.519 E	1340 m	isa1005	Methanol	5M	3.7
					Acetone	5A	1.5
					Water	5W	5.2
<i>Pseudoevernia furfuracea</i>	Lake Seben Province	40°31.421 N, 031°36.973 E	1460 m	isa1008	Methanol	6M	8.2
					Acetone	6A	3.7
					Water	6W	0.1
<i>Ramalina fastigiata</i>	Lake Abant Natural Park	40°36.562 N, 031°17.555 E	1330 m	isa1004	Methanol	7M	4
					Acetone	7A	1.4
					Water	7W	1.9

Tables 3.1. Tested lichen species, localities, extraction types, designation and yields (continued).

Lichen Species	Location	Coordinate	Altitude	Collection number	Extract	Designation	Yield (%)
<i>Evernia divaricata</i>	Lake Golcuk Natural Park	40°39.215 N, 031°37.688 E	1233 m	isa1011	Methanol	8M	10
					Acetone	8A	4
					Water	8W	7
<i>Letharia vulpina</i>	Aladaglar Province	40°36.014 N, 031°16.655 E	1353 m	isa1007	Methanol	9M	8.4
					Acetone	9A	3.8
					Water	9W	2.6
<i>Platismatia glauca</i>	Lake Seben Province	40°31.684 N, 031°36.805 E	1451 m	isa1010	Methanol	10M	3.3
					Acetone	10A	2.2
					Water	10W	0.8
<i>Usnea florida</i>	Lake Abant Natural Park	40°36.589 N, 031°17.519 E	1341 m	isa1001	Methanol	11M	4.8
					Acetone	11A	3
					Water	11W	1.8
<i>Lobaria pulmonaria</i>	Yedigoller National Park	40°56.334 N, 031°44.428 E	874 m	isa1013	Methanol	12M	12.1
					Acetone	12A	8.3
					Water	12W	3.8
<i>Ramalina farinacea</i>	Lake Abant Natural Park	40°36.745 N, 031°17.100 E	1338 m	İsa1002	Methanol	13M	4.8
					Acetone	13A	3
					Water	13W	3.5

3.2 Biological Activity of Lichen Samples

3.2.1 Antibacterial Activity

Antibacterial activities of lichens; *Bryoria capillaris* (1A,1M,1W), *Ramalina fraxinea* (2A,2M,2W), *Evernia prunastri* (3A,3M,3W), *Cetraria islandica* (4A,4M,4W), *Anaphthycia ciliaris* (5A,5M,5W), *Pseudoevernia furfuracea* (6A,6M,6W), *Ramalina fastigiata* (7A,7M,7W), *Evernia divaricata* (8A,8M,8W) (9A,9M,9W), *Platismatia glauca* (10A,10M,10W), *Usnea florida* (11A,11M,11W), *Lobaria pulmonaria* (12A,12M,12W) and *Ramalina farinacea* (13A,13M,13W) were evaluated.

The disc diffusion assay (Kirby-Bauer Method) was used to screen for antibacterial activity (Prescott et al. 1990). Ten bacterial strains were used in the bioassay: *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 14028), *Serratia marcescens* (ATCC 8100), *Proteus vulgaris* (ATCC 13315), *Enterobacter cloacae* (ATCC 23355) and *Klebsiella pneumoniae* (ATCC 13883) which are gram-negative bacteria and *Streptococcus pyogenes* (ATCC 19615), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228) which are gram-positive bacteria. Bacteria used in antibacterial assay and their representative diseases are represented in Table 3.2. (Levinson and Jawertz, 2002).

Each lyophilized bacteria discs (BD, Microtrol Discs - Becton Dickinson Laboratories, France) were transferred to test tubes containing 2 ml of Tryptic Soy Broth (TSB) and incubated overnight at 37°C. After incubation, one bacteriological loop from each broth was streaked on Tryptic Soy Agar (TSA) plates and incubated for 2 days at 37°C. After 2 days, a single colony was removed and streaked on a new TSA plate for pure culture and incubated 37°C for 2 additional days. After 2 days, 1-2 loops of pure culture were transferred to 10 ml of 9 % saline suspension in a test tube for each bacterial strain. The turbidity of the each suspension is adjusted to obtain turbidity visually comparable to that of a 0.5 McFarland standard (1.5x10⁸ cfu/ml). A sterile cotton swab was dipped into the standardized bacterial suspension

and used to evenly streak the entire surface of a (60 X 15 mm) Mueller-Hinton (M-H) agar plate (One test tube was used for 10 agar plate). Agar plates were streaked four times, each time turning the plate at a 90° angle and finally rubbing the swab through the edge of the plate.

All extracts were dissolved in sterile distilled water in order to obtain a final concentration of 100 mg/ml. Then, all dissolved extracts were sterilized by filtering through a 0.22 µm filter (Pal-Gelman Laboratory) and sterile filter paper discs (Glass Microfibre filters, Whatman®; 6 mm in diameter) were impregnated with 13 µl of extract. There were five replicates in each plate and two plates for each extract tested for each bacterium. Five different antimicrobial susceptibility test discs were used as positive controls which consist of (Bioanalyse®): Erythromycin (15 µg) (E-15), Ampicillin (10 µg) (AM-10), Carbenicillin (100µg) (CB-100), Tetracycline (30 µg) (TE-30) and Chloramphenicol (30 µg) (C-30). Five antibiotic discs were used for each plate and run in duplicate. Negative control consisted of water. Inoculated plates with discs were placed in a 37°C incubator. After 16 to 18 hrs of incubation, diameter (mm) of inhibition zone was measured. All experiments were repeated three times.

All data were analyzed by analysis of variance (ANOVA) and mean values were compared with Duncan's Multiple Range Tests using SPSS vers. 15 (SPSS Inc. Chicago, IL, USA).

Table 3.2. Bacterial strains used in antibacterial assay and their representative diseases.

	Pathogen	Representative Diseases	Habitat	Drugs of choice
Gram (-) Bacteria	<i>Escherichia coli</i>	Urinary tract infection (UTI), sepsis, neonatal meningitis, 'traveler's diarrhea'	Human colon, colonize the vagina and urethra.	Ampicillin or sulfonamides for UTI, Cephalosporin for meningitis and sepsis, trimethoprim- sulfamethoxazole for diarrhea
	<i>Proteus vulgaris</i>	Wound infection, UTI and sepsis	Human colon and environment (soil and water)	Trimethoprim-sulfamethoxazole or ampicillin, cephalosporin for serious infections.
	<i>Enterobacter cloacae</i>	Hospital-acquired pneumonia, Urinary Tract Infection, ophthalmic infections, and sepsis.	Enteric tract	Highly antibiotic-resistant.
	<i>Salmonella typhimurium</i>	Enterocolitis, diarrhea	Enteric tract	Ampicillin, ciprofloxacin or ceftriaxone
	<i>Serratia marcescens</i>	Conjunctivitis, Nosocomial infections and hospital-acquired pneumonia, Urinary Tract Infections, wound infections, and sepsis.	Enteric tract also soil and water	Gentamicine and cephalosporin
	<i>Klebsiella pneumoniae</i>	Pneumonia, Urinary Tract Infections (cytitis), wound infections, and sepsis.	Human upper respiratory and enteric tracts.	Cephalosporin or with aminoglycosides
	<i>Pseudomonas aeruginosa</i>	Wound infections, Urinary Tract Infections, pneumonia, eye infections, and sepsis.	Environmental water sources, hospital respirators and humidifiers and also skin, upper respiratory tract and colon	Antipseudomonal penicillin and aminoglycoside eg. gentamicin or amikacin
Gram (+) Bacteria	<i>Streptococcus pyogenes</i>	Suppurative (pus-producing) diseases, cellulitis, immunologic diseases such as rheumatic fever and acute glomerulonephritis	Human throat and skin	Penicilin G
	<i>Staphylococcus aureus</i>	Abscesses of many organs, endocarditis, gastroenteritis, toxic shock syndrome, sepsis, hospital-acquired pneumonia, and wound infections	Human nose and also human skin.	Penicilin G, Nafcillin
	<i>Staphylococcus epidermidis</i>	Endocarditis, neonatal sepsis, prosthetic hip infection, wound infections, and cytitis (UTI)	Human skin and mucous membranes.	Vancomycin plus either rifampin or an aminoglycoside.

3.2.2 Antioxidant Activity

3.2.2.1 Free Radical Scavenging Activity DPPH (2,2-diphenyl-picrylhydrazil) Method

Free radical scavenging activities of the methanolic extracts of lichen samples were determined spectrophotometrically by monitoring the disappearance of 2,2-diphenyl-1-picrylhydrazil (DPPH•, Sigma-Aldrich Chemie, Steinheim, Germany) at 517 nm, according to the method described by Brand-Williams et al. (1995).

All methanolic extracts of lichen samples were weighted as 0.003 g (1M, 2M, 3M, 4M, 5M, 6M, 7M, 8M, 9M, 10M, 11M, 12M and 13M) and they were dissolved in 3 ml of methanol. Finally, these stock solutions were composed as 1000 µg/ml concentration. The extract solutions were diluted with methanol (25, 50, 100, 200 µg/ml) from the stock extract solutions. Ascorbic acid was used and a standard curve was prepared using different concentrations of ascorbic acid (200, 100, 50, 25 and 12.5 µg/ml).

0.1 mM solution of DPPH• was prepared in methanol by daily before UV measurements. 1.5 ml of the diluted samples or ascorbic acid (200, 100, 50, 25 and 12.5 µg/ml) was mixed vigorously with 0.5 ml of 0.1 mM DPPH in methanol. These solutions were shaken vigorously and incubated at room temperature in dark for 30 minutes. 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. The blank sample includes only 1.5 ml of the methanol and 0.5 ml of the 0.1 mM DPPH which was used as a positive control (Milardovic et al., 2006; Brand-Williams et al., 1995). All analyses were made in triplicate. The DPPH• scavenging capacity of the extracts was calculated using the following equation:

DPPH• Scavenging Effect (% inhibition) = $[(A_0 - A_1/A_0) \times 100]$ (Gülçin et al., 2003), where A_0 is the absorbance of the positive control

A_1 is the absorbance of tested extracts.

3.2.2.2 Total Phenolic Assay (Folin-Ciocalteu Method For Total Phenolic Content)

Total soluble phenolics in the methanolic extracts of lichen samples were determined with Folin-Ciocalteu reagent according to the method of Slinkard and Singleton (1977) using gallic acid as a standard phenolic compound.

Preparation of extract solution: 0.01 g each methanolic extract of lichen samples (1M, 2M, 3M, 4M, 5M, 6M, 7M, 8M, 9M, 10M, 11M, 12M and 13M) was weighted. Then, 2 ml of distilled water was added to extracts. These extract solutions were stock solution (5 mg/ml). Later, these solutions were diluted as 1 mg/ml.

Preparation of gallic acid stock solution: Gallic acid was used as phenol control. In a 100 ml volumetric flask, 0.5 g of dry gallic acid was dissolved in 100 ml distilled water (5 mg/ml) and diluted as 0, 50, 100, 150, 200, 250 and 500 mg/ml gallic acid. Then, a calibration curve was obtained by using these gallic acid solutions to determine phenol concentration of lichen extracts.

Na₂CO₃ Solution: 20 g of anhydrous sodium carbonate was dissolved in 100 ml of water in a 100 ml volumetric flask.

20 µl from each calibration solution (gallic acid), sample, or blank (water) was added into separate centrifuge tubes, and 1.58 ml water was added into each, and then 100 µl of the Folin-Ciocalteu reagent was added and mixed well. After 2 minutes, 300 µl of Na₂CO₃ solution was added and shaken very well. The solutions were incubated at 200C for 2 hours and the absorbance of each solutions were measured at 765 nm against the 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 concentration of phenolic compounds in the samples was determined as microgram of gallic acid equivalent by using an equation that was obtained from standard gallic acid graph. The results were expressed in mg of gallic acid per g (GEA) of dry weight of lichen. All analyses were made in triplicate.

3.2.2.3 Total Flavonoid Assay (Aluminum chloride (AlCl₃) Colorimetric Assay For Total Flavonoids)

The total flavonoid contents in methanolic extracts of lichens (1M, 2M, 3M, 4M, 5M, 6M, 7M, 8M, 9M, 10M, 11M, 12M and 13M) were measured by aluminum chloride (AlCl₃) colorimetric assay. Catechol was used as a reference flavonoid.

The preparation of extract solution: 0.005 g each methanolic extract of tested lichens was weighted and dissolved in 2ml ethanol (2500 mg/ml). Later, this stock solution will be diluted to 1250 mg/ml and 500 mg/ml concentrations.

The catechol solution preparation: 0.0125 g of catechol was weighted. Then, it was dissolved in 25 ml ethanol. Thus, this stock solution was adjusted as 500 mg/ml concentration. Different concentrations of catechol (20, 40, 60, 80 and 100 mg/ml) were prepared in order to obtain standard calibration curve of catechol.

500 µl of extract solution or standard solution of catechol was added to a 10 ml test tube containing 2 ml distilled water. Then, 150 µl 5% NaNO₂ was added to the test tubes. After 5 minutes, 150 µl of 10 % AlCl₃ was added. 1 minute later, 1000 µl of 1M NaOH was added to the mixture. Immediately, the reaction tube was diluted to volume 5 ml with the addition of 1200 µl distilled water and thoroughly mixed. Absorbance of the mixture was determined at 510 nm versus a blank. Samples were analyzed in three replications (Chang et al., 2002). The total flavonoid contents of lichen samples were expressed as mg catechol equivalents (CE) of dried weight of lichen.

3.2.3 Antiproliferation Activity

Anticancer activities of 18 different extracts (2A,2M,2W; 4A,4M,4W; 9A,9M,9W; 10A,10M,10W; 11A,11M,11W; 12A,12M,12W) were evaluated.

Anticancer activity test of lichen extracts were carried out on Human breast adenocarcinoma (MCF-7) and human hepatocellular carcinoma (HepG2/C3A). Cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). This cell lines were continuously maintained from the stock cultured at Abant Izzet Baysal University, Faculty of Medicine, Department of Pharmacology. The cells were maintained in Dulbecco's modified eagle's medium (DMEM, Invitrogen) containing 10% fetal bovine serum (FBS) and 100 ng/ml of penicillin and streptomycin (Sigma). Cells were allowed to grow in tissue culture flasks and were kept in CO₂ incubator at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. For experimental purpose, cells from exponentially growing culture were used. All experiments were repeated three times.

The MTT (3-[4-5 dimethylthiozol-2-yl] 2-5 diphenyl-tetrazolium bromide) assay was used in this study to assess growth inhibition. For the assay, the MCF-7 cells from exponential-phase culture were detached with 0.1% trypsin-EDTA (Sigma Chemical Co.) to make single-cell suspensions, and viable cells were counted using a Coulter counter. About 1×10^4 viable cells in 100 μ l of culture medium (DMEM) were added to each well in of a 96-well cell culture plate and incubated for 24 h at 37 °C under 5 % CO₂ in a humidified incubator to allow cells to attach to wells of 96 well cell culture plates. After 24 h or 48 h, the cell attachment was checked and the cells were treated with the serial concentrations of lichen extracts and then incubated for 24 h or 48 h.

Acetone and methanolic extracts were initially dissolved in an amount of 100 % DMSO. Aqueous extracts were initially dissolved in an amount of water (80 mg/ml) and further diluted in medium to produce six concentrations. 100 μ l per well of each concentration was added to the plates to obtain final concentration of 200, 100, 50, 25, 12.5 and 6.25 μ g/ml for the extracts. The final concentration of DMSO for the tested dilutions was not greater than 0.25 % (v/v) to avoid solvent toxicity, the

same as in solvent control wells. The final volume in each well was 200 μ l. The plates were incubated for 24 h or 48 h.

After incubation of 24 h or 48 h, the culture medium was removed and replaced with 90 μ l fresh culture medium, DMEM. Then, 10 μ l of MTT solution (5 mg/ml) in phosphate buffered saline (PBS, pH 7.4) was added to each well and the final concentration of MTT reached 0.5 mg/l. The culture was incubated at 37 °C in 5% CO₂ for additional 4 h. After 4 h, 100 μ l DMSO/well were added to all samples for dissolving of formazan that is the final product of MTT reaction and were allowed to incubate at 37 °C, in a 5% CO₂ humidified incubator for a night. After incubation, absorbance of formazan was measured spectrophotometrically in a Multiskan FC microplate photometer reader (Thermo Fisher Scientific Inc.) at 570 nm. Each experiment was carried out three times in triplicate. The percent cytotoxicity of extracts (cell viability) was calculated according to their control groups as;

$$100 \times A_{570 \text{ nm}} (\text{sample}) / A_{570 \text{ nm}} (\text{control}).$$

Quantitative data were expressed as mean absorbance $\pm$ S.E.M. The significance of differences between the means was assessed using one-way ANOVA using SPSS 15 software.

3.3 Chemical Analysis of the Lichen Samples

3.3.1 High Pressure Liquid Chromatography (HPLC)

Methanol and acetone extracts were analyzed using a HPLC system (VWR-Hitachi LaChrom Elite®) equipped with a Hitachi L-2455 Diode-Array Detector (DAD), Hitachi L-2130 Pump, Hitachi L-2200 Autosampler. Chromatographic separation was achieved using Hitachi column oven L-2300 and Venusil XBP C18 column (Bonna-Agela Technologies, particle size 5 μm , 4.6 x 250 mm). Flow rate was 1 ml/min with 25 °C oven and injection volume was 20 μl . All solvents were HPLC grade (Merck) and mobile phase was composed of methanol/water/phosphoric acid (90/10/0.9 v/v/v) (Kosanic et al. 2014). Mobile phases and ultrapure water (SG Labostar) were filtered through a 0.45 μm hydrophilic polypropylene membrane filter (47 mm) (Pall Corporation) prior to HPLC injection. Spectra data were recorded from 200 to 400 nm during the entire run. The chromatograms were obtained at 254 nm.

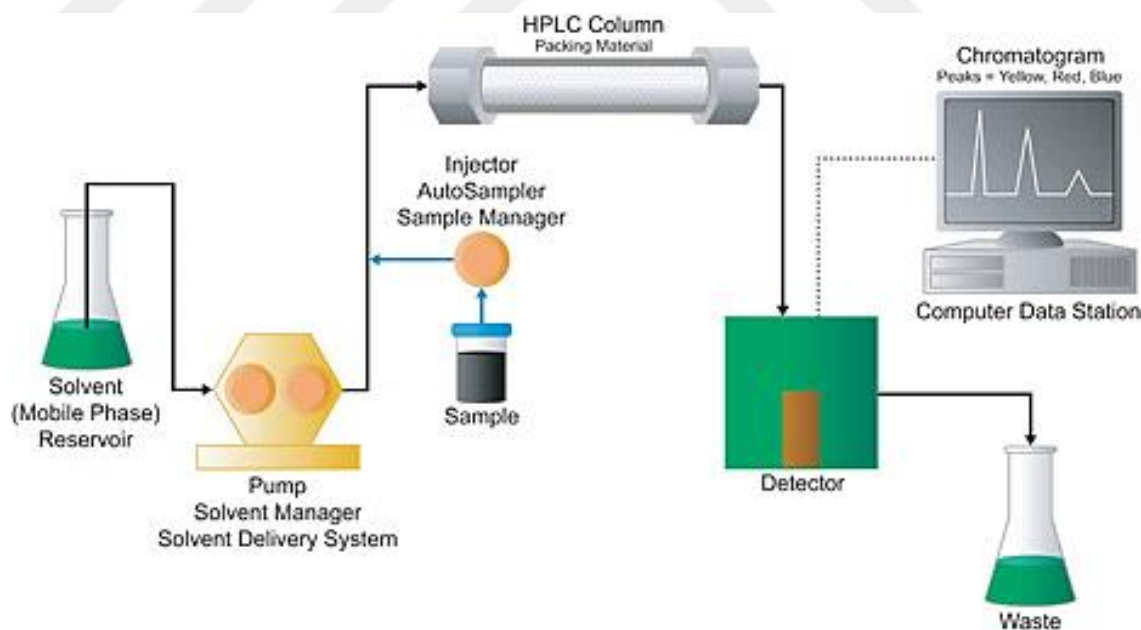


Figure 3.1. High-Performance Liquid Chromatography (HPLC) System (www.waters.com).

3.3.1.1 Sample Preparation for HPLC Analysis

MeOH and acetone extracts of all lichen samples (10 mg) were dissolved in 10 ml MeOH (1 mg/ml) in volumetric flasks. Five different lichen substances were used as reference standards which consist of usnic acid (Sigma[®]), evernic acid (Sigma[®]), Stictic acid (Chromadex[®]), Atranorin (Chromadex[®]) and Fumarprotocetraric acid (BOC sciences[®]). All standards were separately prepared at 1 mg/ml in MeOH (stictic acid, evernic acid and usnic acid) or acetone (atranorin and fumarprotocetraric acid) and diluted five different concentrations (6,25, 12,5, 25, 50, 100 and 200 µg/ml) to calibrate the standard curve. All extracts and standards were filtered through a 0.2 µm GHP Acrodisc (25 mm) (Pall Corporation) into 2 ml HPLC vials. Procedures were repeated 3 times for each sample tested.

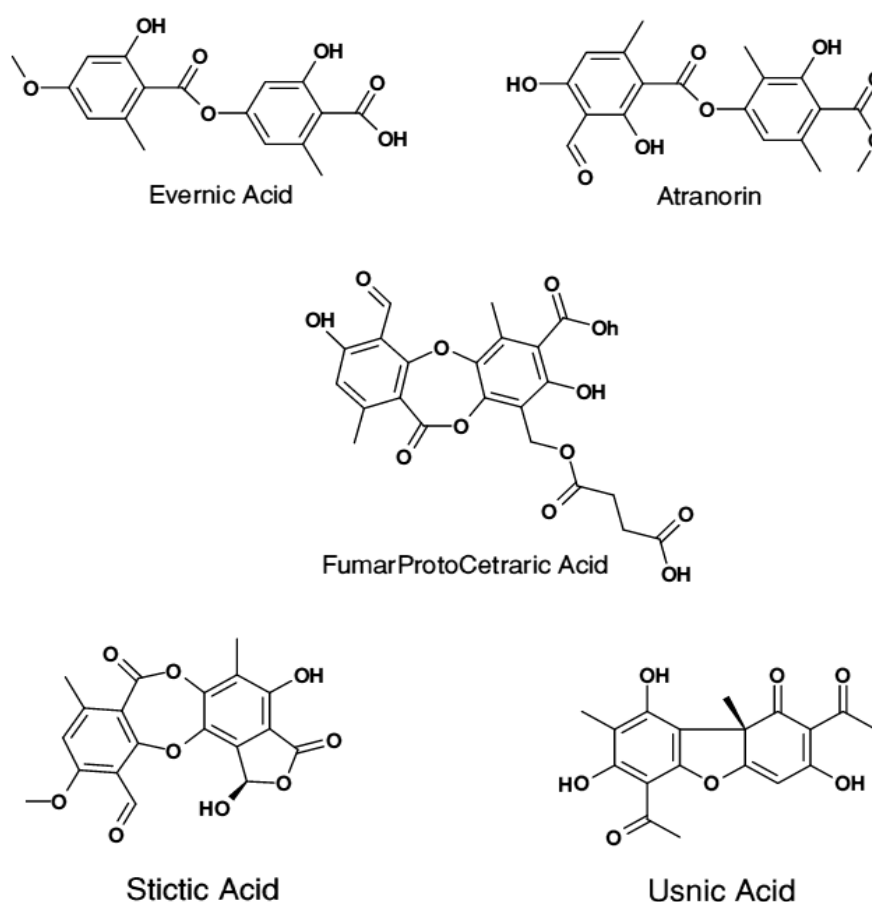


Figure 3.2. Structure of lichen compounds used as standard in HPLC-DAD.

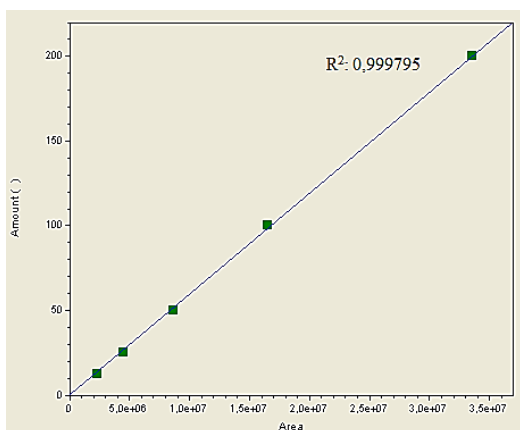


Figure 3.3. Calibration curve of usnic acid.

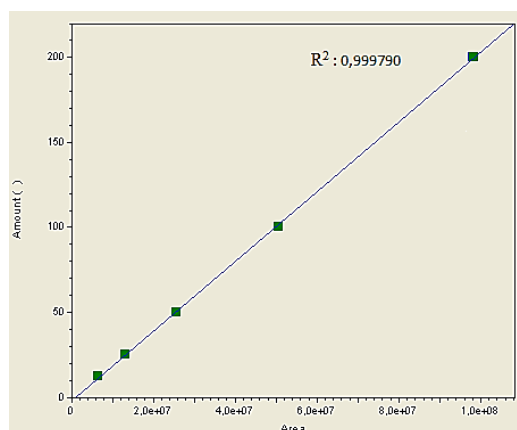


Figure 3.4. Calibration curve of atranorin.

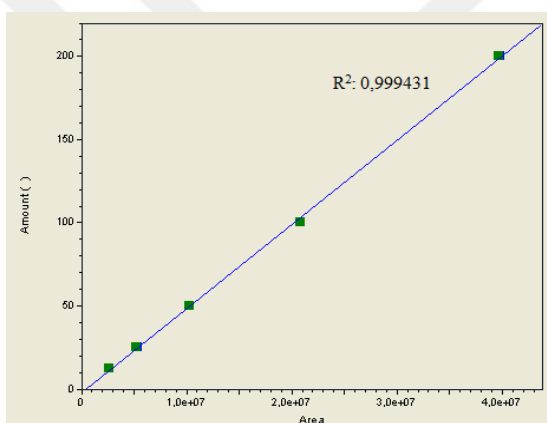


Figure 3.5. Calibration curve of stictic acid.

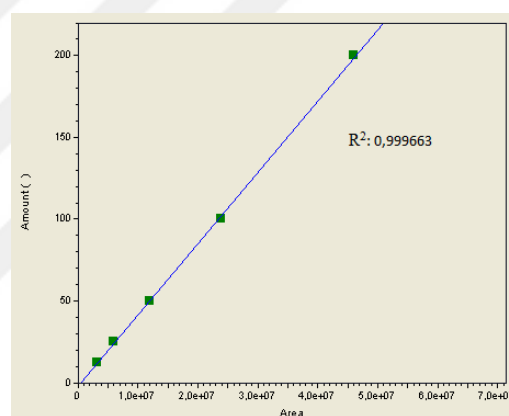


Figure 3.6. Calibration curve of evernic acid.

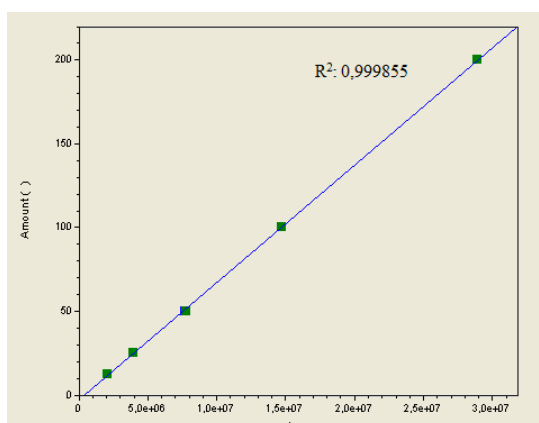


Figure 3.7. Calibration curve of fumarprotocetraric acid.

4. RESULT AND DISCUSSION

4.1 Biological Activity of Lichen Samples

4.1.1 Antibacterial activity

The diameters of inhibition zone for acetone, methanol and aqueous extractions of the 13 lichen species against ten different bacterial strains were reported in Table 4.1. Generally, Gram-positive (+) bacterial strains were more susceptible to the inhibitory effects of the lichens than Gram-negative (-) bacterial strains (Table 3.3). The change of sensitivity between Gram positive (+) and negative (-) bacteria could be described as structural differences between these organisms. Gram-positive (+) bacteria includes peptidoglycans and teichoic acids, while Gram negative bacteria (-) consists of peptidoglycans, lipopolysaccharides and lipoproteins in the cell wall (Nostro et al., 2000). Positive controls; antibiotics exhibited antibacterial activity to all tested strains. Since final concentration of all extracts were adjusted with distilled water. It was used as negative control and didn't show any activity against ten bacterial strains.

Among the all lichen extracts, methanol extracts had better antibacterial activity than acetone and aqueous extracts. The best inhibitory activity was obtained with methanol extracts of *Ramalina fraxinea* (2M) against eight out of ten tested bacterial strains in the range of 9.5 ± 0.3 – 30.5 ± 1.3 mm in diameter. The weakest antibacterial activity was showed by methanol extract of *Anaphthycia ciliaris* (5M) against only two strains; *S.epidermidis* (10.8 ± 0.5 mm) and *S.pyogenes* (12.3 ± 0.6 mm). Except *E. divaricata*, *U. florida* and *R. farinacea*, water extracts of all lichen species didn't exhibit any activity against bacterial strains (Table 4.1).

Among the extracts of *Bryoria capillaris*, acetone extract (1A) had antibacterial activity against eight bacterial strains with the inhibition zones between 8.0 ± 0.4 mm – 19.0 ± 0.4 mm. Only five bacteria were inhibited by methanol extract

(1M) of *B. capillaris*, while there was no effect of water extract (1W) against neither Gram positive(+) nor Gram negative (-) bacteria. The acetone extract (1A) showed higher inhibition zone (14.8 ± 0.3 mm) than reference antibiotic Erythromycin in the case of *P. vulgaris* (13.6 ± 0.3 mm). Also, it exhibited one of the highest antibacterial effects against *S. marcescens* with the inhibition zone of 9.5 ± 0.5 mm. While *S. epidermidis* was the most susceptible bacteria with the inhibition zones of 19 ± 0.4 mm, *K. pneumonia* and *E. coli* had no susceptibility against any extracts of *B. capillaris* (Table 4.1). Antibacterial activity of *Bryoria capillaris* against *S. aureus*, *S. epidermidis*, *S. pyogenes*, *S. typhimurium*, *P. aereginosa*, *P. vulgaris* and *E. cloaca* was evaluated for the first time in this study.

Methanol extract (2M) of *Ramalina fraxinea* showed inhibitory activity against all bacterial strains except *E. coli* and *K. pneumonia*, while acetone extract (2A) exhibited the antibacterial activity against only Gram-positive (+) bacteria. Any inhibition was not obtained by water extract (2W). As compared to all the lichens, MeOH extract (2M) exhibited the best antibacterial activity against *P. aeruginosa* with the inhibition zones of 18.8 ± 0.8 mm. This result was even better than all reference antibiotics; ampicillin (no inhibition), carbenicillin (10.0 ± 0.0 mm), chloramphenicol (9.0 ± 0.0 mm), erythromycin (no inhibition), tetracycline (16.0 ± 0.0 mm). Against *E. cloacae* and *S. typhimurium*, MeOH extract (2M) demonstrated the best antibacterial activity with the 23.0 ± 0.0 mm and 14.8 ± 0.3 mm in diameters, respectively and these results were better than inhibitions of Erythromycin (9.6 ± 0.3 mm and 10.8 ± 0.5 mm, respectively) (Table 4.1). While Cansaran et al. (2007) and Şahin et al. (2007) revealed the inhibition effects against *E. coli* and *S. aureus*, it is the first report that methanol extract of *R. fraxinea* showed antibacterial activity against *S. marcescens*, *S. pyogenes*, *S. typhimurium*, *P. aereginosa*, *P. vulgaris* and *E. cloaca* in our study. Also, Yamamoto et al. (1994) determined the inhibition activity against *S. aureus* and *S. epidermidis*.

Methanol extract (3M) of *Evernia prunastri* was more effective than acetone extract (3A). While both extracts showed strong antibacterial activity against Gram positive (+) bacterial strains, they had weak effect against Gram-negative bacteria. No inhibition was obtained with water extract (3W) against Gram-positive (+) or Gram-negative (-) bacteria. Both acetone and methanol extract of *E. prunastri*

demonstrated the best activity against *S. pyogenes* (25.0 ± 1.3 and 27.5 ± 0.9 mm, respectively) and *S. epidermidis* (23.5 ± 0.5 and 24.5 ± 0.3 mm, respectively). While acetone extract didn't show any inhibition against *S. marcescens*, methanol extract had weak antibacterial activity with the inhibition zone of 7.5 ± 0.3 mm (Table 4.1). Aslan et al. (2006) demonstrated that methanol extract of *E. prunastri* had weak antibacterial activity, but we found that the methanol extract of this lichen species had inhibitory activity especially against Gram-positive bacteria (*S. aureus*, *S. epidermidis* and *S. pyogenes*).

Both acetone (4A) and methanol (4M) extracts of *Cetraria islandica* were found to be active against Gram-positive (+) bacteria; *S. aureus*, *S. epidermidis* and *S. pyogenes*, but acetone extract exhibited best antibacterial activity with the inhibition zone of 20.3 ± 0.6 , 25.5 ± 0.3 and 28.0 ± 1.2 mm, respectively. There was no effect of water extract (4W) against any kind of bacteria. Additionally to Gram-positive (+) bacteria, acetone extract of *C. islandica* showed antibacterial activity against some Gram-negative (-) bacteria; *P. aeruginosa* and *P. vulgaris* (10.0 ± 0.4 and 8.3 ± 0.3 mm, respectively). This was the second best effect against *P. aeruginosa* as compared to all lichen extracts and also better than ampicillin (no inhibition), chloramphenicol (9.0 ± 0.0 mm) and erythromycin (no inhibition) (Table 4.1). Dülger et al. (1998) revealed ethyl acetate, acetone, chloroform and ethanol extracts of *C. islandica* against different microorganism by disc diffusion method. They found that *C. islandica* exhibited antibacterial activity against some Gram positive bacterial strain, but had no activity against Gram negative bacteria. However, we determined the antibacterial activity of acetone extract of *C. islandica* against *P. aeruginosa* and *P. vulgaris*.

Among the extract of *Anaphthycia ciliaris*, acetone extract (5A) showed better activity than methanol extract (5M) against Gram positive (+) bacteria. None of the Gram-negative (-) bacterial strain was susceptible to *A. ciliaris*. Also, water extract (5W) didn't demonstrate antibacterial activity against neither Gram-negative (-) nor Gram-positive (+). Methanol extract of *A. ciliaris* showed antibacterial activity against *S. epidermidis* (10.8 ± 0.5 mm) and *S. pyogenes* (12.3 ± 0.6 mm), while acetone extract showed antibacterial activity against *S. aureus* (9.5 ± 0.3 mm), *S. epidermidis* (8.3 ± 0.3 mm) and *S. pyogenes* (14.8 ± 0.3 mm). Methanol extract had the weakest

antibacterial activity as compared to all the lichen samples (Table 4.1). Rankovic et al. (2010) reported that the methanol extract of *Anaptychia ciliaris* had moderate antibacterial activity, inhibited the growth of five out of six bacterial strains. However, we couldn't find similar effect in our study.

Both acetone (6A) and methanol (6M) extracts of *Pseudoevernia furfuracea* were found to have strong activity against Gram positive (+) bacteria. Water extract (6W) didn't exhibit any activity against neither Gram positive (+) nor Gram negative (-) bacteria. Against *S. aureus* and *S. epidermidis*, acetone extract showed better antibacterial effect (22.3 ± 0.5 mm and 24.5 ± 0.3 mm, respectively) than methanol extract (20.3 ± 0.3 and 22.5 ± 0.3 mm, respectively). But against *S. pyogenes*, methanol extract (30.5 ± 0.5 mm) was more effective as compared to acetone extract (27.0 ± 1.3 mm). There was no inhibition against Gram negative (-) bacteria except the inhibition of the methanol extract to *P. vulgaris* with inhibition zone of 13.5 ± 0.3 mm (Table 4.1). In previous study, *P. furfuracea* exhibited antibacterial activity against *E. coli*, *K. pneumonia*, *S. aureus* and *P. vulgaris* (Kosanovic et al., 2013; Güvenç et al., 2012; Türk et al., 2006). Additionally these bacterial strains, *S. epidermidis* and *S. pyogenes* were inhibited by both methanol and acetone extract of *P. furfuracea* for the first time in the present study.

Among the all extracts of *Ramalina fastigiata*, methanol extract (7M) showed a significantly higher antibacterial activity than acetone extract (7A). Both extracts showed strong antibacterial activity against especially Gram positive (+) bacteria. Water extract (7W) didn't exhibit any activity against neither Gram positive (+) nor Gram negative (-) bacteria. The inhibition zone of acetone and methanol extracts were 19.3 ± 0.3 mm and 16.5 ± 0.3 mm, respectively against *S. aureus*, while 26.0 ± 0.4 mm and 29.5 ± 0.5 mm, respectively against *S. epidermidis*. Also, they showed antibacterial activity against *S. pyogenes* (29.5 ± 0.5 mm and 29.3 ± 0.5 mm, respectively). Methanol extract of *R. fastigiata* also demonstrated the inhibition activity against Gram negative (-) bacteria; *P. vulgaris* (11.3 ± 0.3 mm), *K. pneumonia* (13.3 ± 0.3 mm) and *E. cloacea* (19.3 ± 0.5 mm). *K. pneumonia* was only inhibited by methanol extract of *R. fastigiata* as compared to all the lichen samples. It also exhibited better activity than some reference antibiotics; ampicillin (8.4 ± 1.1 mm), carbenicillin (8.0 ± 1.4 mm) and erythromycin (13.0 ± 0.0 mm) (Table 4.1). Cansaran

et al. (2007) and Şahin et al. (2015) revealed that acetone extract of *R. fastigiata* exhibited the effect against *E. coli* and *S. aureus*. In addition to these results, we revealed the antibacterial activity against *S. epidermidis*, *S. pyogenes*, *P. vulgaris*, *K. pneumonia* and *E. cloacae* for the first time.

Both acetone (8A) and methanol (8M) extracts of *Evernia divaricata* showed relatively strong antibacterial activity against Gram positive (+) bacterial strains; *S. aureus*, *S. epidermidis* and *S. pyogenes*. While acetone extract manifested antibacterial activity with the inhibition zones of 20.0 ± 0.0 mm, 31.0 ± 0.6 mm and 32.0 ± 0.0 mm, respectively, methanol extract exhibited with the inhibition zone of 20.5 ± 0.3 mm, 30.3 ± 0.3 mm and 35.0 ± 0.0 mm (better than chloramphenicol), respectively. Water extract (8W) showed weak inhibition activity against *S. aureus* (7.5 ± 0.3 mm) and *S. epidermidis* (8.3 ± 0.5 mm). Gram negative (-) bacterial strains was resistant against all extracts of *E. divaricata* (Table 4.1). Antibacterial activity of *E. divaricata* against *S. aureus*, *S. epidermidis*, *S. pyogenes*, *P. vulgaris*, *P. aeruginosa*, *K. pneumonia* and *E. coli* was reported by previous studies (Aslan et al., 2006; Çobanoğlu et al., 2010; Zhao et al., 2010). However, our results showed that *E. divaricata* exhibited the strong inhibition effect against only Gram positive (+) bacterial strains.

Acetone extract (9A) of *Letharia vulpina* inhibited the growth of the 4 bacterial strains, while methanol extract (9M) showed antibacterial activity against the growth of 3 bacterial strains. No inhibition was obtained by water extract (9W). Methanol extract only inhibited the growth of *P. vulgaris* (10.0 ± 0.0 mm) as a Gram negative (-) bacteria. Acetone extract showed antibacterial activity against *P. vulgaris* (10.5 ± 0.5 mm) and *E. cloacae* (7.5 ± 0.3 mm). Among the all lichens tested, acetone extract of *L. vulpina* exhibited the strongest antibacterial activity against *S. aureus* with the inhibition zone of 31.5 ± 0.5 mm. This value was even better than reference antibiotics; chloramphenicol (25.0 ± 0.0 mm) and erythromycin (30.0 ± 0.0 mm). Only *L. vulpina* couldn't demonstrate inhibition activity against *S. pyogenes*, all other species inhibited the growth with effective zones (Table 4.1). Kırmızıgül et al. (2003) determined the inhibitory effect of methanol extract against *S. aureus*. In addition to this bacterium, *S. epidermidis* and *P. vulgaris* were inhibited by methanol extract of *L. vulpina* for the first time in this research.

Although both the acetone (10A) and methanol (10M) extractions of *Platismatia glauca* exhibited activity against all of the Gram positive (+) bacterial strains, the acetone extractions was more active than methanol extraction. Water extract (10W) had no antibacterial activity. Gram negative (-) bacterial strains were resistant to all extracts of *P. glauca*. Against *S. aureus* and *S. epidermidis*, acetone extract showed best antibacterial activity with the inhibition zone of 12.0 ± 0.6 mm and 13.8 ± 0.5 mm, respectively while methanol extract demonstrated the best activity against *S. pyogenes* (19.5 ± 5 mm) (Table 4.1). Mitrovic et al. (2014) reported that *P. glauca* exhibited inhibition effect against *S. aureus*, *P. aurescens*, *E. coli* and *S. typhimurium*. Also, we demonstrated the antibacterial activity of *P. glauca* against only Gram positive strains. The inhibition of *S. epidermidis* and *S. pyogenes* was investigated for the first time with this study.

While Gram positive (+) bacteria were inhibited by all extracts of *Usnea florida*, none of the extracts didn't show antibacterial activity against Gram negative (-) bacterial strains. Methanol extract (11M) demonstrated the strongest inhibition activity against *S. aureus* (14.5 ± 0.3 mm) and *S. epidermidis* (31.5 ± 0.5 mm). Against *S. pyogenes*, acetone extract (11A) exhibited the best antibacterial activity with 27.0 ± 0.6 mm in diameters. Water extract (11W) was active less than the other extracts against *S. aureus*, *S. epidermidis* and *S. pyogenes* with the inhibition zones of 6.8 ± 0.3 mm, 17.5 ± 0.3 mm and 22.5 ± 1.3 mm, respectively. Observed antibacterial activity of methanol extract against *S. epidermidis* was the best activity when compared the all other lichen species. Also, it was better than some of the reference antibiotics; ampicillin (31.0 ± 0.0 mm) and tetracycline (8.0 ± 0.0 mm) (Table 4.1). Cansaran et al. (2006) determined the antibacterial activity of *U. florida*. But, the inhibition of Gram positive bacterial strains; *S. aureus*, *S. pyogenes* and *S. epidermidis* was evaluated for the first time with this study.

Both the acetone and methanol extract of *Lobaria pulmonaria* were determined to be active against Gram positive (+) bacteria. Acetone extraction (12A) was more effective than methanol extraction (12M). Water extract (12W) didn't inhibit any type of bacterial strains. There was no effect of any extracts against Gram negative (-) bacteria except *P. vulgaris* with 8.5 ± 0.3 mm and 7.8 ± 0.5 mm zones for acetone and methanol extract, respectively. Acetone extract of *L. pulmonaria* was

very active against *S. epidermidis* (21.8 ± 0.3 mm) and *S. pyogenes* (36.5 ± 0.5 mm) but it was moderately active against *S. aureus* (14.8 ± 0.3 mm). When compared with all lichen species, the acetone extract of *L. pulmonaria* showed the best activity against *S. pyogenes*. This result was better than chloramphenicol (25.0 ± 0.0), erythromycin (30.0 ± 0.0) and tetracycline (32.0 ± 0.0) (Table 4.1). While Çobanoğlu et al. (2005) reported that *L. pulmonaria* inhibited the growth of *B. subtilis*, *B. cereus* and *S. aureus*, we revealed the antibacterial activity against *S. epidermidis*, *S. pyogenes* and *P. vulgaris*.

Antibacterial activity of lichen *Ramalina farinacea* was manifested by the acetone (13A), methanol (13M) and water (13W) extracts. The acetone and methanol extracts had strong antibacterial activity, inhibiting all of the Gram positive (+) strains. The methanol extract showed maximal activity against *S. aureus* (15.3 ± 0.3 mm), *S. epidermidis* (30.3 ± 0.3 mm) and *S. pyogenes* (29.8 ± 0.3) as compared to other extracts. Except for Gram positive (+), only acetone extract of *R. farinacea* exhibited weak antibacterial activity against *P. vulgaris* (7.5 ± 0.3 mm) as Gram negative (-) bacteria. Both water and methanol extracts had no inhibitory activity against Gram negative bacteria (-) (Table 4.1.). It was reported that *R. farinacea* demonstrated inhibition effect against *S. aureus*, *S. epidermidis*, *E. coli* and *P. vulgaris* (Şahin et al., 2015; Karagöz et al., 2009; Tay et al., 2005). Similarly, *R. farinacea* also inhibited the same bacterial strains in our study.

Table 4.1. Antibacterial activity of lichen samples

Mean diameter of inhibitory zones (mm $\pm$ SE)										
Treatment	<i>S.auerus</i>	<i>S. epidermidis</i>	<i>S. marcescens</i>	<i>S.pyogenes</i>	<i>S.typhimurium</i>	<i>P.aeruginosa</i>	<i>P. vulgaris</i>	<i>K. pneumonia</i>	<i>E. cloacae</i>	<i>E.coli</i>
1A	16.0 $\pm$ 1.1	19.0 $\pm$ 0.4	9.5 $\pm$ 0.3	14.8 $\pm$ 0.5	9.8 $\pm$ 0.5	8.0 $\pm$ 0.4	14.8 $\pm$ 0.3	-	8.5 $\pm$ 0.3	-
1M	8.5 $\pm$ 0.3	11.5 $\pm$ 0.5	-	12.8 $\pm$ 0.8	-	-	9.8 $\pm$ 0.3	-	8.0 $\pm$ 0.4	-
1W	-	-	-	-	-	-	-	-	-	-
2A	15.0 $\pm$ 0.4	30.5 $\pm$ 0.5	-	27.0 $\pm$ 1.3	-	-	-	-	-	-
2M	16.3 $\pm$ 0.3	28.3 $\pm$ 0.6	9.5 $\pm$ 0.3	30.5 $\pm$ 1.3	14.8 $\pm$ 0.3	18.8 $\pm$ 0.8	12.3 $\pm$ 0.3	-	23.0 $\pm$ 0.0	-
2W	-	-	-	-	-	-	-	-	-	-
3A	16.0 $\pm$ 0.6	23.5 $\pm$ 0.5	-	25.0 $\pm$ 1.3	-	-	10.0 $\pm$ 0.0	-	-	-
3M	16.3 $\pm$ 0.3	24.5 $\pm$ 0.3	7.5 $\pm$ 0.3	27.5 $\pm$ 0.9	-	-	11.5 $\pm$ 0.3	-	-	-
3W	-	-	-	-	-	-	-	-	-	-
4A	20.3 $\pm$ 0.6	25.5 $\pm$ 0.3	-	28.0 $\pm$ 1.2	-	10.0 $\pm$ 0.4	8.3 $\pm$ 0.3	-	-	-
4M	11.0 $\pm$ 0.0	16.5 $\pm$ 0.3	-	24.0 $\pm$ 1.2	-	-	-	-	-	-
4W	-	-	-	-	-	-	-	-	-	-
5A	9.5 $\pm$ 0.3	8.3 $\pm$ 0.3	-	14.8 $\pm$ 0.3	-	-	-	-	-	-
5M	-	10.8 $\pm$ 0.5	-	12.3 $\pm$ 0.6	-	-	-	-	-	-
5W	-	-	-	-	-	-	-	-	-	-
6A	22.3 $\pm$ 0.5	24.5 $\pm$ 0.3	-	27.0 $\pm$ 1.3	-	-	-	-	-	-
6M	20.3 $\pm$ 0.3	22.5 $\pm$ 0.3	-	30.5 $\pm$ 0.5	-	-	13.5 $\pm$ 0.3	-	-	-
6W	-	-	-	-	-	-	-	-	-	-
7A	19.3 $\pm$ 0.3	26.0 $\pm$ 0.4	-	29.5 $\pm$ 0.5	-	-	10.5 $\pm$ 0.3	-	-	-
7M	16.5 $\pm$ 0.3	29.5 $\pm$ 0.5	-	29.3 $\pm$ 0.5	-	-	11.3 $\pm$ 0.3	13.3 $\pm$ 0.3	19.3 $\pm$ 0.5	-
7W	-	-	-	-	-	-	-	-	-	-

Table 4.1. Antibacterial activity of lichen materials (continued).

Treatment	Mean diameter of inhibitory zones (mm $\pm$ SE)									
	<i>S.auerus</i>	<i>S. epidermidis</i>	<i>S. marcescens</i>	<i>S.pyogenes</i>	<i>S.typhimurium</i>	<i>P.aeruginosa</i>	<i>P. vulgaris</i>	<i>K. pneumonia</i>	<i>E. cloacae</i>	<i>E.coli</i>
8A	20.0 $\pm$ 0.0	31.0 $\pm$ 0.6	-	32.0 $\pm$ 0.0	-	-	-	-	-	-
8M	20.5 $\pm$ 0.3	30.3 $\pm$ 0.3	-	35.0 $\pm$ 0.0	-	-	-	-	-	-
8W	7.5 $\pm$ 0.3	8.3 $\pm$ 0.5	-	-	-	-	-	-	-	-
9A	31.5 $\pm$ 0.5	30.0 $\pm$ 0.9	-	-	-	-	10.5 $\pm$ 0.5	-	7.5 $\pm$ 0.3	-
9M	30.8 $\pm$ 0.8	30.5 $\pm$ 0.5	-	-	-	-	10.0 $\pm$ 0.0	-	-	-
9W	-	-	-	-	-	-	-	-	-	-
10A	12.0 $\pm$ 0.6	13.8 $\pm$ 0.5	-	18.0 $\pm$ 0.4	-	-	-	-	-	-
10M	10.0 $\pm$ 0.0	10.0 $\pm$ 0.0	-	19.5 $\pm$ 0.3	-	-	-	-	-	-
10W	-	-	-	-	-	-	-	-	-	-
11A	13.8 $\pm$ 0.3	24.8 $\pm$ 1.4	-	27.0 $\pm$ 0.6	-	-	-	-	-	-
11M	14.5 $\pm$ 0.3	31.5 $\pm$ 0.5	-	25.5 $\pm$ 0.5	-	-	-	-	-	-
11W	6.8 $\pm$ 0.3	17.5 $\pm$ 0.3	-	22.5 $\pm$ 1.3	-	-	-	-	-	-
12A	14.8 $\pm$ 0.3	21.8 $\pm$ 0.3	-	36.5 $\pm$ 0.5	-	-	8.5 $\pm$ 0.3	-	-	-
12M	9.3 $\pm$ 0.3	11.3 $\pm$ 0.3	-	17.0 $\pm$ 0.6	-	-	7.8 $\pm$ 0.5	-	-	-
12W	-	-	-	-	-	-	-	-	-	-
13A	14.8 $\pm$ 0.3	24.5 $\pm$ 0.3	-	29.0 $\pm$ 0.6	-	-	7.5 $\pm$ 0.3	-	-	-
13M	15.3 $\pm$ 0.3	30.3 $\pm$ 0.3	-	29.8 $\pm$ 0.3	-	-	-	-	-	-
13W	-	12.0 $\pm$ 0.6	-	20.8 $\pm$ 0.5	-	-	-	-	-	-
Ampicillin (10 mg)	39.8 $\pm$ 0.8	31.0 $\pm$ 0.0	14.6 $\pm$ 0.3	48.6 $\pm$ 2.5	27.4 $\pm$ 0.3	-	27.0 $\pm$ 1.4	8.4 $\pm$ 1.1	27.0 $\pm$ 0.0	21.2 $\pm$ 0.5
Carbenicillin (100mg)	43.8 $\pm$ 0.8	40.0 $\pm$ 1.4	28.0 $\pm$ 0.0	42.8 $\pm$ 3.3	25.2 $\pm$ 2.2	10.0 $\pm$ 0.0	36.8 $\pm$ 1.9	8.0 $\pm$ 1.4	32.0 $\pm$ 1.4	24.4 $\pm$ 0.3
Chloramphenicol (30 mg)	25.0 $\pm$ 0.0	32.6 $\pm$ 1.7	26.8 $\pm$ 0.8	32.0 $\pm$ 1.4	28.8 $\pm$ 0.8	9.0 $\pm$ 0.0	27.0 $\pm$ 1.4	29.2 $\pm$ 0.8	28.8 $\pm$ 0.5	28.2 $\pm$ 0.5
Erythromycin (15mg)	30.0 $\pm$ 0.0	40.0 $\pm$ 0.0	11.0 $\pm$ 0.0	35.2 $\pm$ 3.6	10.8 $\pm$ 0.5	-	13.6 $\pm$ 0.3	13.0 $\pm$ 0.0	9.6 $\pm$ 0.3	12.0 $\pm$ 0.0
Tetracycline (30 mg)	32.0 $\pm$ 0.0	8.0 $\pm$ 0.0	23.8 $\pm$ 0.8	36.2 $\pm$ 1.2	25.2 $\pm$ 0.5	16.0 $\pm$ 0.0	37.6 $\pm$ 1.6	29.8 $\pm$ 0.5	30.6 $\pm$ 0.3	28.8 $\pm$ 0.8
Water	-	-	-	-	-	-	-	-	-	-

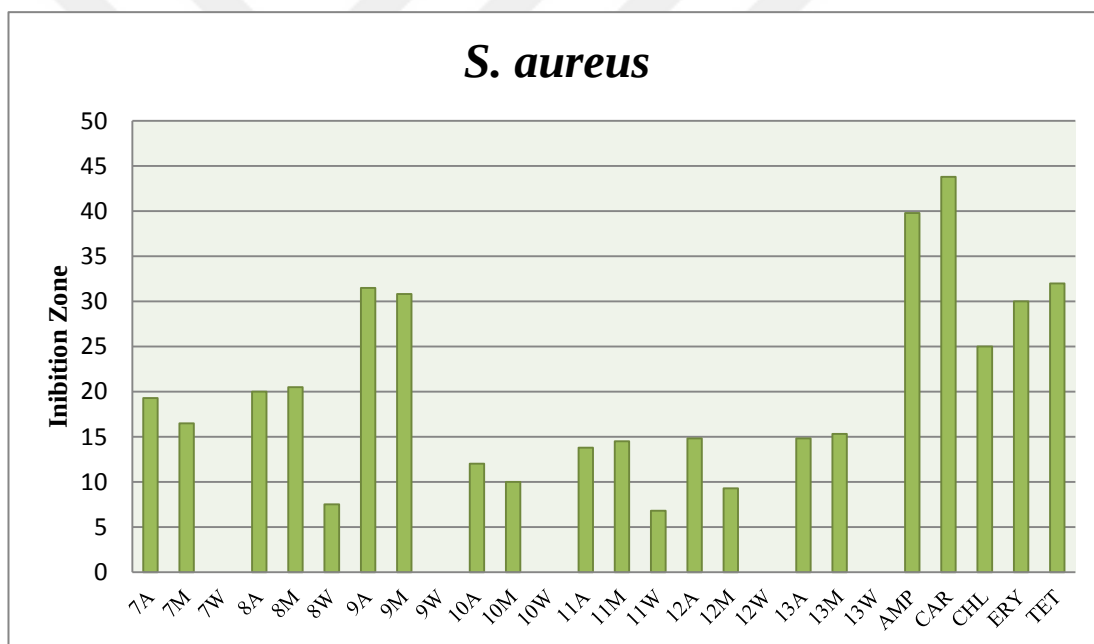
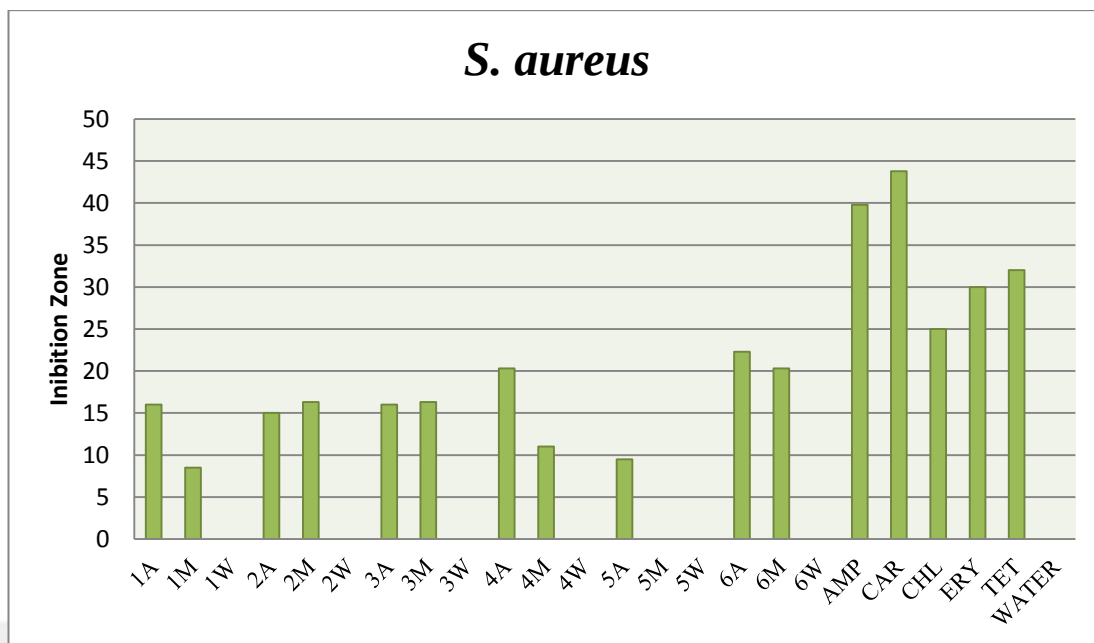


Figure 4.1. Antibacterial activities of lichen extracts and antibiotics against *S. aureus*.

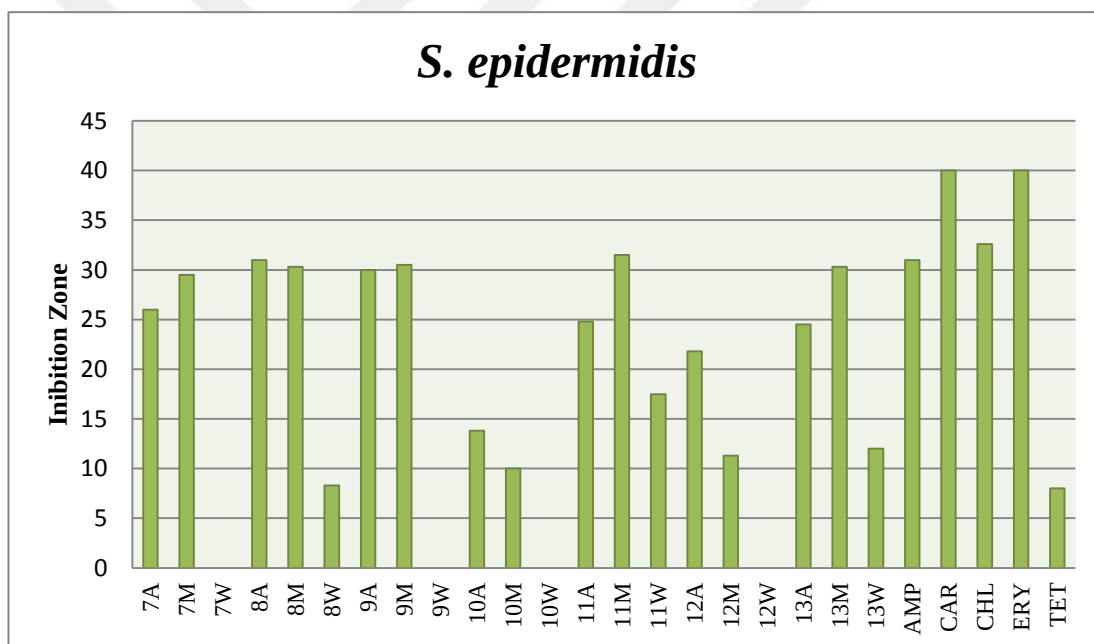
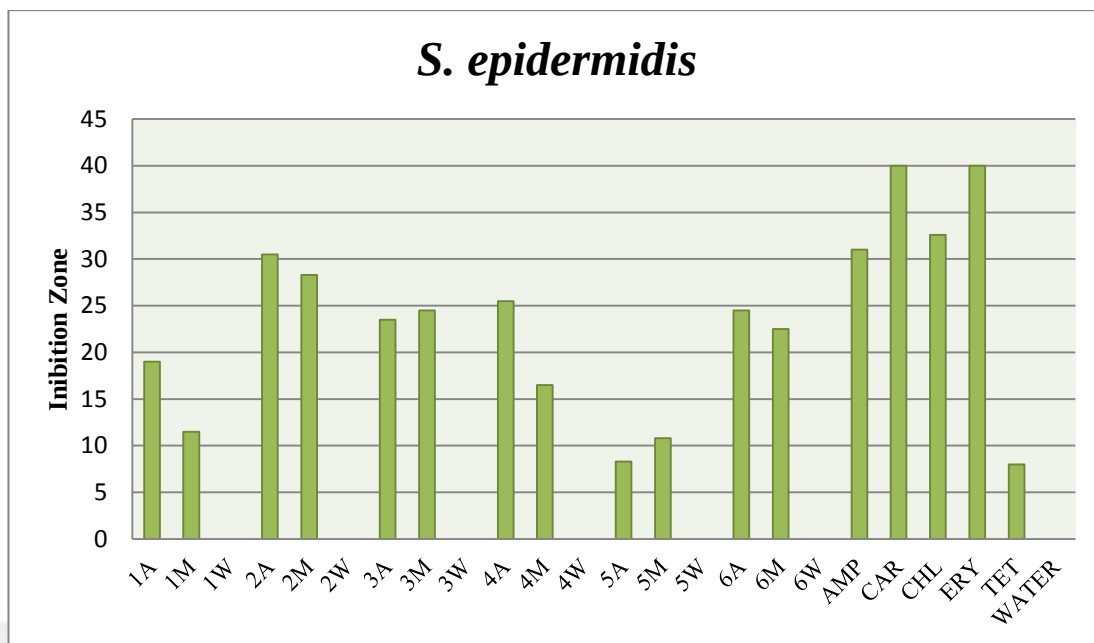


Figure 4.2. Antibacterial activities of lichen extracts and antibiotics against *S.epidermidis*.

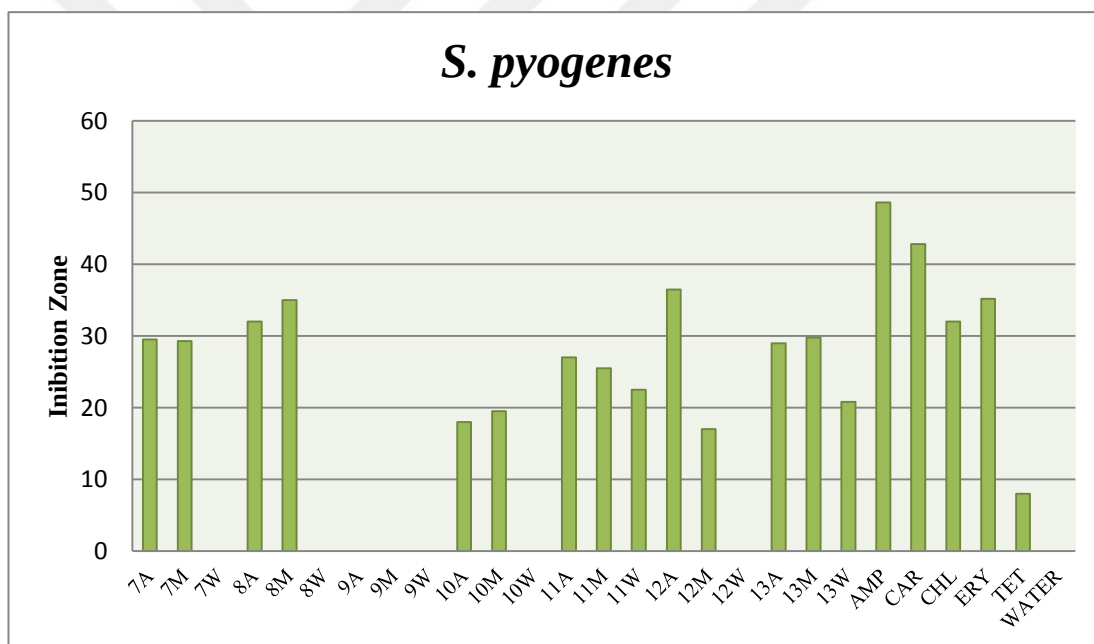
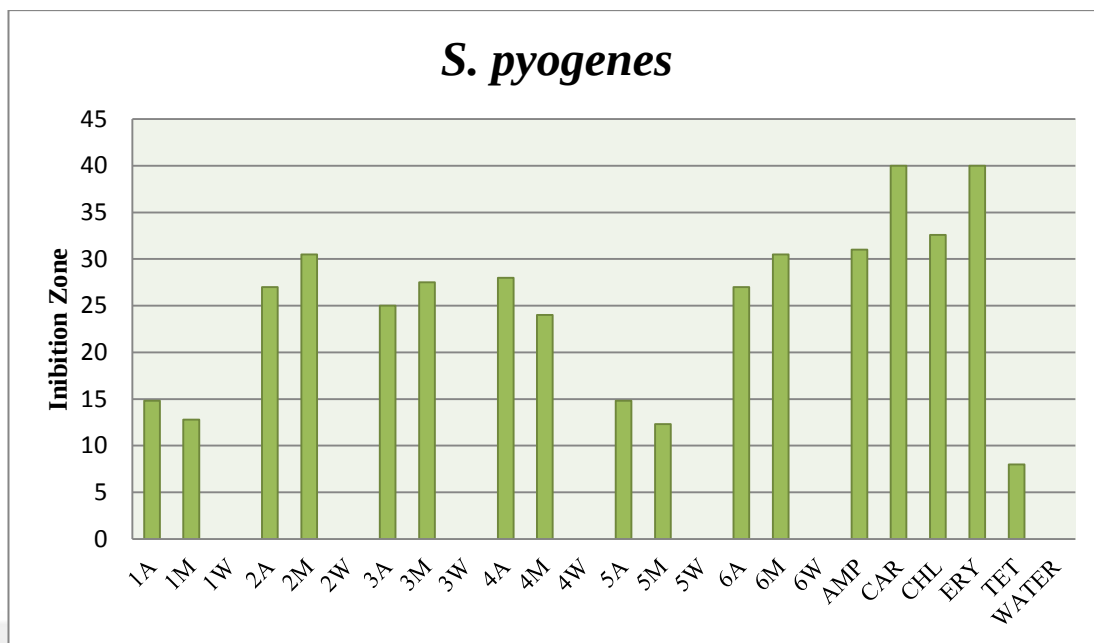


Figure 4.3. Antibacterial activities of lichen extracts and antibiotics against *S.pyogenes*.

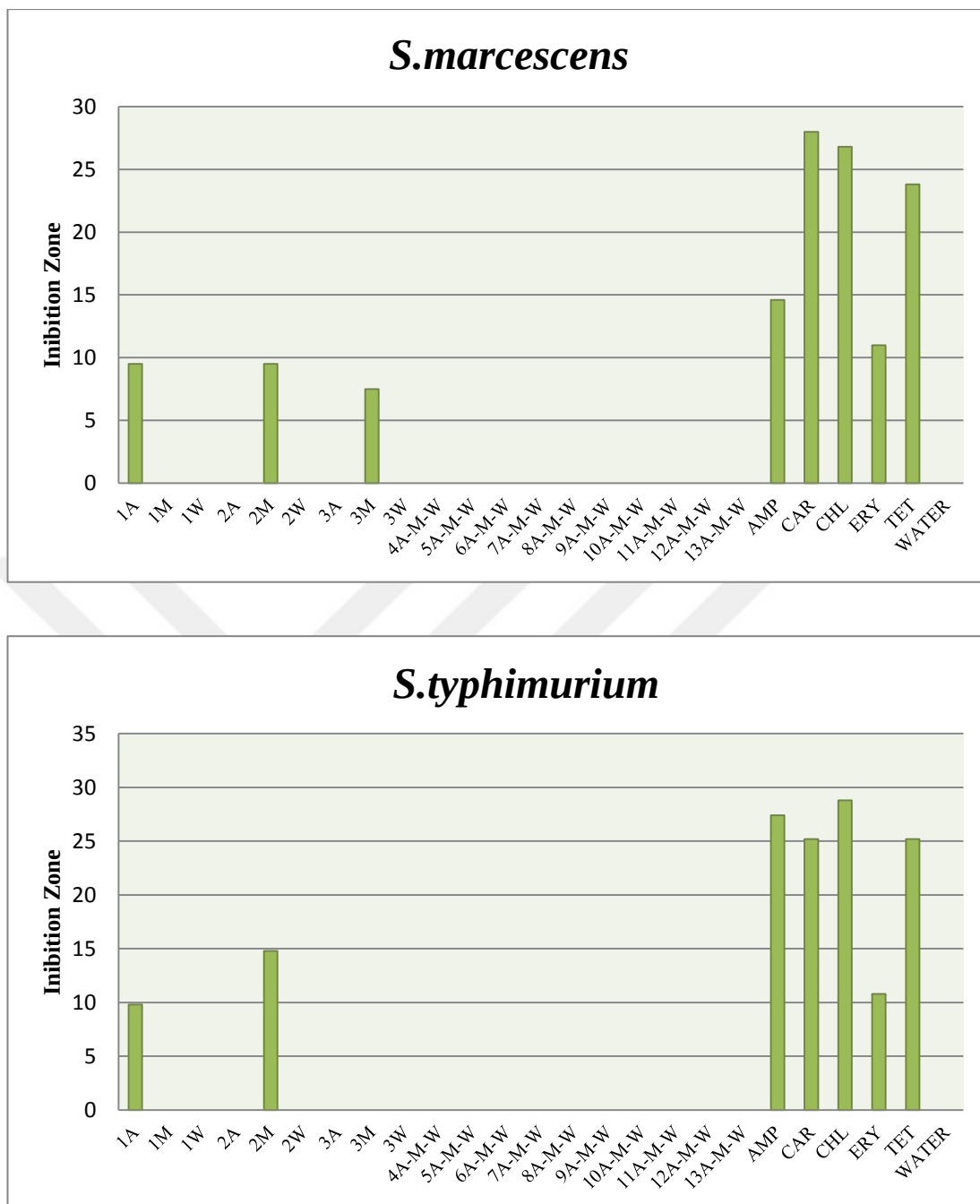


Figure 4.4. Antibacterial activities of lichen extracts and antibiotics against *S.marcescens* and *S.typhimurium*.

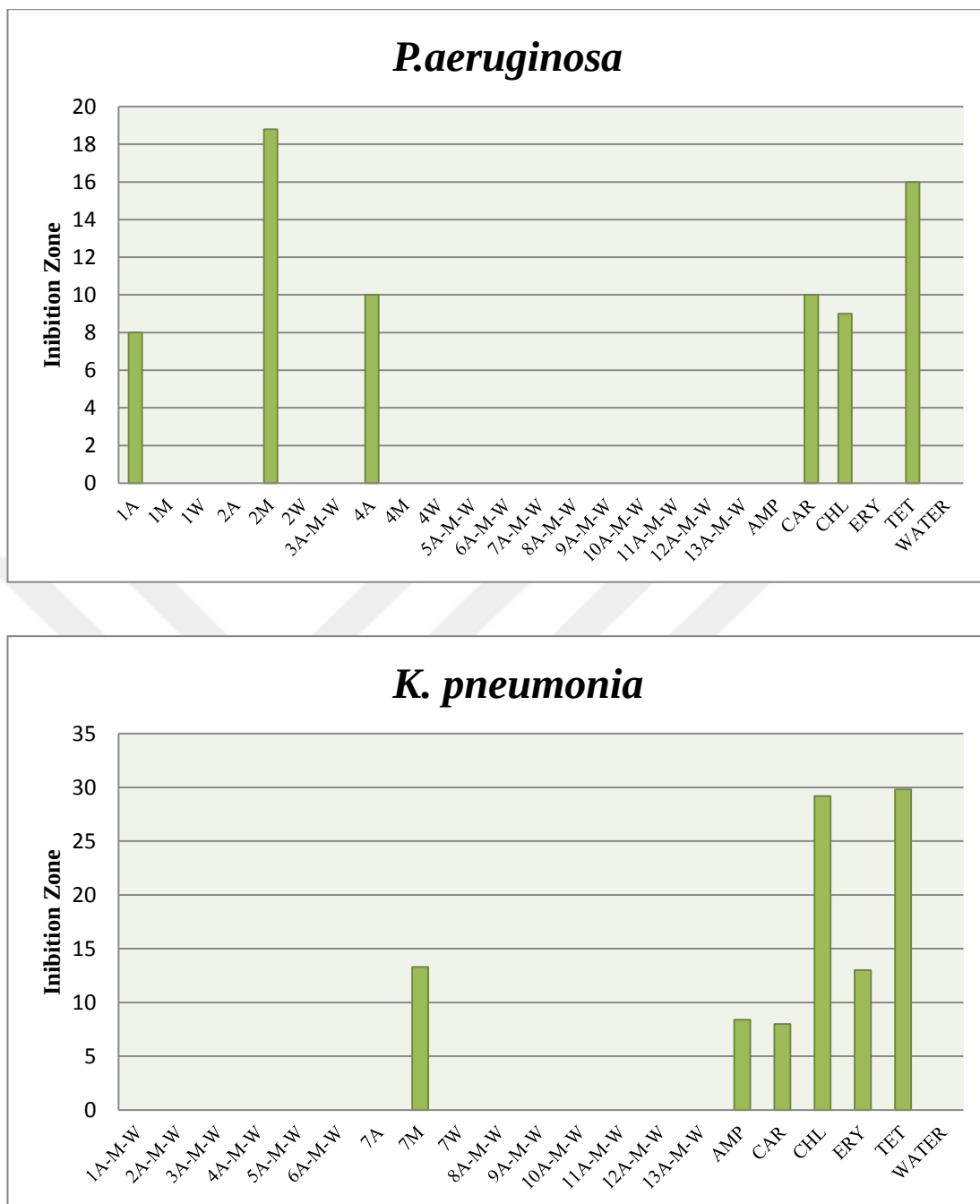


Figure 4.5. Antibacterial activities of lichen extracts and antibiotics against *K.pneumonia* and *P.aeruginosa*.

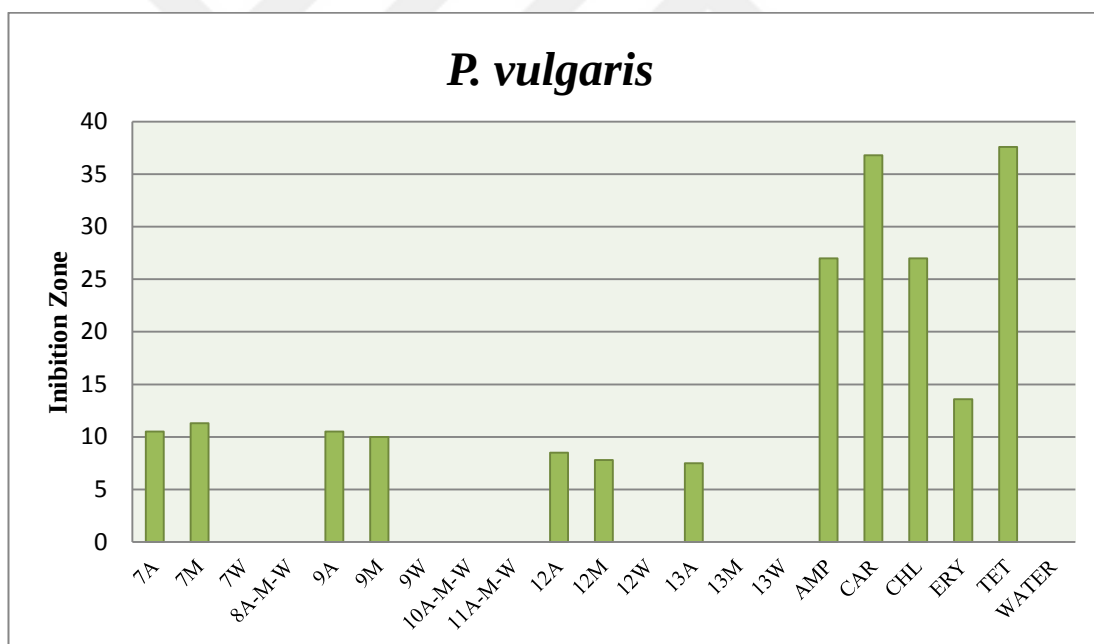
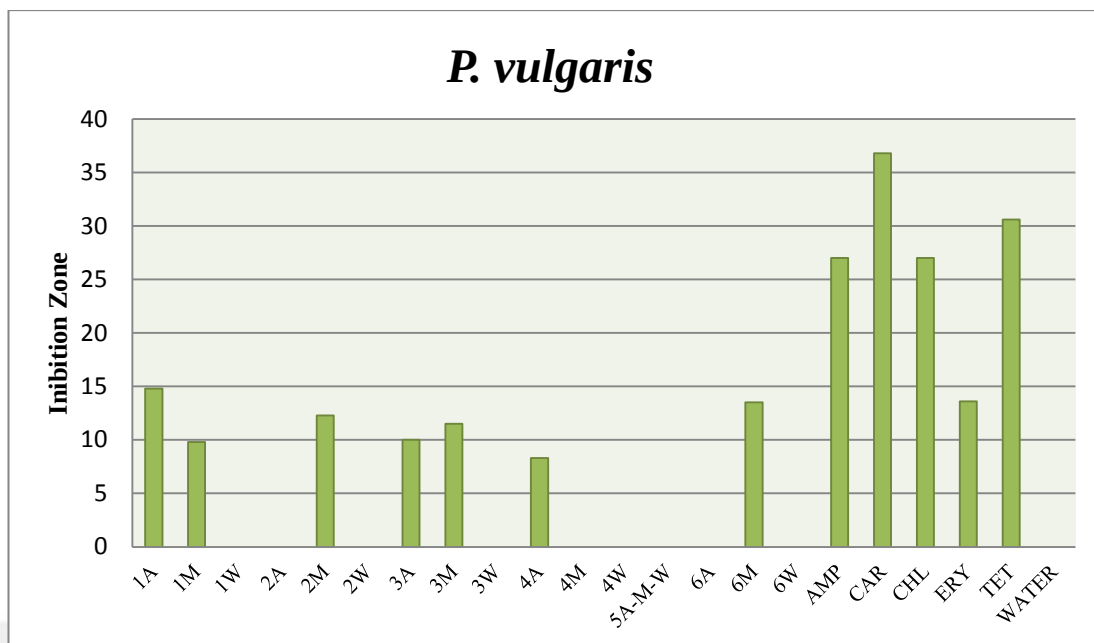


Figure 4.6. Antibacterial activities of lichen extracts and antibiotics against *P.vulgaris*.

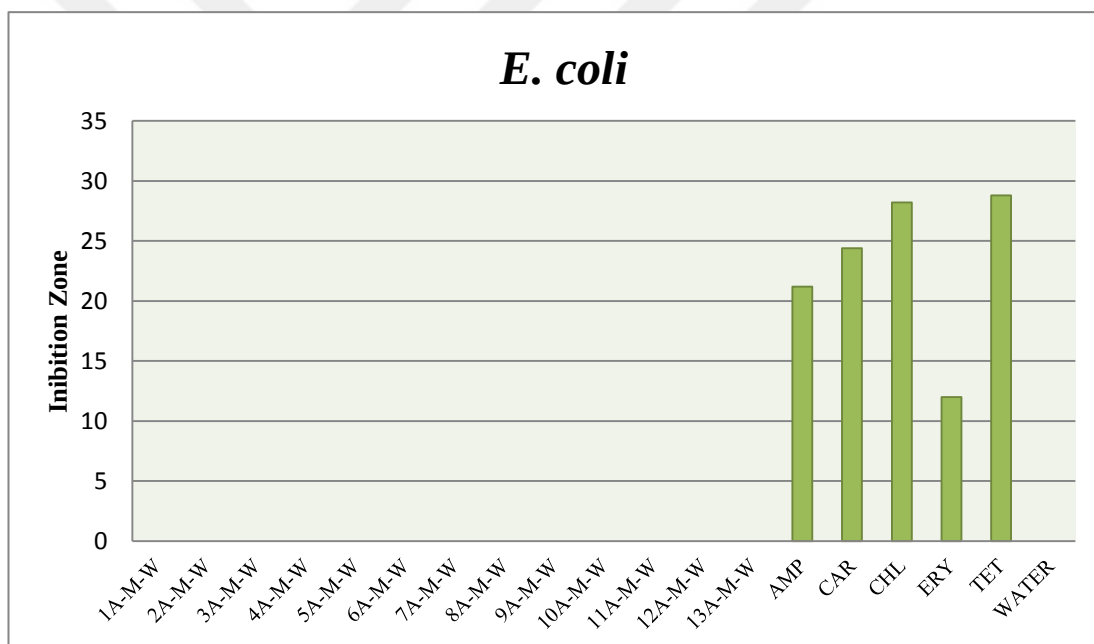
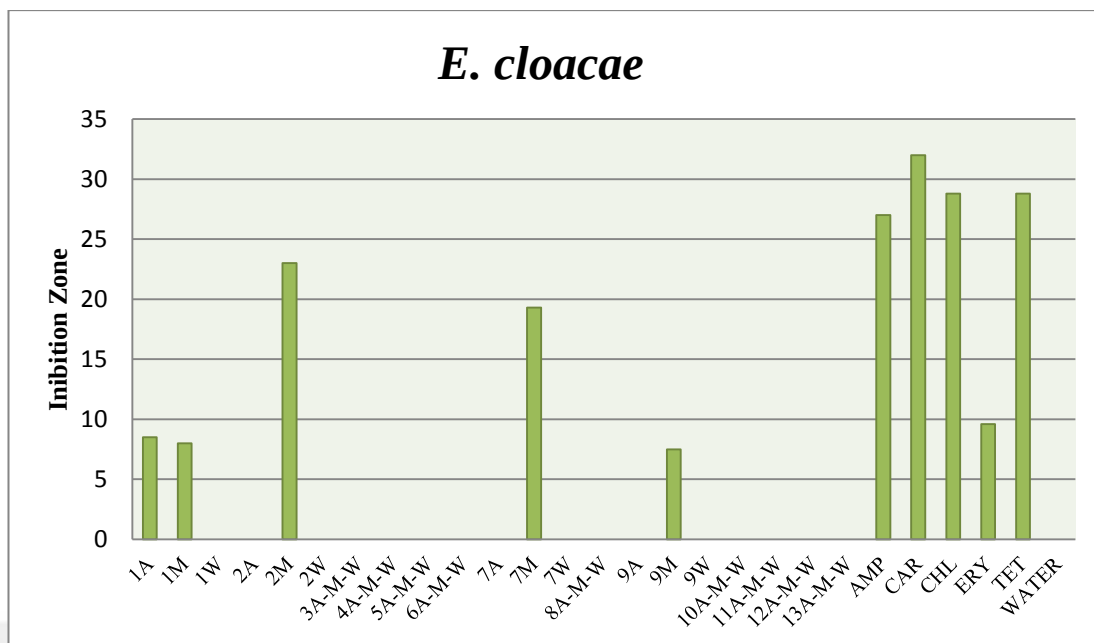


Figure 4.7. Antibacterial activities of lichen extracts and antibiotics against *E. coli* and *E. cloacae*.

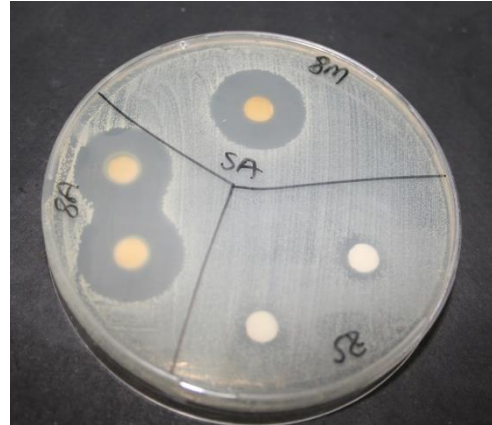


Figure 4.8. Antibacterial activity of acetone, methanol and water extracts of some lichens extracts; *Letharia vulpina* (9) and *Evernia divaricata* (8), against *S. aureus*.

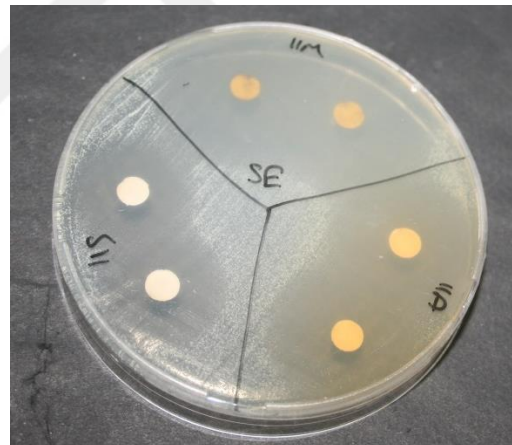
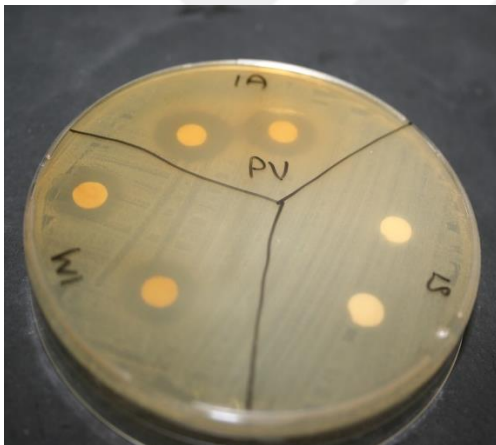


Figure 4.9. Antibacterial activity of acetone, methanol and water extracts of some lichens; *Bryoria capillaris* (1) and *Usnea florida* (11), against *P. vulgaris* and *S. epidermidis*.

4.1.2 Antioxidant Activity

4.1.2.1 Free radical Scavenging Activity - DPPH (2,2-diphenyl-picrylhydrazil) Method

In the present study, antioxidant activity of methanolic extracts of lichen samples was assessed. Free radical scavenging activities (%) of lichens were expressed in Table 4.2.

The results showed that at different concentrations, good antioxidant activities were obtained by lichen extracts. At 50 µg/ml (minimum active concentration), methanol extract of *Pseudoevernia furfuracea* (6M) exhibited the strongest DPPH radical scavenging activity (81.1 %). Then, *Lobaria pulmonaria* (12M) was effective with 64.4% DPPH radical scavenging activity at the same concentration. Except *Bryoria capillaris* (1M) and *Lobaria pulmonaria* (12M), all extracts of lichen indicated gradually antioxidant activities at 100 µg/ml and 200 µg/ml (Table 4.2).

When antioxidant activities at 100 µg/ml concentration were compared, *Letharia vulpina* (9M) showed good DPPH radical scavenging activity with 74.8% in addition to 6M (81.4 %) and 12M (73.8%). At 200 µg/ml, 6M and 9M showed the similar DPPH radical scavenging activity with 85.1%. Even the highest concentrations of lichen samples didn't exhibit better activity than ascorbic acid that was used as control antioxidant (Table 4.2).

Table 4.2. Free radical scavenging activity of methanolic extracts of lichen samples and ascorbic acid (positive control).

Treatments	% Inhibition of DPPH Concentrations				
	12.5 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml	200 µg/ml
Ascorbic acid	92.83	97.08	97.49	98.10	98.12
1M	38.9	45.8	54.3	67.4	60.4
2M	6.9	10.5	23.9	37.7	66.5
3M	11.8	13.8	15.2	28.5	43.4
4M	17.5	27.4	41.5	58.1	65.6
5M	1.4	0.1	0.7	19.3	28.9
6M	7.2	64.4	81.1	81.4	85.1
7M	1.0	1.9	23.9	41.5	75.2
8M	5.6	2.3	11.6	15.7	31.1
9M	6.4	11.6	17.4	74.8	85.1
10M	5.4	15.3	23.4	34.7	45.9
11M	10.9	10.1	9.9	19.4	32.9
12M	32.9	39.9	64.4	73.8	51.9
13M	11.3	10.1	11.5	28.5	39.1

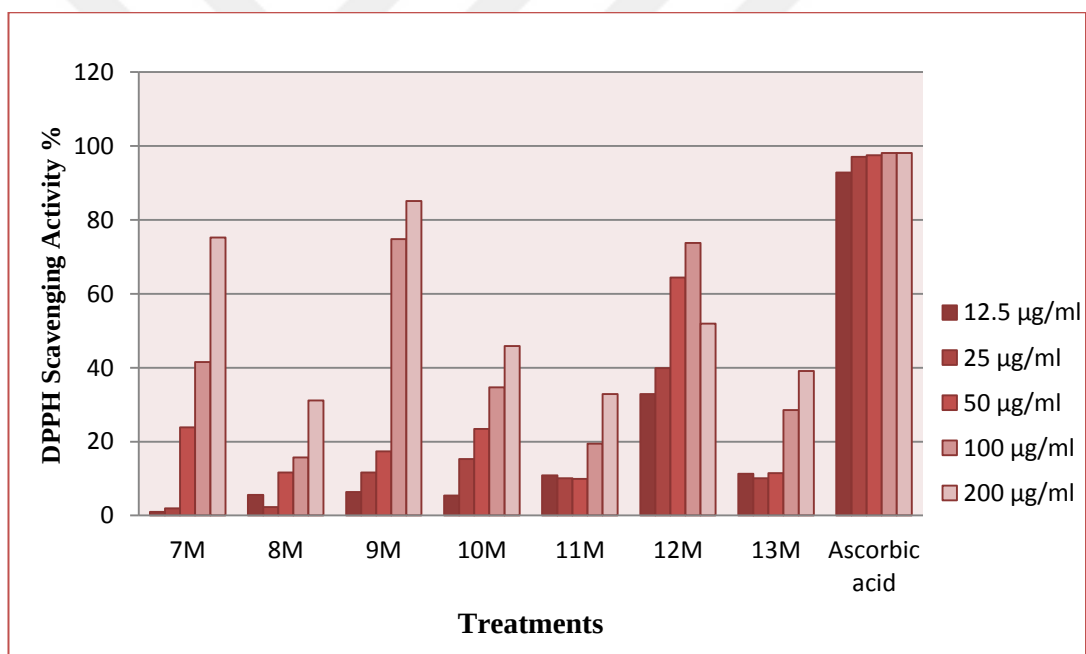
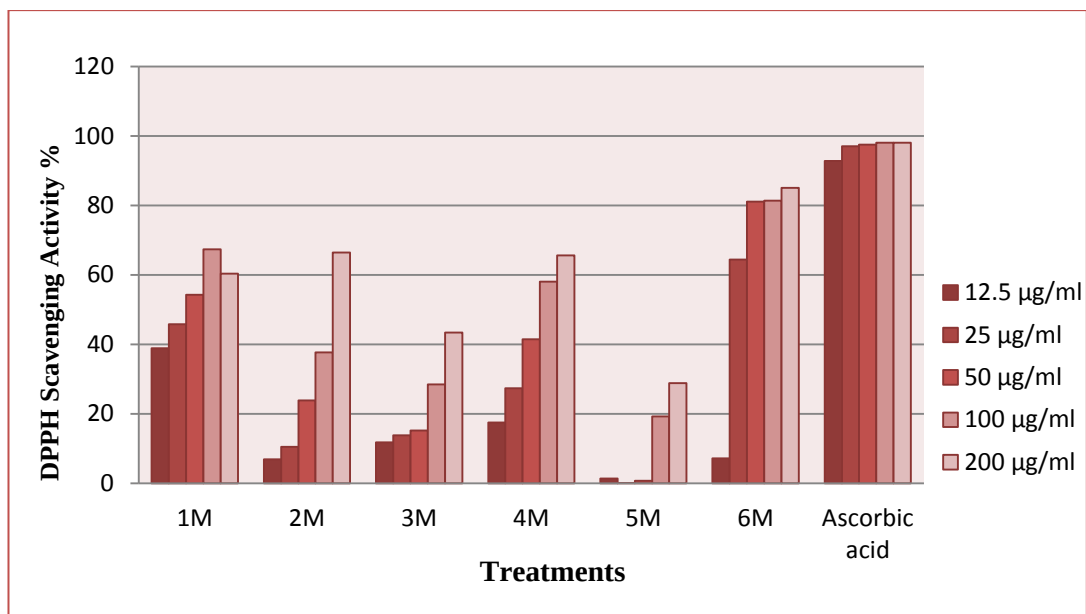


Figure 4.10. Free radical scavenging activity (%) of methanol extracts of lichen samples and ascorbic acid at different concentration.

4.1.2.1 Total Phenolic Assay (Folin-Ciocalteu Method For Total Phenolic Content)

Total phenolic contents of lichen extracts were assessed using the Folin Ciocalteu reagent. Calibration curves were plotted using known quantities of standard Gallic acid in order to determine the phenolic contents of the extracts (Figure 4.11). Total phenolic contents of all methanol extracts were indicated in Table 4.3.

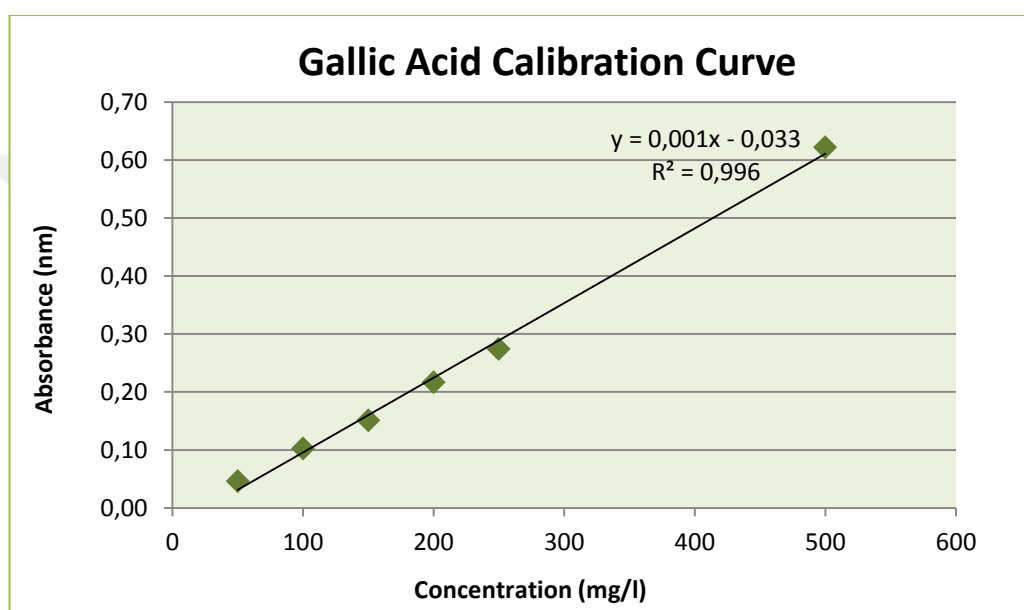


Figure 4.11. Calibration curve for gallic acid.

Among the all extracts, *Bryoria capillaris* (1M) included the most phenolic content with 163.3 ± 0.00 mg Gallic acid equivalent/ g dried extract. After then, *Evernia prunastri* (3M) and *Evernia divaricata* (8M) contained high phenolic constituents according to other extracts with 153.1 ± 0.00 mg Gallic acid equivalent/ g dried extract and 118.0 ± 0.00 mg Gallic acid equivalent/ g dried extract, respectively (Table 4.3 and Figure 4.13).

There was no significant phenolic contents in the methanol extracts of *Ramalina fraxinea* (2M), *Anapthygia ciliaris* (5M), *Ramalina fastigiata* (7M), *Letharia vulpina* (9M) and *Usnea florida* (11M) (36.4 ± 0.00 , 29.2 ± 0.00 , 39.4 ± 0.00 , 27.5 ± 0.00 and 37.5 ± 0.00 mg Gallic acid equivalent/ g dried extract, respectively) (Table 4.3 and Figure 4.13).

4.1.2.1 Total Flavonoid Assay (Aluminum chloride (AlCl₃) Colorimetric Assay For Total Flavonoids)

Aluminum chloride (AlCl₃) was used for the colorimetric determination of total flavonoid content in lichen extracts. Calibration curve of standard Catechol solutions were plotted after measuring the absorbance at 510 nm (Figure 4.12). Total flavonoid contents of all lichen extracts were shown in Table 4.3 and Figure 4.13.

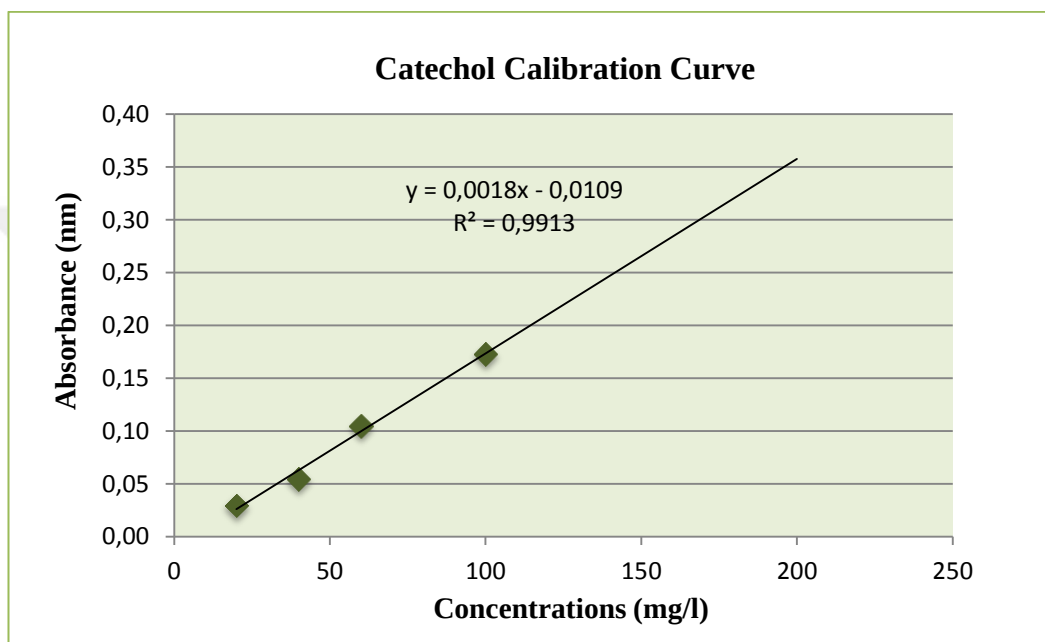


Figure 4.12. Calibration curve for catechol standards.

The results in the studied extracts showed that *Bryoria capillaris* (1M) contained the highest flavonoid contents as 154.5 ± 0.001 mg catechol equivalent/ g dried extract and followed by *Ramalina farinacea* (13M) extract (134.9 ± 0.00 mg catechol equivalent/ g dried extract). Moreover, methanol extract of *Cetraria islandica* (4M) included the lowest flavonoid constituent with 55.6 ± 0.00 mg catechol equivalent/ g dried extract (Table 4.3 and Figure 4.13).

Although some extracts showed correlation between phenolic and flavonoid contents, most of them have no relation as shown in the Table 4.3 and Figure 4.13.

Antioxidant capacity of *Bryoria capillaris* was evaluated for the first time in this study. Although moderate DPPH radical scavenging activity (67.4%) of *B.*

capillaris was demonstrated, the highest total phenolic and flavonoid contents was found with this study. Şahin et al. (2015) reported the *R. fraxinea* had low antioxidant activity and total phenolic contents among the all tested lichen species. Similarly, we revealed the same results. Moreover; the total flavonoid content of *R. fraxinea* was determined for the first time in our study. Mitrovic et al. (2013) demonstrated that *E. prunastri* has low amount of total phenol and flavonoid (80.73 ± 1.25 mg/g dried extract and 27.46 ± 0.78 mg/g dried extract, respectively), but in our study the antioxidant assays exhibited that the methanol extract of *Evernia prunastri* has higher amounts (153.1 ± 0.00 mg/g dried extract and 88.5 ± 0.00 mg/g dried extract, respectively). This result also supported the data of HPLC analysis in our study.

While Kosanic et al. (2013) determined weak DPPH radical scavenging activity of *P. furfuracea*, we investigated strongest DPPH radical scavenging activity among the all tested lichen species. We reported the total flavonoid amount of *P. furfuracea* (104.0 ± 0.001 mg CE/ g dried lichen extract) for the first time. Şahin et al. (2015) determined the high antioxidant capacity and total phenolic content of *R. fastigiata*. However, we investigated moderate antioxidant activity and low phenolic content in our study. Also, this is the first report about total flavonoid content of *R. fastigiata*.

Total phenolic and flavonoid contents of *E. divaricata* were exhibited as 79.1 ± 0.00 mg CE/g dried lichen extract and 118.0 ± 0.00 mg GAE /g dried lichen extract, respectively. The data of total flavonoid was reported for the first time in this study. Antioxidant capacity of *L. vulpina* was investigated for the first time with our study. High DPPH radical scavenging activity (%85), moderate amount of total flavonoid (88.5 ± 0.00 mg CE/g dried lichen extract) and low amount of total phenolic (27.5 ± 0.00 mg GAE/g dried lichen extract) contents were revealed with this study.

Odabaşoğlu et al. (2004) determined that *U. florida* had low antioxidant activity and total phenolic contents. Also, we obtained similar result for *U. florida*. Moreover, it was reported for the first time that *U. florida* had a moderate amount of total flavonoid content (116.0 ± 0.00 mg CE/g dried lichen extract). Atalay et al. (2011) and Odabaşoğlu et al. (2004) reported that *L. pulmonaria* had high DPPH activity and total phenolic content. Also, we determined the similar results.

Moreover, Total flavonoid amount of *L. pulmonaria* reported for the first time with this study.

Table 4.3. Total phenol and flavonoid contents of methanolic extracts of the lichens. Gallic acid equivalent was used as mg GAE/g of dried mass of lichen material. Catechol equivalent was used as mg CE/g of dried mass of lichen material. Results were expressed as means $\pm$ SD.

Treatments	Total Phenolics mg GAE/ g dried mass	Total Flavonoids mg CE/ g dried mass
1M	163.3 $\pm$ 0.00	154.5 $\pm$ 0.001
2M	36.4 $\pm$ 0.00	94.3 $\pm$ 0.001
3M	153.1 $\pm$ 0.00	88.5 $\pm$ 0.00
4M	78.3 $\pm$ 0.00	55.6 $\pm$ 0.00
5M	29.2 $\pm$ 0.00	93.3 $\pm$ 0.00
6M	97.4 $\pm$ 0.00	104.0 $\pm$ 0.001
7M	39.4 $\pm$ 0.00	94.8 $\pm$ 0.001
8M	118.0 $\pm$ 0.00	79.1 $\pm$ 0.00
9M	27.5 $\pm$ 0.00	88.5 $\pm$ 0.00
10M	47.2 $\pm$ 0.00	106.9 $\pm$ 0.001
11M	37.5 $\pm$ 0.00	116.0 $\pm$ 0.00
12M	82.4 $\pm$ 0.00	114.2 $\pm$ 0.00
13M	51.2 $\pm$ 0.00	134.9 $\pm$ 0.00

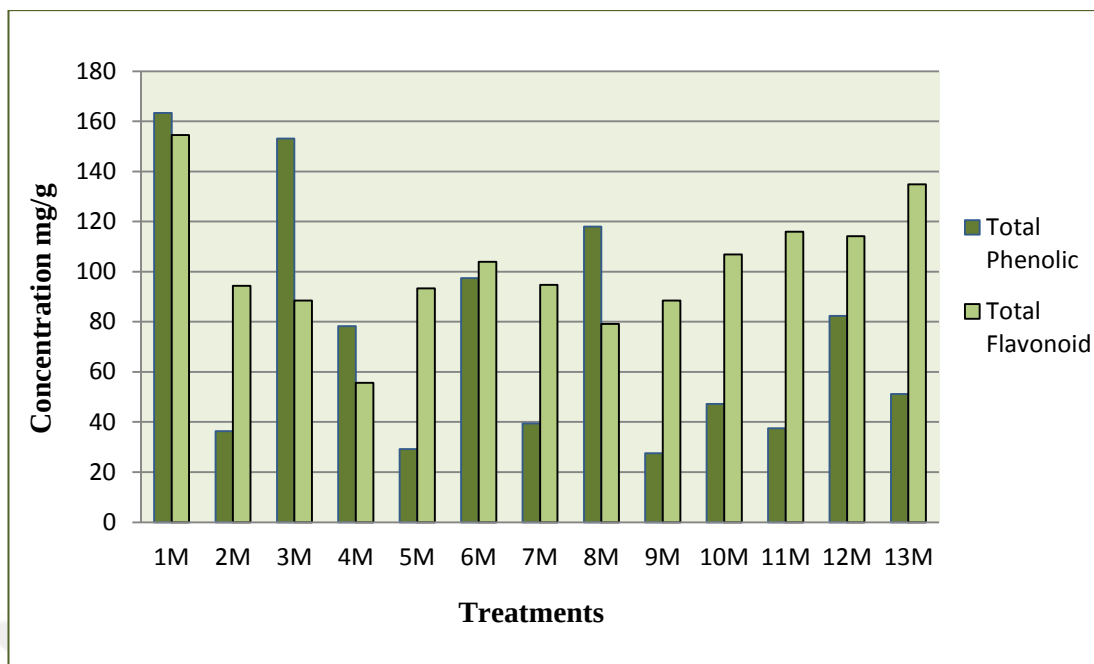


Figure 4.13. Total phenol and flavonoid contents of methanolic extracts of lichens.

4.1.3 Antiproliferative Activity

The cytotoxic effects of 18 crude extracts (acetone, methanol and water) of *R. fraxinea*, *C. islandica*, *L. vulpina*, *P. glauca*, *U. florida* and *L. pulmonaria* at various concentrations were evaluated with *in vitro* cytotoxicity assay against MCF-7 (human breast cancer) and HepG2/C3A (human hepatocellular carcinoma) cell lines (Table 4.4). A plant extract is usually regarded as interesting for *in vitro* cytotoxic activity $IC_{50} < 100 \mu\text{g/ml}$ (Boyd, 1997)

While none of the extracts exhibited the antiproliferative activity against MCF-7 cell line, the proliferation of HepG2/C3A cell line was only inhibited by acetone extracts of lichen samples. Among the acetone extracts, *L. pulmonaria* exhibited the best antiproliferative activity against HepG2/C3A cell line with IC_{50} value of $90.2 \pm 1.2 \mu\text{g/ml}$. The next effective activity was obtained by *P. glauca* with the IC_{50} value of $111.7 \pm 1.7 \mu\text{g/ml}$ (Table 4.4).

However, acetone extracts of *R. fraxinea*, *L. vulpina* and *U. florida* showed moderate antiproliferative activity against HepG2/C3A cell line with IC_{50} value of $148.3 \pm 2.2 \mu\text{g/ml}$, $175.7 \pm 1.4 \mu\text{g/ml}$, $181.9 \pm 2.1 \mu\text{g/ml}$, respectively. Acetone extract of *C. islandica* showed any antiproliferative activity against neither MCF-7 cell line nor HepG2/C3A cell line (Table 4.4).

In our study, while methanol or water extract of *R. fraxinea* didn't show any cytotoxic activity, acetone extract exhibited antiproliferative activity against HepG2/c3A cell line ($148.3 \pm 2.2 \mu\text{g/ml}$). This is the first report for antiproliferative activity of *R. fraxinea*. Grujicic et al. (2014) showed the anticancer activity of *Cetraria islandica*, against Femx (human melanoma) and LS174 (human colon carcinoma) cell lines. However, we could not find the same activity against MCF-7 and Hep G2/ C3A cell lines in our study.

Antiproliferative activity of acetone extract of *L. vulpina* were evaluated for the first time on HepG2/C3A cell line ($175.7 \pm 1.4 \mu\text{g/ml}$) in our study. Bezivin et al. (2003) investigated that *P. glauca* demonstrated cytotoxic activity on murine lymphotic leukemia (L1210), murine Lewis lung carcinoma (3LL) and human glioblastoma (U251). In our study, acetone extract of *P. glauca* exhibited

antiproliferative activity against HepG2/C3A cell line (human hepatocellular carcinoma) ($111.7 \pm 1.7 \mu\text{g/mL}$) and acetone extract of *U. florida* showed antiproliferative activity against HepG2/c3A cell line ($181.9 \pm 2.1 \mu\text{g/mL}$). These are the first reports for antiproliferative activity of *P. glauca* and *U. florida* against HepG2/c3A cell line. It was also reported the antiproliferative effect of *L. pulmonaria* for the first time. It exhibited the highest effect on HepG2/C3A ($90.2 \pm 1.2 \mu\text{g/mL}$) among the all tested lichen species.



Table 4.4. Antiproliferative activities of lichen extracts on human breast cancer (MCF-7) and human hepatocellular carcinoma (HepG2/C3A) cell lines.

Lichen Extracts	IC ₅₀ (µg/mL) ^a	
	MCF-7	HepG2/C3A
<i>R. fraxinea</i>		
Acetone extract (2A)	> 200	148.3 ± 2.2
MeOH extract (2M)	> 200	> 200
Water extract (2W)	> 200	> 200
<i>C. islandica</i>		
Acetone extract (4A)	> 200	> 200
MeOH extract (4M)	> 200	> 200
Water extract (4W)	> 200	> 200
<i>L. vulpina</i>		
Acetone extract (9A)	> 200	175.7 ± 1.4
MeOH extract (9M)	> 200	> 200
Water extract (9W)	> 200	> 200
<i>P. glauca</i>		
Acetone extract (10A)	> 200	111,7 ± 1.7
MeOH extract (10M)	> 200	> 200
Water extract (10W)	> 200	> 200
<i>U. florida</i>		
Acetone extract (11A)	> 200	181.9 ± 2.1
MeOH extract (11M)	> 200	> 200
Water extract (11W)	> 200	> 200
<i>L. pulmonaria</i>		
Acetone extract (12A)	> 200	90.2 ± 1.2
MeOH extract (12M)	> 200	> 200
Water extract (12W)	> 200	> 200

^a Concentration of extract that cause 50% inhibition of cell proliferation.

4.1 Chemical analysis of Lichen Samples

4.1.1 HPLC Analysis

HPLC with diode array detector (DAD) system provides the identification of substances (qualitative analyzes) by comparison of retention time data and UV spectra of peaks, and determination the amount of substances (quantitative analyzes). In this research, Quantification of chosen five standards (usnic acid, atranorin, stictic acid, evernic acid and fumarprotocetraric acid) in thirteen lichen species was performed by HPLC. Also, fourteen different lichen compounds in the lichen species were only identified qualitatively by comparing of the absorbance spectral data in the literatures (Yoshimura et al., 1994).

The values of retention time and absorbance maxima of the used standards were shown in Table 4.5. The chromatograms and UV spectrums of standards was illustrated in Figure 4.14 - Figure 4.21. Amounts of the lichen acids in the examined lichen species were exhibited in Table 4.6.

HPLC analysis showed that acetone was the most efficient solvent for obtaining lichen compounds. The usnic acid levels ranged from 1.23 ± 0.03 to 440.98 ± 0.25 mg/g dried lichen extract among the all species. While acetone extract of *Usnea florida* was detected as usnic acid source significantly, four species (*P. furfuracea*, *L. vulipna*, *P. glauca* and *L. pulmonaria*) didn't possess any amount of usnic acid. The dibenzofuran usnic acid has anti-inflammatory, antimicrobial, anticancer, anti-viral, gastroprotective, antipyretic and non-genotoxic anticancer activities (Odabaşoğlu et al., 2006; Rankovic et al., 2008; Perry et al., 1999; Molnar and Farkas, 2010; Fazio et al., 2007; Okuyama et al., 1995).

Stictic acid was only found in methanol and acetone extracts of *L. pulmonaria* with 117.8 ± 0.36 and 243.30 ± 0.6 , respectively. Depsidone stictic acid has neuroprotective, antioxidant, antifungal and cytotoxic activities (Correche et al. 2002, 2004; Rankovic and Misic, 2010; Lohezic Le Devehat et al. 2007). Moreover, norstictic and constictic acid from similar family were detected. While norstictic acid was detected in a few lichen species (*B. capillaris*, *P. glauca*, *L. pulmonaria* and *R.*

farinacea), constictic acid was only found in *L. pulmonaria*. Also, norstictic acid has antimicrobial, antioxidant and anticancer activity (Tay et al. 2004; Honda et al. 2010; Rankovic et. al., 2014).

Atranorin was detected in five different species; *E. prunastri*, *A. ciliaris*, *P. furfuracea*, *L. vulpine* and *P. glauca*. The highest amount of atranorin was found in acetone extract of *P. furfuracea* (115.98 ± 0.29 mg/ g dried lichen extract). Also, acetone extract of *Evernia prunastri* have excessive amount of atranorin (102.27 ± 0.17 mg/g lichen extract). It was reported that Atranorin shows antimicrobial, antioxidant, anti-inflammatory, antifungal and anticancer effects (Kumar et al., 1999; Yilmaz et al., 2004, Rankovic et al., 2008,2014; Melo et al., 2011). Chloroatranorin also was identified in *E. prunastri*, *P. furfuracea* and *P. glauca*. They were found together in many species. Chloroatranorin has anti-inflammatory, antioxidant, antimicrobial and cytotoxic activities (Bugni et al., 2009; Turk et al., 2006; Valencia-Islas et al., 2007).

Evernic acid was determined in *Evernia prunastri* and *Ramalina fastigiata*. Acetone extract of *E. prunastri* is very important source of evernic acid with amount of 258.81 ± 0.43 mg/g lichen extract. It has antifungal, antibacterial, antioxidant and anticancer activities (Halama and Van Haluvin, 2004; Kosanic et al., 2013;).

Fumarprotocetraric acid was only found in *Cetraria islandica* (Iceland moss). Similarly, acetone extract has higher amount than methanol (233.18 ± 0.93 and 1515.64 ± 2.24 , respectively). Moreover, protocetraric acid was identified in *C. islandica*, while hypoprotocetraric acid was determined in *R. farinacea*. Fumarprotocetraric acid has strong antimicrobial, antioxidant and anticancer activities (Correche et al., 2002, Rankovic and Misic, 2010; Kosanic and Rankovic, 2011)

The detailed constituents of each lichen species were reported in Table 4.7- Table 4.19. The chromatograms and 3D views of lichen samples were shown between Figure 4.16- Figure 4.33. Also, UV spectra of identified compounds were shown in Figure 4.34. Table 4.20 expressed briefly the all detected lichen compounds by HPLC-DAD analysis.

Romagni and Dayan (2002) reported that chemical constituents of *B. capillaris* were barbatolic acid, fumarprotocetraric acid and alectorialic acid. In our study; norstictic acid, psoromic acid and usnic acid were identified in *B. capillaris* and these compounds were reported for the first time (Table 4.7) While Şahin et al. (2015) determined that *R. fraxinea* contained only usnic acid, the results of our analysis showed the same data (Table 4.8). Kosanic et al. (2013) reported that the major metabolites of *Evernia prunastri* were evernic acid, usnic acid, atranorin and chloroatranorin. Additionally to these substances, we detected orcellinic acid in *E. prunastri* in our study (Table 4.9).

Ingolfssdottir and Gudjonsdottir (1997) reported that *Cetraria islandica* contained fumarprotocetraric acid and protocetraric acid. We detected fumarprotocetraric acid, protocetraric acid and usnic acid in our study (Table 4.10). Romagni and While Dayan (2002) reported that chemical constituents of *Anaptychia ciliaris* were atranorin and zeorin. We identified sekikaic acid, gyrophoric acid, usnic acid and atranorin in our study (Table 4.11). it was determined that *Pseudoevernia furfuracea* had physodic acid, physodalic acid, atranorin and chloroatranorin by Kosanic et al. (2013). In the present study, we detected all lichen compounds mentioned in reference (Table 4.12).

R. fastigiata included evernic acid, sekikaic acid, obtusatic acid, homosekikaic acid and usnic acid in our analysis (Table 4.13). However, Şahin et al. 2015 reported that it contained evernic acid, lecanoric acid, fumarprotocetraric acid and usnic acid. While they compared only retention time datas for identification of compounds, we compared both retention time and UV spectra of lichen compounds to identify. Sekikaic acid, homosekikaic acid and obtusatic acid were identified for the first time in *R. fastigiata*. It was reported that *E. divaricata* contained atranorin usnic acid and divaricatic acid (Zhao et al., 2010; Çobanoğlu et al., 2010). In addition to these compounds, sekikaic acid was also detected for the first time in *Evernia divaricata* in present study (Table 4.14).

It was investigated that *Letharia vulpina* consisted of vulpinic acid and atranorin (Yamamoto et al., 1994; Stephenson and Rundel, 1979). We found that *L. vulpina* included vulpinic acid, gyrophoric acid and atranorin. Gyrophoric acid was identified for the first time in *L. vulpina*. (Table 4.15). While caperatic acid and

atranorin were reported in *Platismatia glauca* by Romagni and Dayan (2002), we detected norstictic acid, atranorin and chloroatranorin in *P. glauca*. This study was the first report for identification of chloroatranorin and norstictic acid in *P. glauca* (Table 4.16)

Chemical composition of *U. florida* were determined in detail by Fiscus (1979). Also, Cansaran et al. (2006) revealed the usnic acid content of *U. florida*. Moreover, we detected that usnic acid and vulpinic acid were found in *Usnea florida* by comparison of retention time and UV spectra in HPLC. Vulpinic acid was reported firstly in this study (Table 4.17). Although Şahin et al. (2015) reported that the methanol extract of *Ramalina farinacea* has fumarprotocetraric acid, stictic acid and usnic acid by comparisons of only retention times, we detected hypoprotocetraric acid, norsitictic acid and usnic acid in *R. farinacea* by comparing the retention times and UV spectra (200-400 nm) from HPLC-DAD. Hypoprotocetraric acid and gyrophoric acid were identified in *R. farinacea* for the first time with our study (Table 4.19).

Table 4.5. Retention time of tested lichen standards and their absorbance maxima (nm).

Peaks No	Compound	Substance Class	Retention time (min)	Absorbance maxima UV spectrum (nm)
1	Stictic acid	Depsidone	3.47	212, 306
2	Fumarprotocetraric acid	Depsidone	5.42	212, 240, 318
3	Evernic acid	Depside	6.77	212, 270, 304
4	Usnic acid	Dibenzofuran	13.70	233, 282
5	Atranorin	Depside	14.87	210, 251, 320

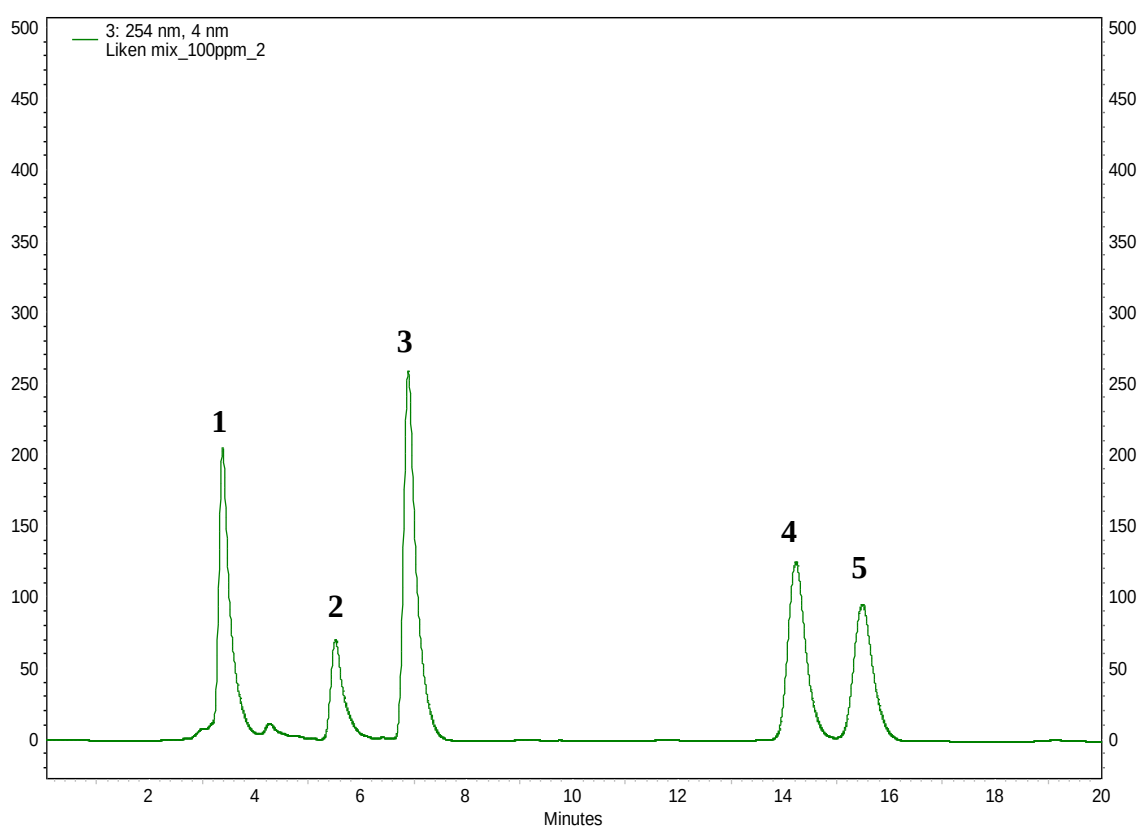


Figure 4.14. Chromatogram of standards used for identification.
(1= Stictic acid, 2=Fumarprotocetraric acid, 3=Evernic acid, 4=Usnic acid, 5=Atranorin)

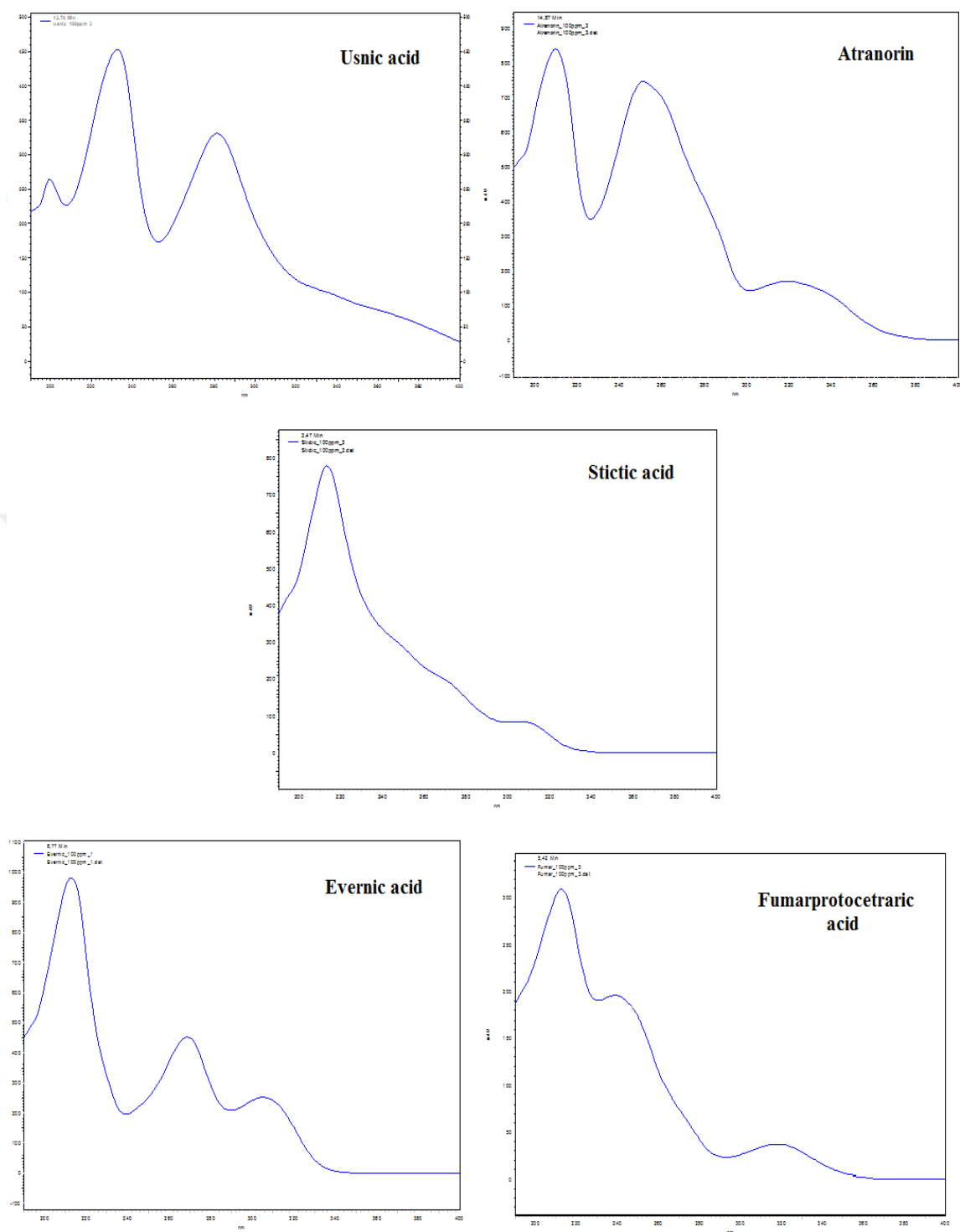


Figure 4.15. UV spectrum of lichen standards used for identification.

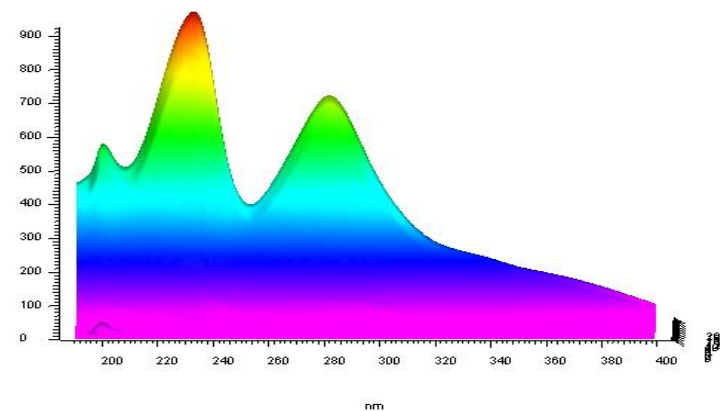
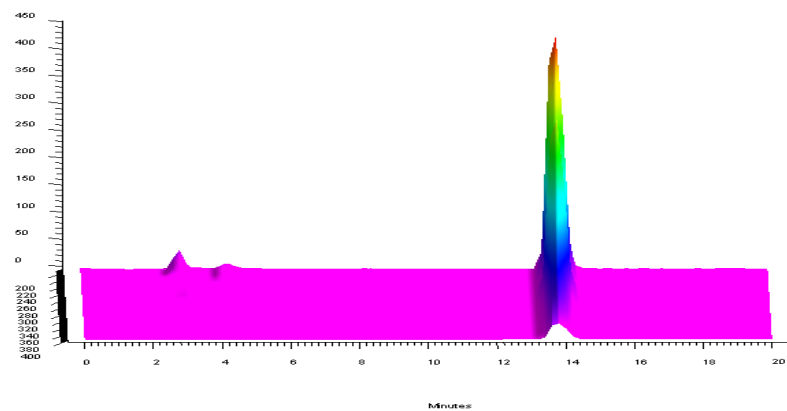
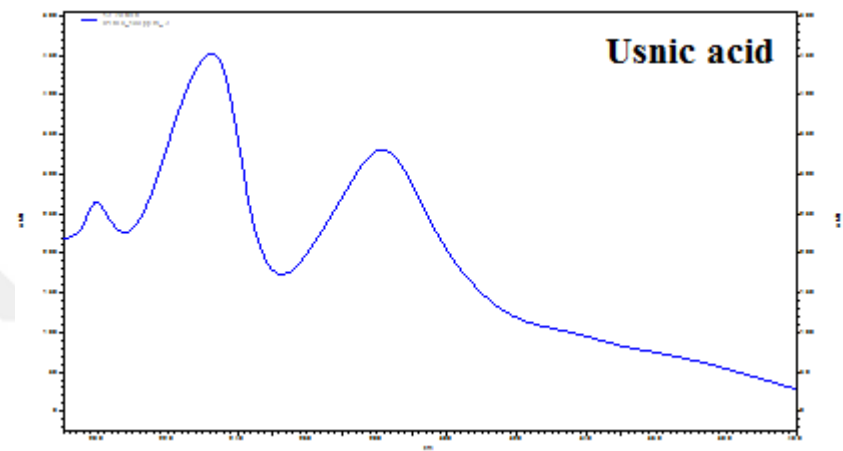
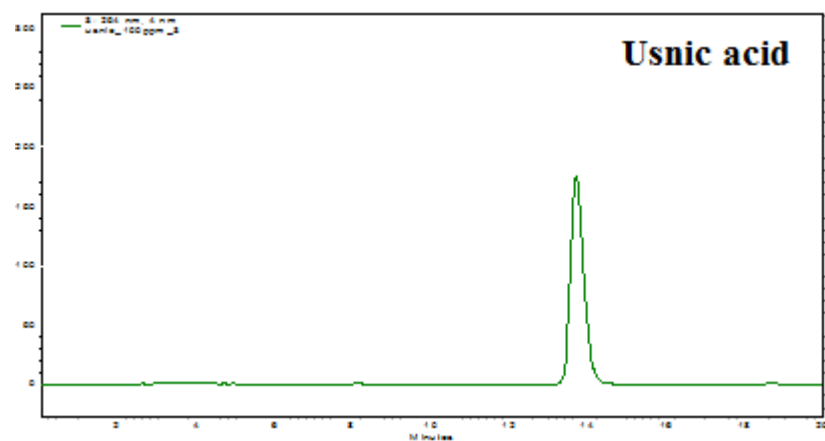


Figure 4.16. Chromatogram shows the view from front of the peaks.

Figure 4.17. Spectrum indicates the side view of the peaks.

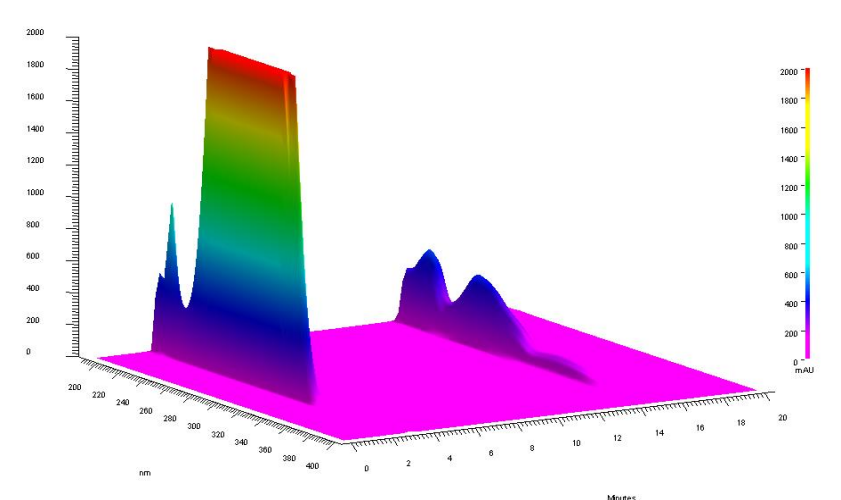
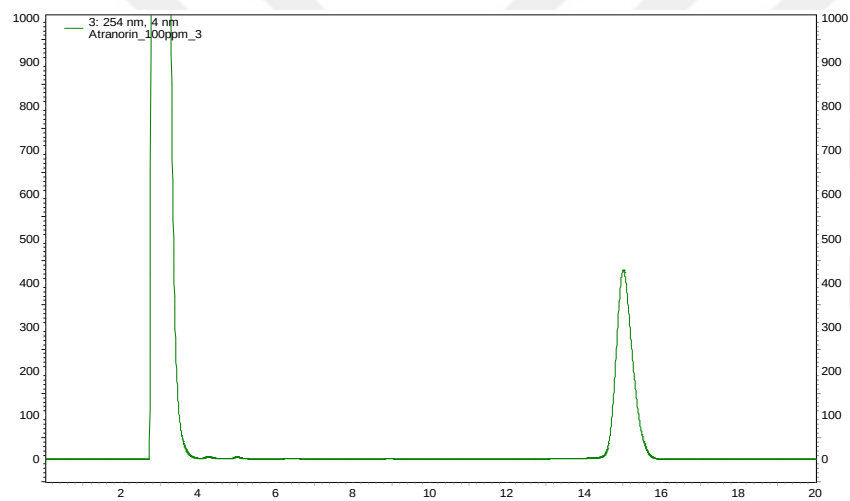


Figure 4.18. HPLC chromatogram of atranorin.

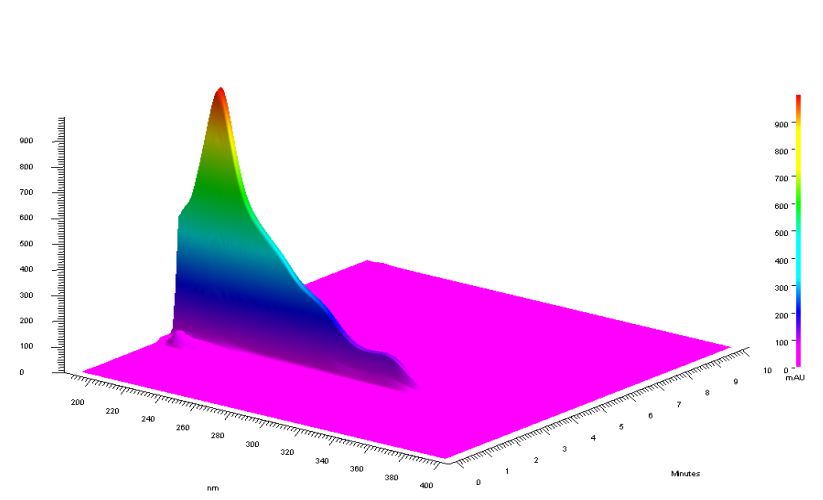
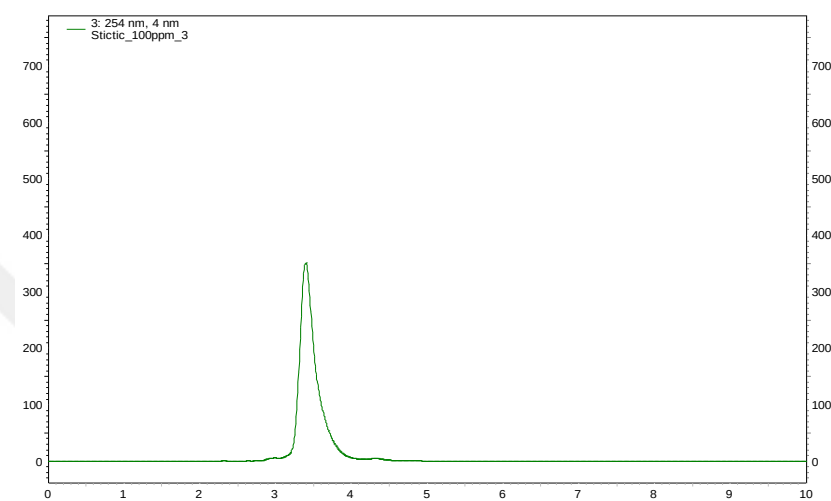


Figure 4.19. HPLC chromatogram of stictic acid.

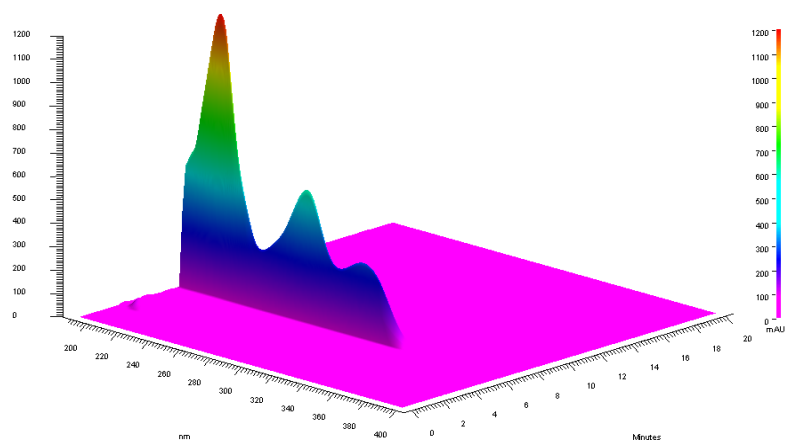
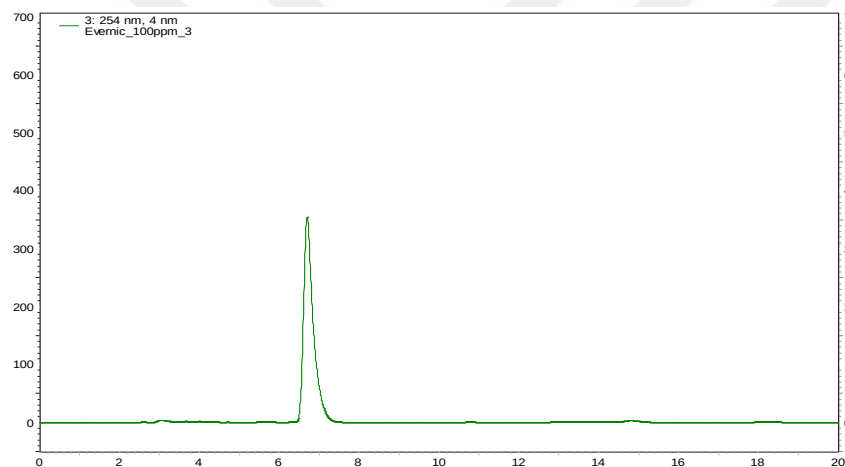


Figure 4.20. HPLC chromatogram of evernic acid.

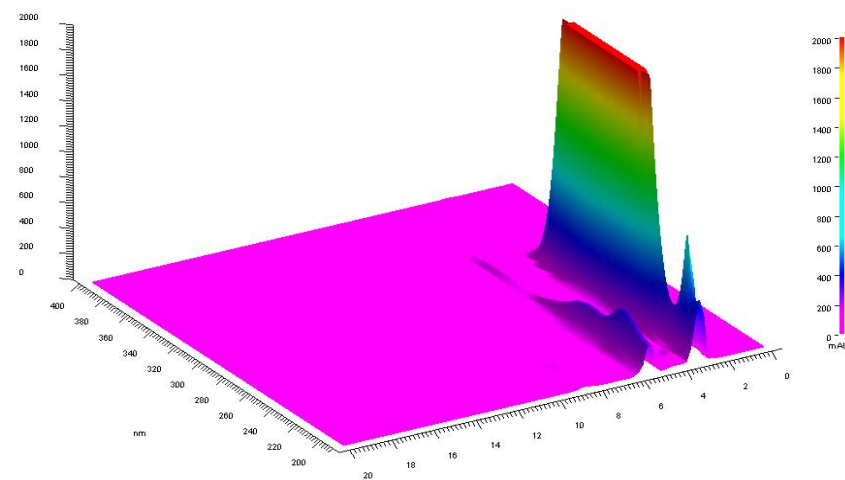
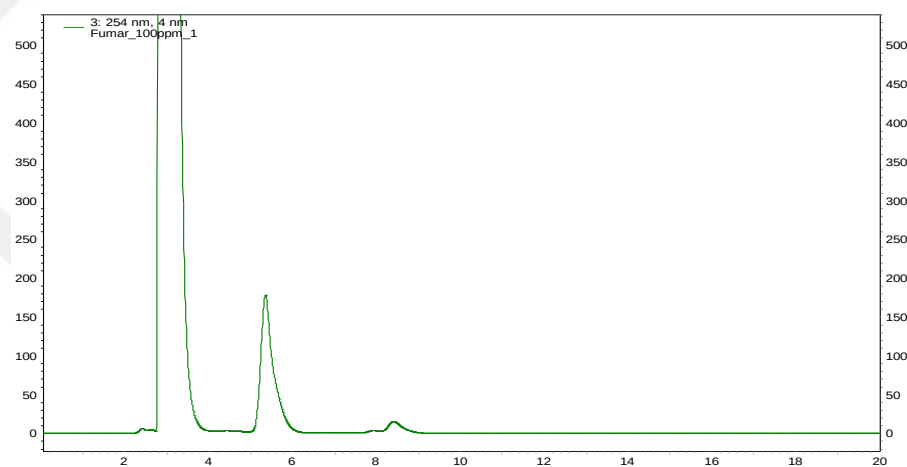


Figure 4.21. HPLC chromatogram of fumarprotocetraric acid.

Table 4.6. Determined concentration of selected acids in tested lichen species. Values are means $\pm$ SE of three measurements.

Lichen Extract		Standart Compounds (mg/g dry extract)									
		Atranorin		Evernic acid		Fumarprotocetraric acid		Stictic acid		Usnic acid	
Name	Designation	Concentration	RT (min)	Concentration	RT (min)	Concentration	RT (min)	Concentration	RT (min)	Concentration	RT (min)
<i>Bryoria capillaris</i>	1A	-		-		-		-		101.43 $\pm$ 0.15	13.74
	1M	-		-		-		-		84.94 $\pm$ 0.78	14.21
<i>Ramalina fraxinea</i>	2A	-		-		-		-		9.24 $\pm$ 0.04	13.71
	2M	-		-		-		-		16.21 $\pm$ 0.12	14.10
<i>Evernia prunastri</i>	3A	102.27 $\pm$ 0.17	14.84	258.81 $\pm$ 0.43	6.70	-		-		22.20 $\pm$ 1.10	13.68
	3M	10.09 $\pm$ 0.07	15.21	111.99 $\pm$ 0.19	6.72	-		-		22.82 $\pm$ 0.82	13.93
<i>Cetraria islandica</i>	4A	-		-		233.18 $\pm$ 0.93	5.47	-		4.30 $\pm$ 0.01	14.01
	4M	-		-		151.64 $\pm$ 2.24	5.52	-		-	
<i>Anaptychia ciliaris</i>	5A	-		-		-		-		-	
	5M	-		-		-		-		-	
<i>Pseudoevernia furfuracea</i>	5A	115.98 $\pm$ 0.29	14.84	-		-		-		1.26 $\pm$ 0.03	14.14
	6M	3.03 $\pm$ 0.02	15.38	-		-		-		-	
<i>Ramalina fastigiata</i>	7A	-		112.9 $\pm$ 0.92	6.71	-		-		65.24 $\pm$ 0.02	13.70
	7M	-		41.5 $\pm$ 0.00	6.88	-		-		57.76 $\pm$ 1.00	13.73

Table 4.6. Determined concentration of selected acids in tested lichen species. Values are means $\pm$ SE of three measurements (continued).

Lichen Extract		Standart Compounds (mg/g dry extract)									
		Atranorin		Evernic acid		Fumarprotocetraric acid		Stictic acid		Usnic acid	
Name	Designation	Concentration	RT (min)	Concentration	RT (min)	Concentration	RT (min)	Concentration	RT (min)	Concentration	RT (min)
<i>Evernia divaricata</i>	8A	-		-		-		-		49.10 $\pm$ 0.02	13.727
	8M	-		-		-		-		50.71 $\pm$ 0.86	14.147
<i>Letharia vulpina</i>	9A	8.45 $\pm$ 0.17	14.88	-		-		-		-	
	9M	-		-		-		-		-	
<i>Platismatia glauca</i>	10A	59.88 $\pm$ 0.02	14.84	-		-		-		-	
	10M	11.31 $\pm$ 0.38	15.43	-		-		-		-	
<i>Usnea florida</i>	11A	-		-		-		-		440.98 $\pm$ 0.25	13.73
	11M	-		-		-		-		99.41 $\pm$ 0.26	14.20
<i>Lobaria pulmonaria</i>	12A	-		-		-		243.30 $\pm$ 0.60	3.40	-	
	12M	-		-		-		117.80 $\pm$ 0.36	3.36	-	
<i>Ramalina farinacea</i>	13A	-		-		-				57.61 $\pm$ 0.14	13.72
	13M	-		-		-				38.02 $\pm$ 0.07	14.12

Table 4.7. Chemical constituents of *Bryoria capillaris* and their identification

Peaks no	Compound	Retention time min)	Absorbance maxima (nm) UV spectrum		
			Experimental values	Data from standards	Data from reference
1	Norstictic acid	3.38	211, 237, 309		212, 238, 310
2	Psoromic acid	6.48	207, 240		208, 240
3	Usnic acid	14.08	200, 232, 280	200, 233, 282	234, 282

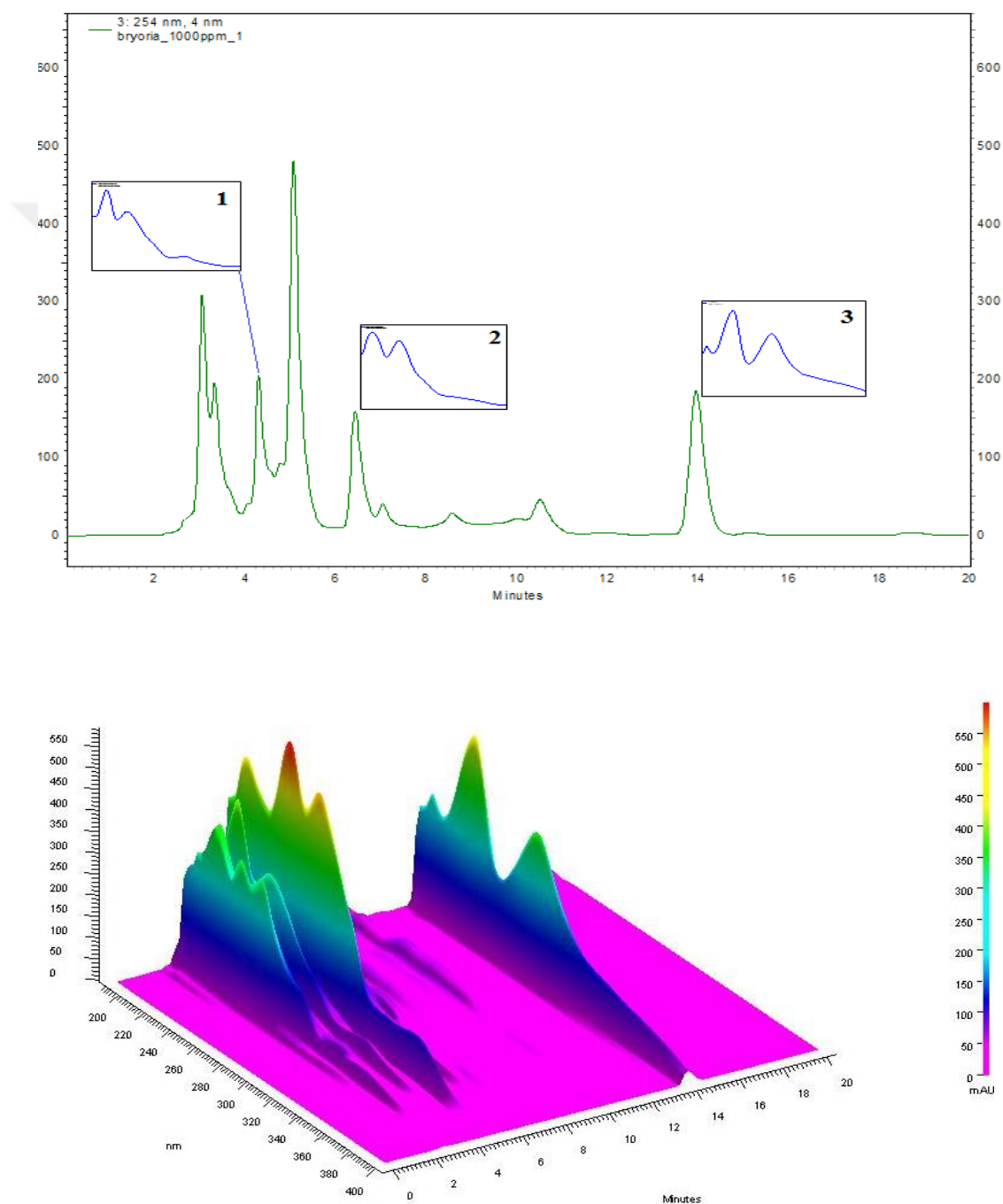


Figure 4.22. HPLC chromatogram of *B. capillaris* with UV spectrum and 3D view.

Table 4.8. Chemical constituents of *Ramalina fraxinea* and their identification.

Peaks no	Compound	Retention time (min)	Absorbance maxima (nm) UV spectrum		
			Experimental values	Data from standards	Data from reference
1	Usnic acid	14.04	200, 232, 281	200, 233, 282	234, 282

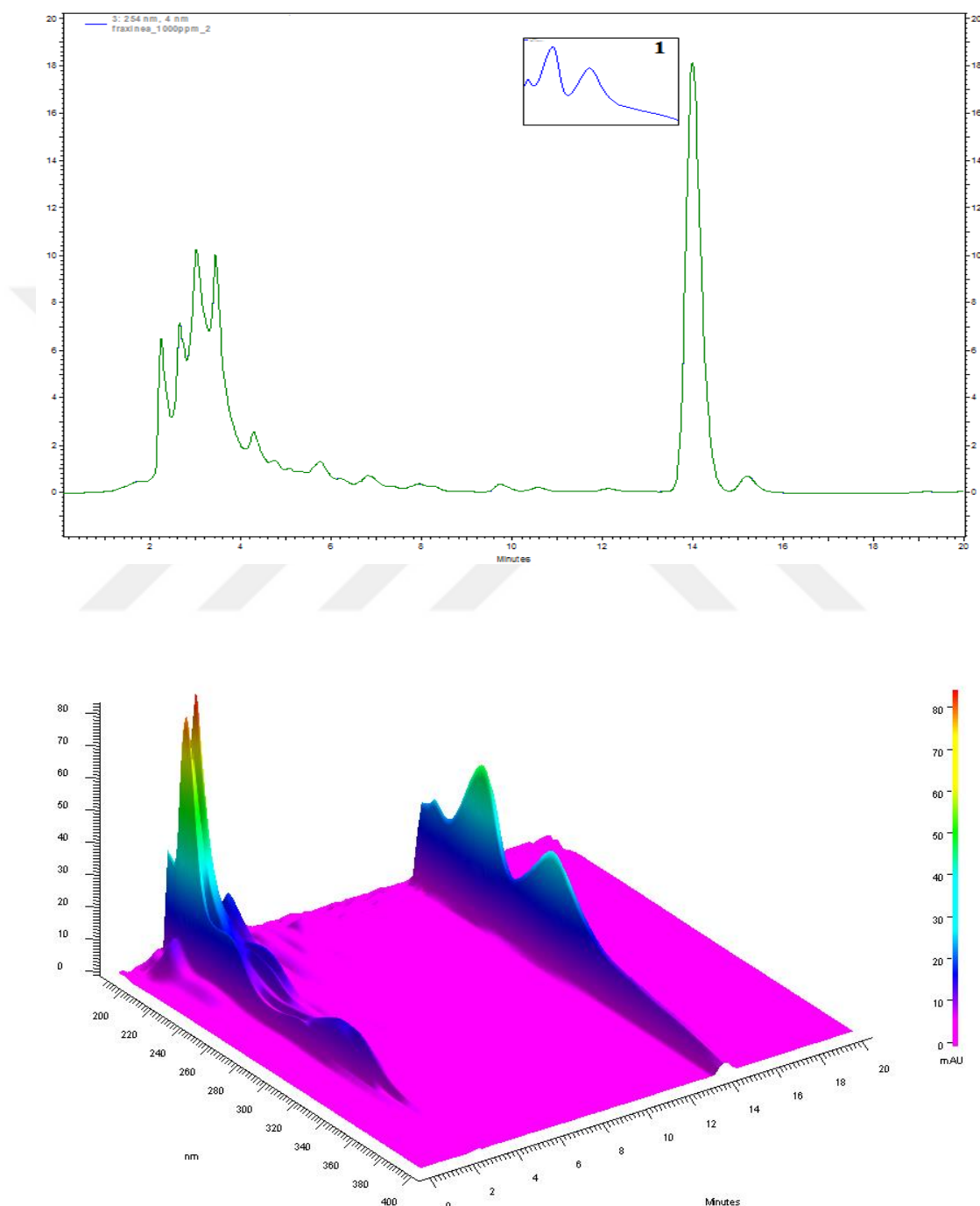


Figure 4.23. HPLC chromatogram of *R. fraxinea* with UV spectrum and 3D view.

Table 4.9. Chemical constituents of *Evernia prunastri* and their identification.

Peaks no	Compound	Retention time (min)	Absorbance maxima (nm) UV spectrum		
			Experimental values	Data from standards	Data from reference
1	Orsellinic acid	3.10	214, 260, 299		212, 262, 300
2	Evernic acid	6.86	213, 269, 305	212, 270, 304	212, 270, 304
3	Usnic acid	14,05	199, 233, 280	200, 233, 282	234, 282
4	Atranorin	15.25	210, 251, 320	210, 251, 320	210, 252, 262s, 320
5	Chloroatranorin	18.78	211, 249, 315, 348		212, 250, 314, 350

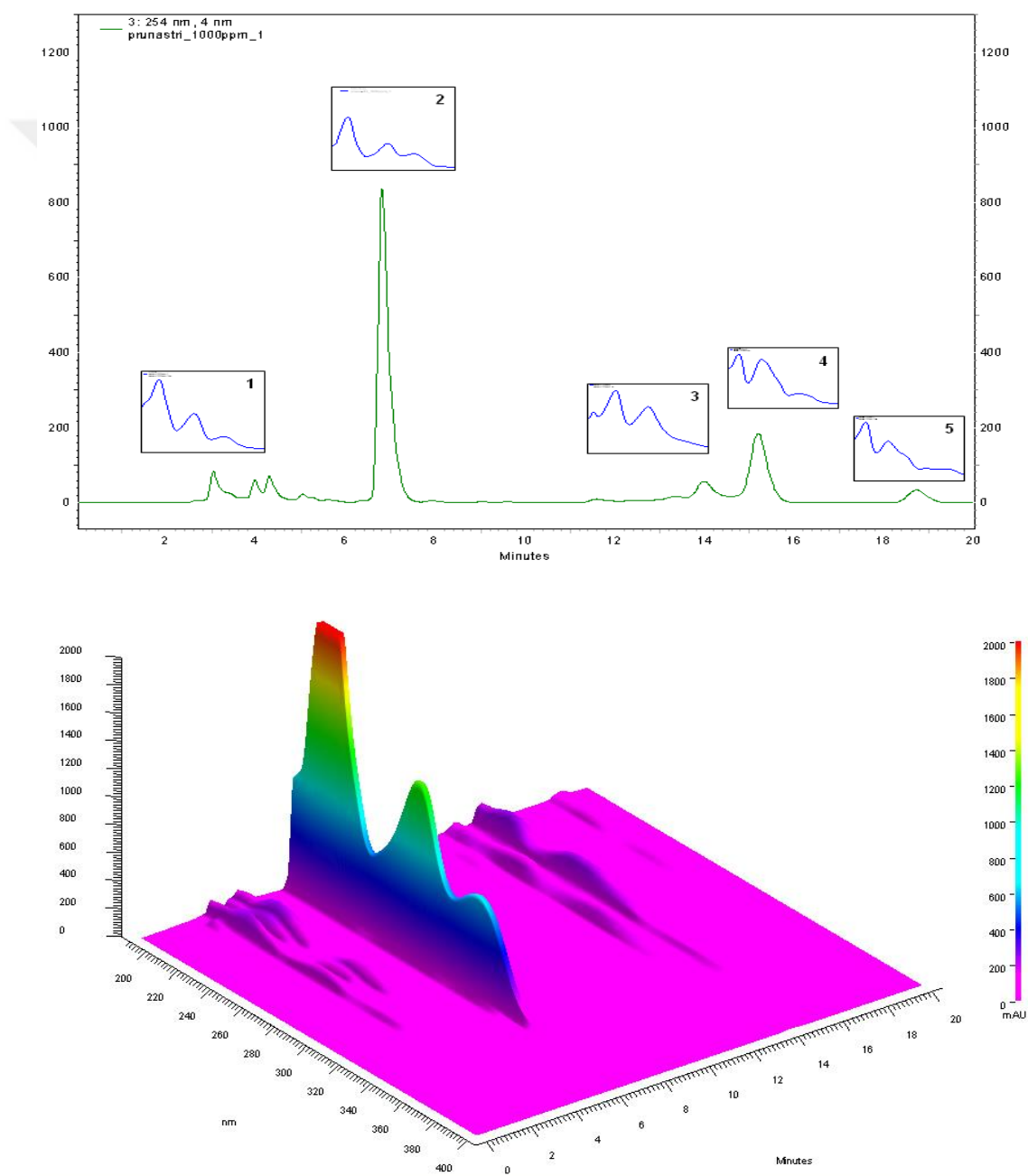


Figure 4.24. HPLC chromatogram of *E. prunastri* with UV spectrum and 3D view.

Table 4.10. Chemical constituents of *Cetraria islandica* and their identification.

Peaks no	Compound	Retention time (min)	Absorbance maxima (nm) UV spectrum		
			Experimental values	Data from standards	Data from reference
1	Protocetraric acid	4.58	212, 241, 318		212, 242, 320
2	Fumarprotocetraric acid	5.54	212, 239, 318	212, 240, 318	212, 240, 318
3	Usnic acid	14.08	200, 232, 280	200, 233, 282	234, 282

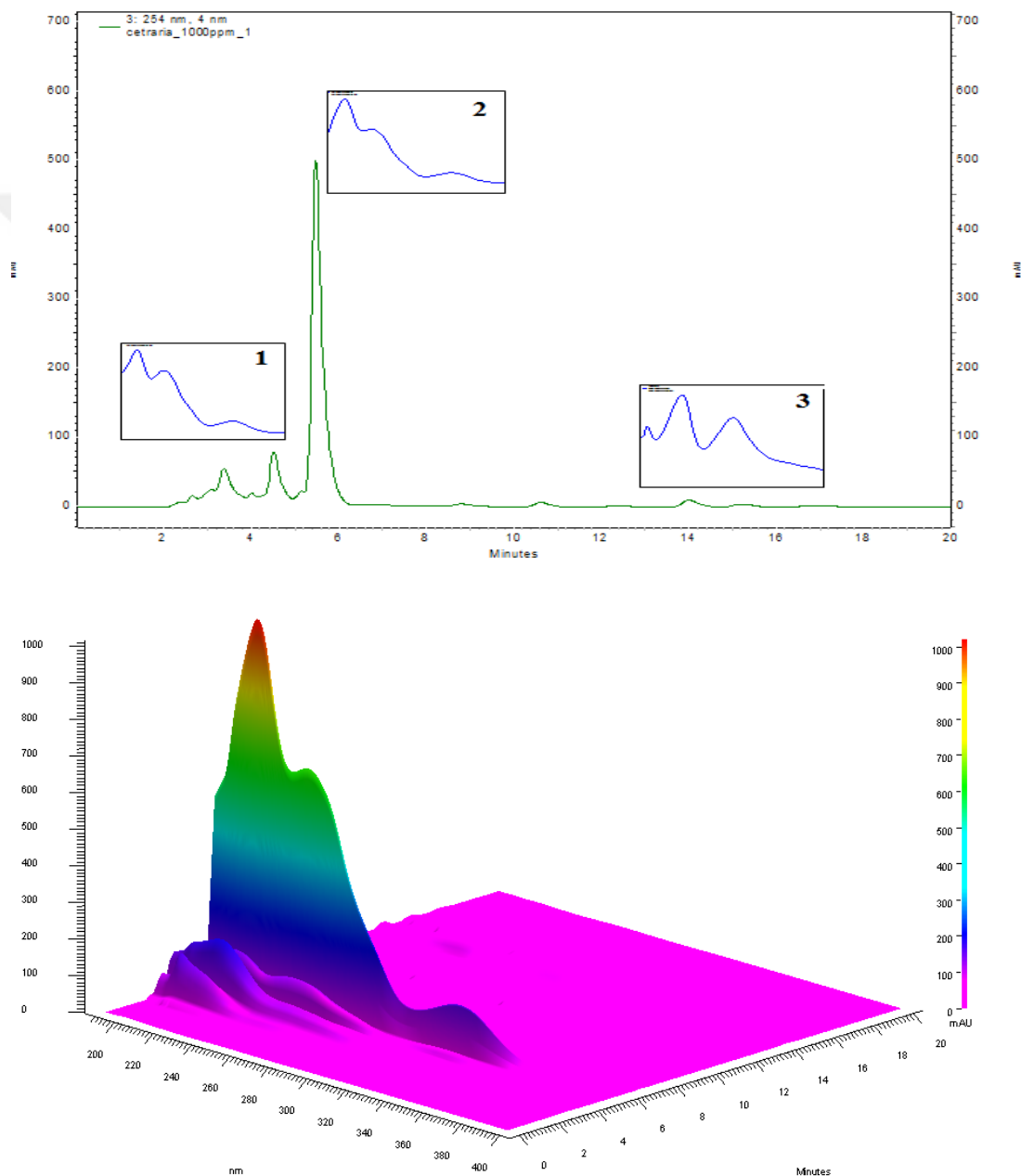


Figure 4.25. HPLC chromatogram of *C. islandica* with UV spectrum and 3D view.

Table 4.11. Chemical constituents of *Anaptychia ciliaris* and their identification

Peaks no	Compound	Retention time (min)	Absorbance maxima (nm) UV spectrum		
			Experimental values	Data from standards	Data from reference
1	Sekikaic acid	8.02	217, 263, 303		218, 264, 304
2	Gyrophoric acid	10.65	212, 270, 306		212, 270, 304
3	Usnic acid	13.96	200, 232, 280	200, 233, 282	234, 282
4	Atranorin	15.18	210, 250, 320	210, 251, 320	210, 252, 262s, 320

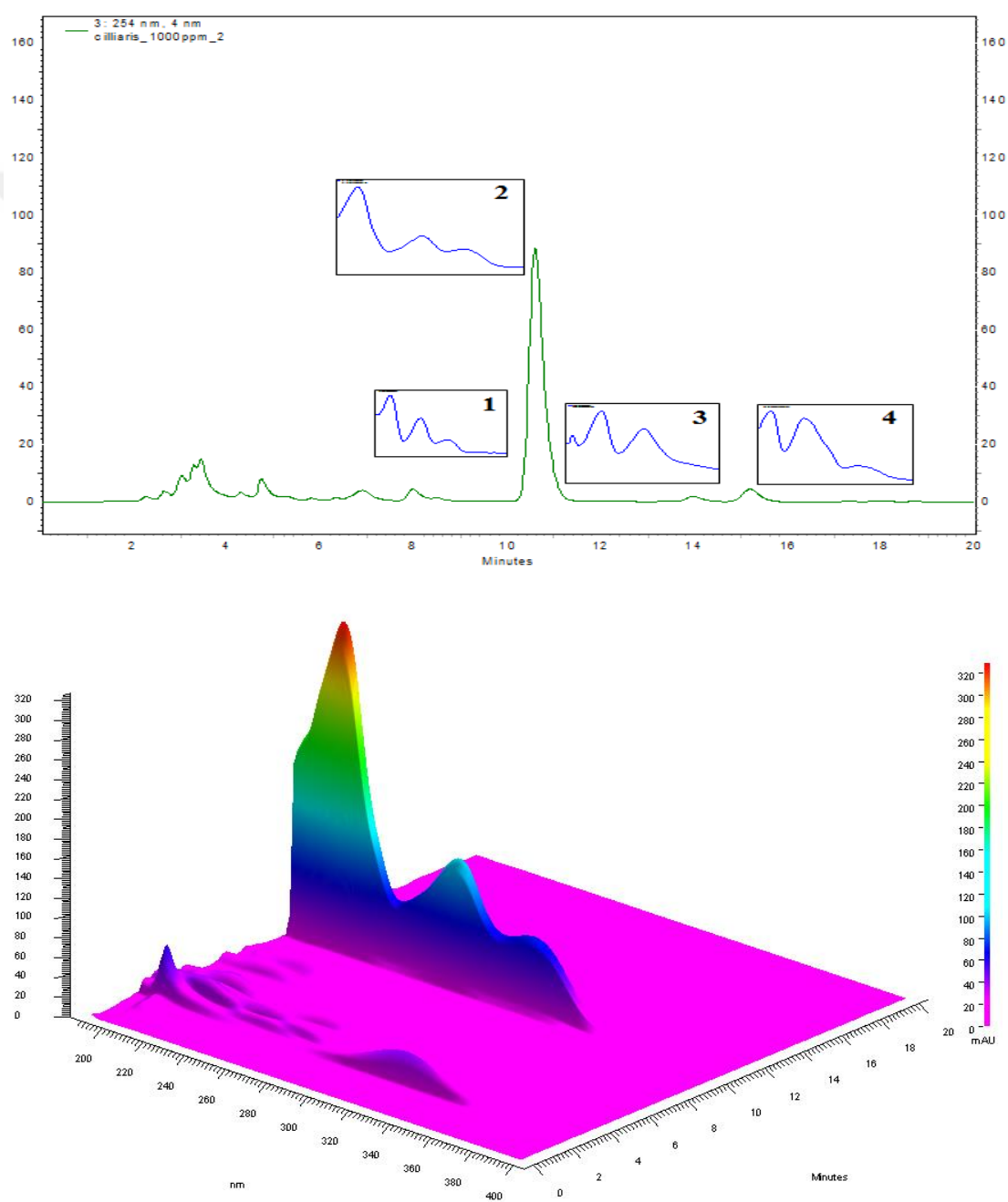


Figure 4.26. HPLC chromatogram of *A. ciliaris* with UV spectrum and 3D view.

Table 4.12. Chemical constituents of *Pseudoevernia furfuracea* and their identification.

Peaks no	Compound	Retention time (min)	Absorbance maxima (nm) UV spectrum		
			Experimental values	Data from standards	Data from reference
1	Physodic acid	6.99	210, 256, 314		212, 263, 314
2	Atranorin	15.25	210, 251, 320	210, 251, 320	210, 252, 262s, 320
3	Chloroatranorin	18.78	211, 249, 315, 348		212, 250, 314, 350

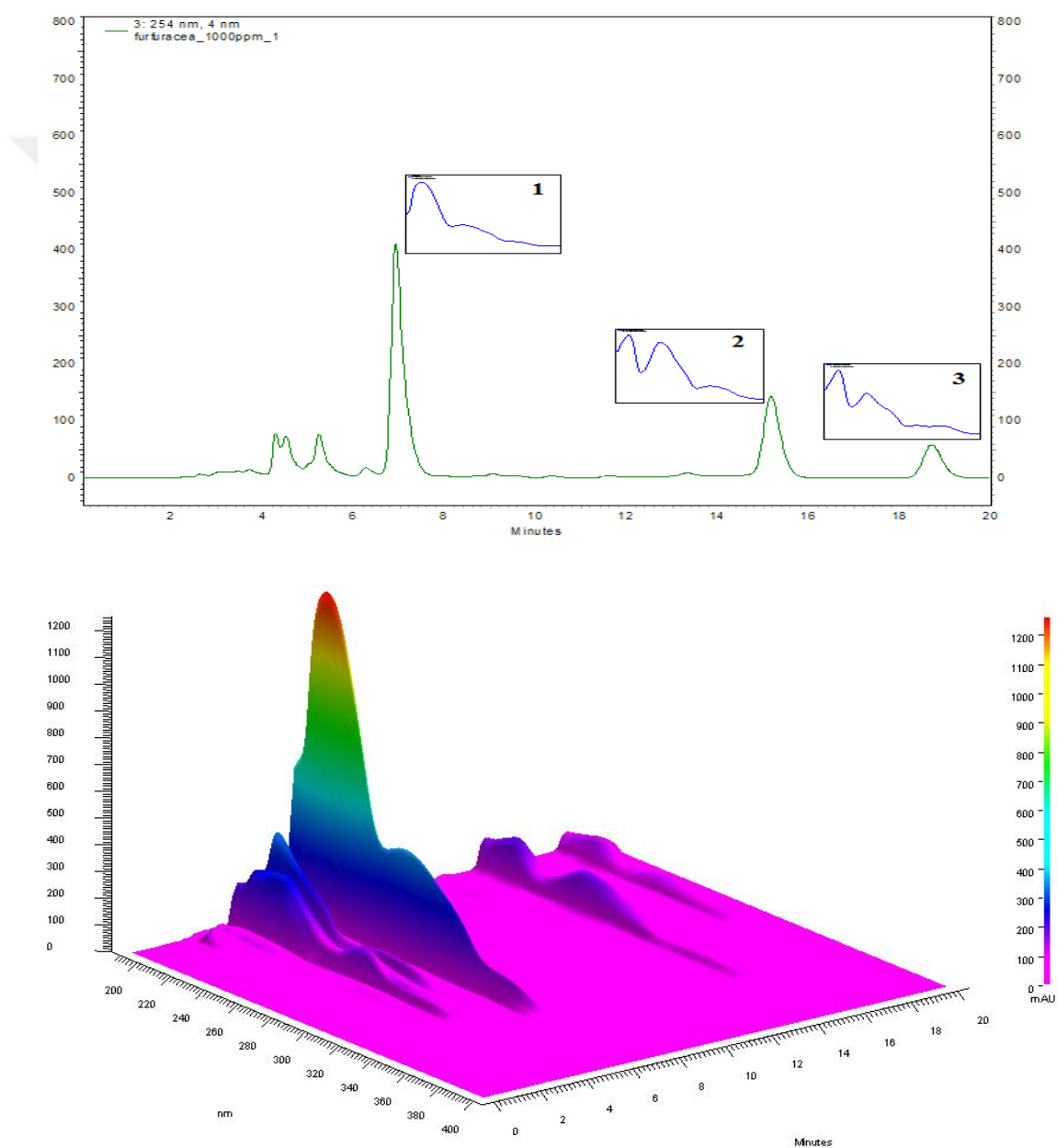


Figure 4.27. HPLC chromatogram of *P. furfuracea* with UV spectrum and 3D view.

Table 4.13. Chemical constituents of *Ramalina fastigiata* and their identification.

Peaks no	Compound	Retention time (min)	Absorbance maxima (nm) UV spectrum		
			Experimental values	Data from standards	Data from reference
1	Evernic acid	6.86	212, 268, 304	212, 270, 304	212, 270, 304
2	Sekikaic acid	8.04	217, 263, 304		218, 264, 304
3	Obtusatic acid	9.63	213, 277, 310		212, 278, 312s
4	Homosekikaic acid	12.16	216, 264, 304		215, 264, 304
5	Usnic acid	14.08	199, 232, 281	200, 233, 282	234, 282

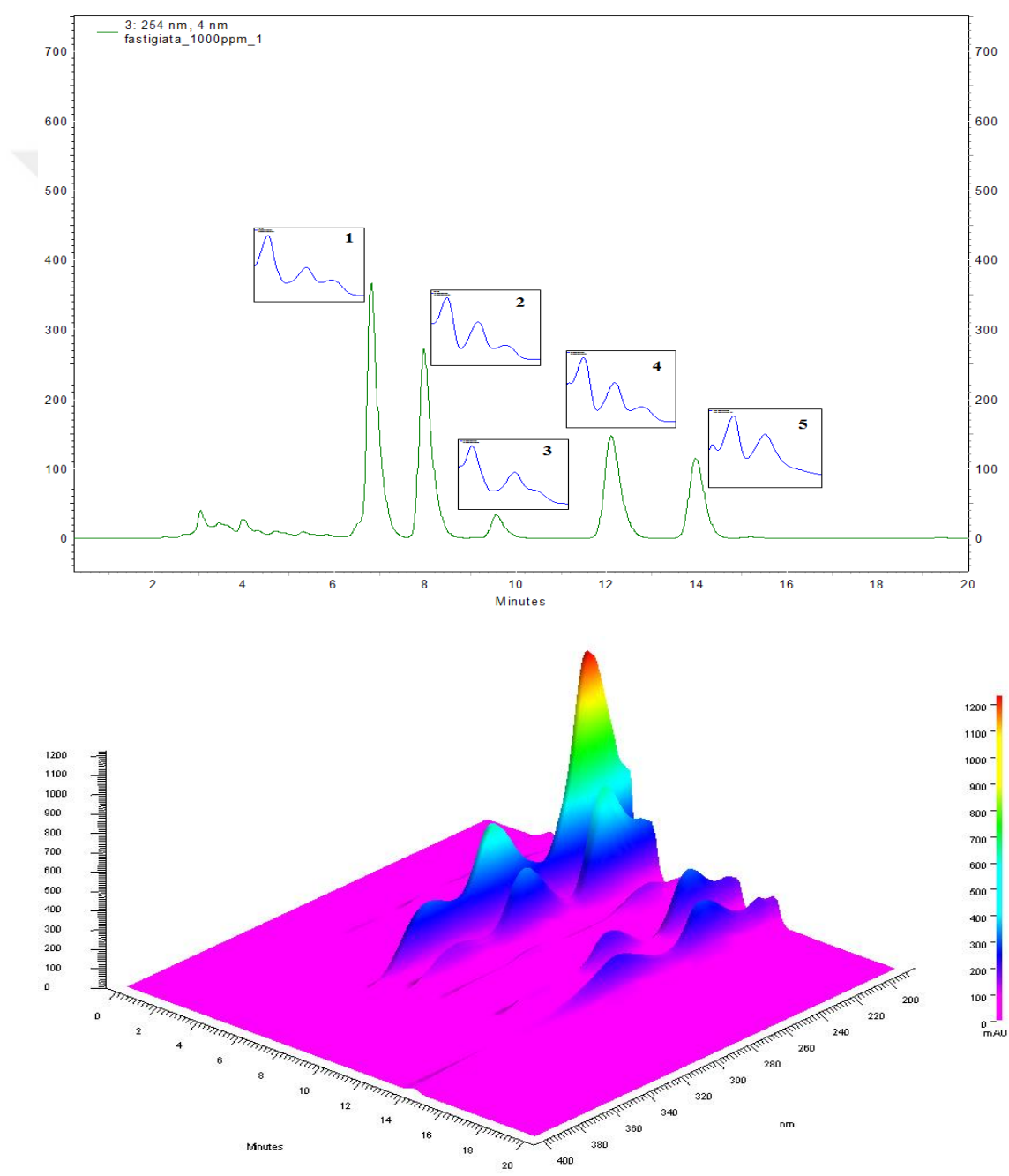


Figure 4.28. HPLC chromatogram of *R. fastigiata* with UV spectrum and 3D view.

Table 4.14. Chemical constituents of *Evernia divaricata* and their identification.

Peaks no	Compound	Retention time (min)	Absorbance maxima (nm) UV spectrum		
			Experimental values	Data from standards	Data from reference
1	Sekikaic acid	8.04	217, 263, 304		218, 264, 304
2	Divaricatic acid	10.78	213, 269, 305		212, 270, 304
3	Usnic acid	14.08	199, 232, 281	200, 233, 282	234, 282

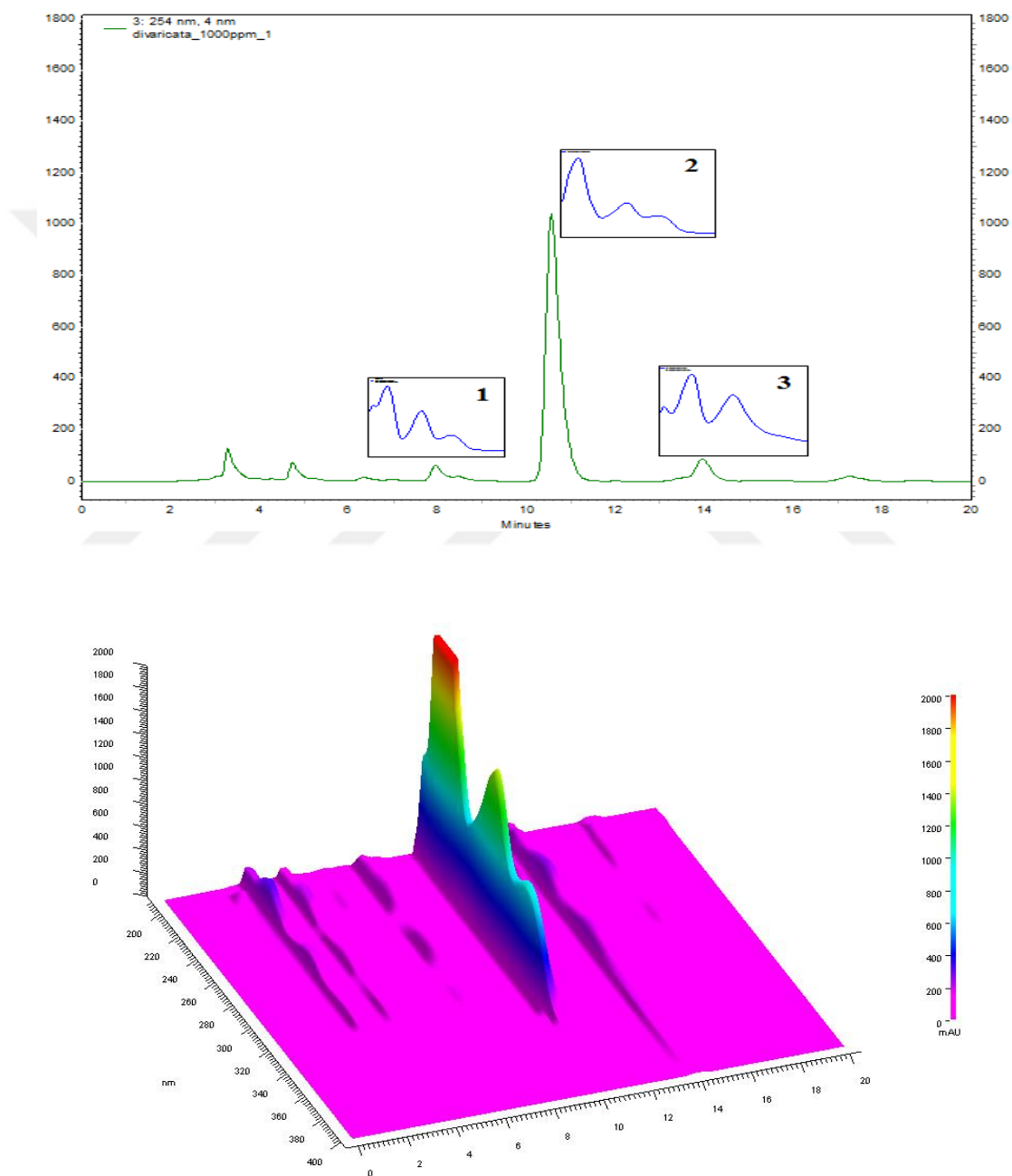


Figure 4.29. HPLC chromatogram of *E. divaricata* with UV spectrum and 3D view.

Table 4.15. Chemical constituents of *Letharia vulpina* and their identification.

Peaks no	Compound	Retention time (min)	Absorbance maxima (nm) UV spectrum		
			Experimental values	Data from standards	Data from reference
1	Vulpinic acid	6.70	202, 233, 274, 354		204, 234, 276, 354
2	Gyrophoric acid	10.65	212, 270, 306		212, 270, 304
3	Atranorin	15.18	209, 251, 320	210, 251, 320	210, 252, 262s, 320

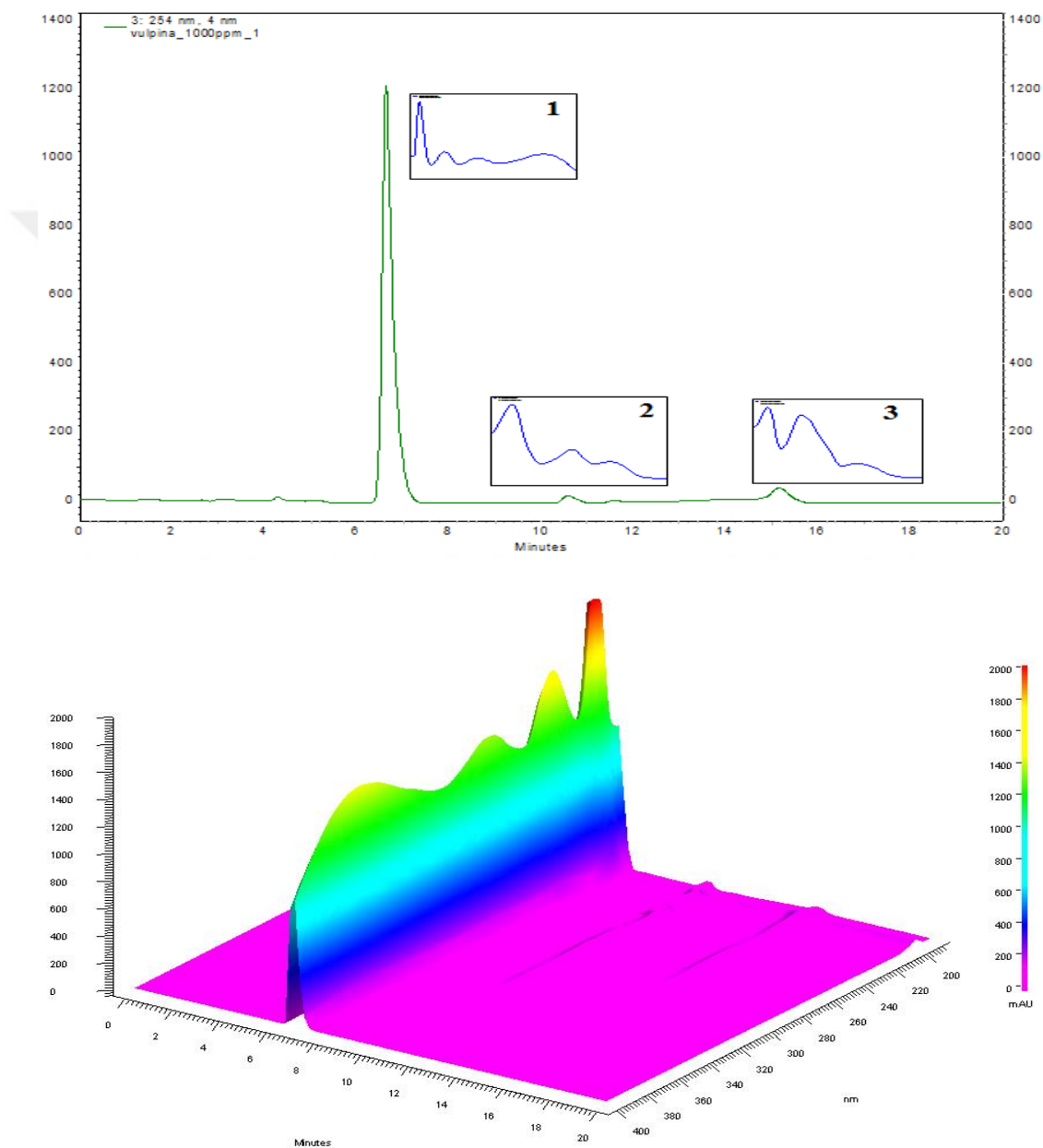


Figure 4.30. HPLC chromatogram of *L. vulpina* with UV spectrum and 3D view.

Table 4.16. Chemical constituents of *Platismatia glauca* and their identification.

Peaks no	Compound	Retention time (min)	Absorbance maxima (nm) UV spectrum		
			Experimental values	Data from standards	Data from reference
1	Norstictic acid	3.38	211, 237, 309		212, 238, 310
2	Atranorin	15.23	210, 251, 320	210, 251, 320	210, 252, 262s, 320
3	Chloroatranorin	18.76	211, 249, 316, 348		212, 250, 314, 350

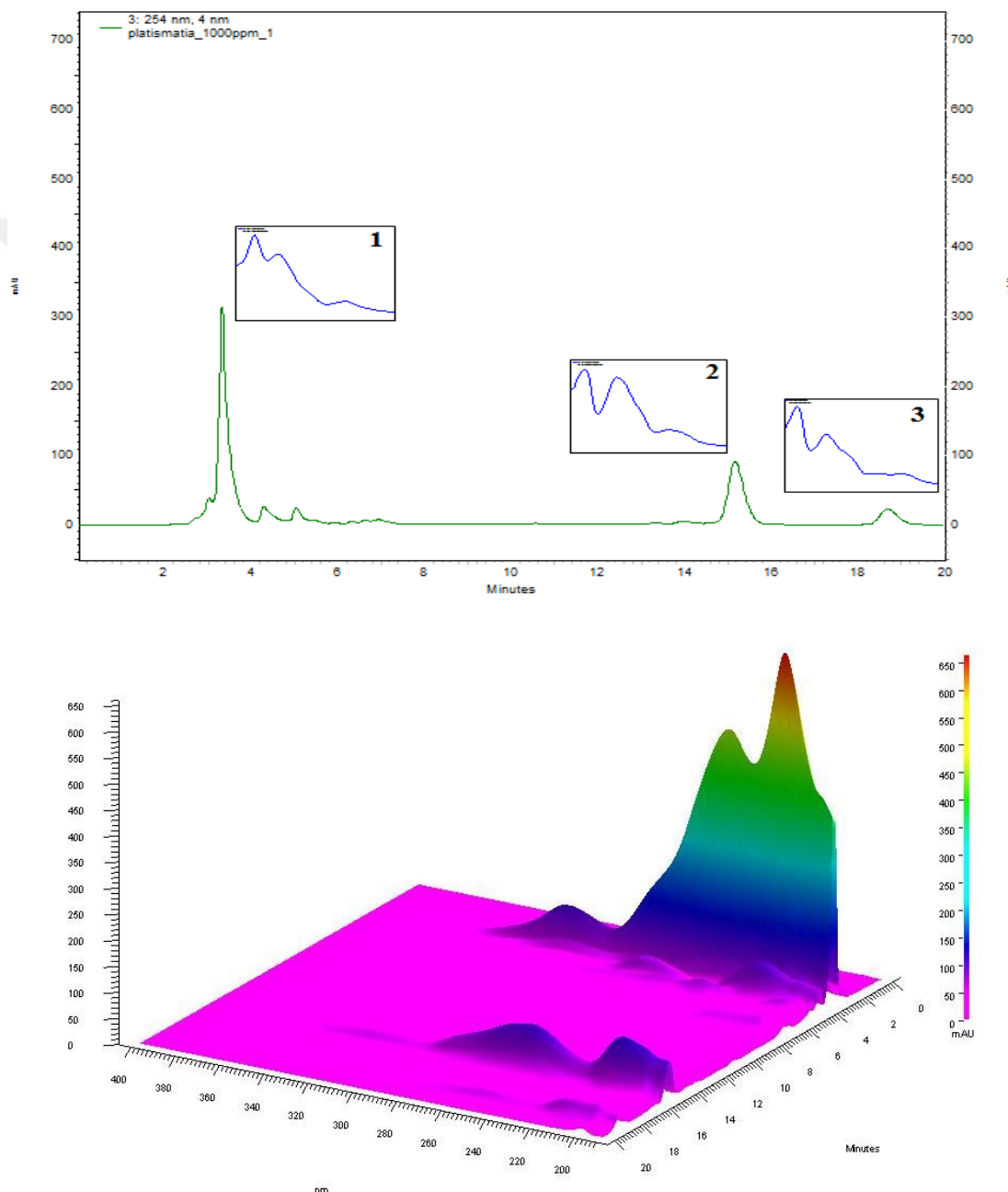


Figure 4.31. HPLC chromatogram of *P. glauca* with UV spectrum and 3D view.

Table 4.17. Chemical constituents of *Usnea florida* and their identification.

Peaks no	Compound	Retention time (min)	Absorbance maxima (nm) UV spectrum		
			Experimental values	Data from standards	Data from reference
1	Vulpinic acid	6.70	202, 233, 274, 354		204, 234, 276, 354
2	Gyrophoric acid	10.65	212, 270, 306		212, 270, 304
3	Usnic acid	13.79	200, 232, 281	200, 233, 282	234, 282

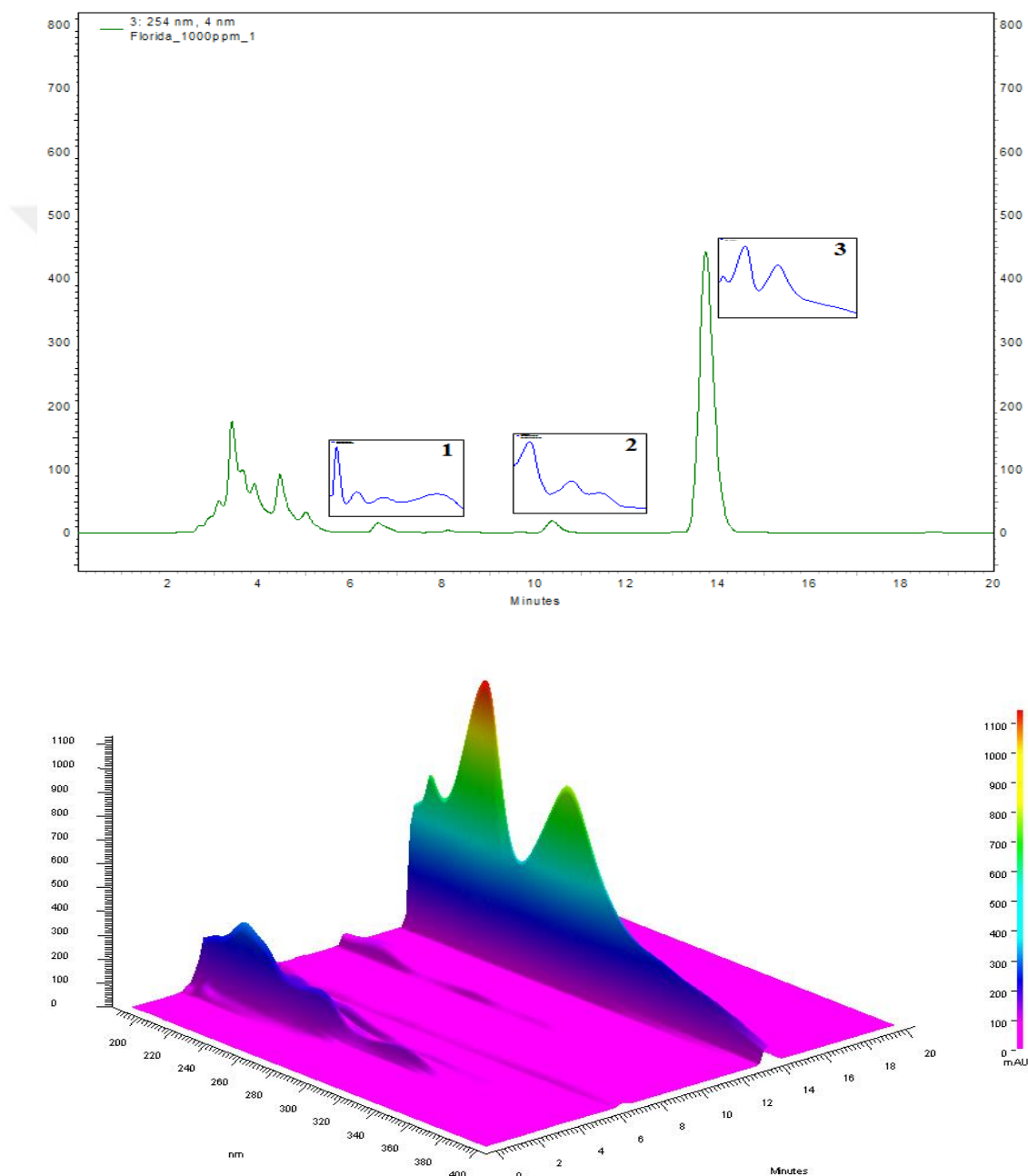


Figure 4.32. HPLC chromatogram of *U. florida* with UV spectrum and 3D view.

Table 4.18. Chemical constituents of *Lobaria pulmonaria* and their identification.

Peaks no	Compound	Retention time (min)	Absorbance maxima (nm) UV spectrum		
			Experimental values	Data from standards	Data from reference
1	Constictic acid	2.95	213, 310		212, 310
2	Stictic acid	3.42	213, 306	212, 306	212, 306
3	Norstictic acid	4.38	211, 237, 309		212, 238, 310

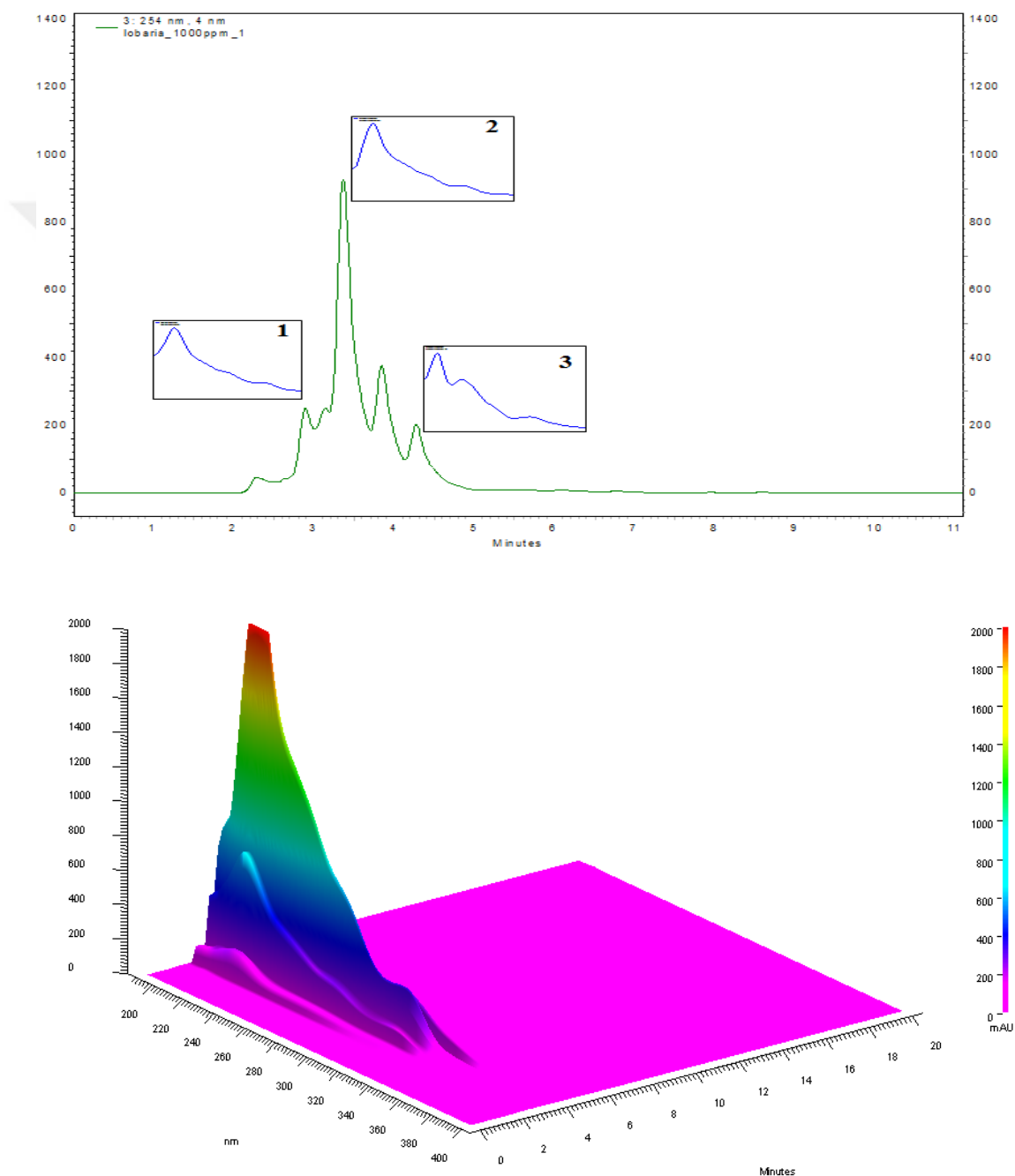


Figure 4.33. HPLC chromatogram of *L. Pulmonaria* with UV spectrum and 3D view.

Table 4.19. Chemical constituents of *Ramalina farinacea* and their identification.

Peaks no	Compound	Retention time (min)	Absorbance maxima (nm) UV spectrum		
			Experimental values	Data from standards	Data from reference
1	Norstictic acid	4.34	212, 237, 308		212, 238, 310
2	Hypoprotocetraric acid	5.36	215, 257		216, 258
3	Gyrophoric acid	10.65	212, 269, 305		212, 270, 304
4	Usnic acid	13.96	200, 232, 280	200, 233, 282	234, 282

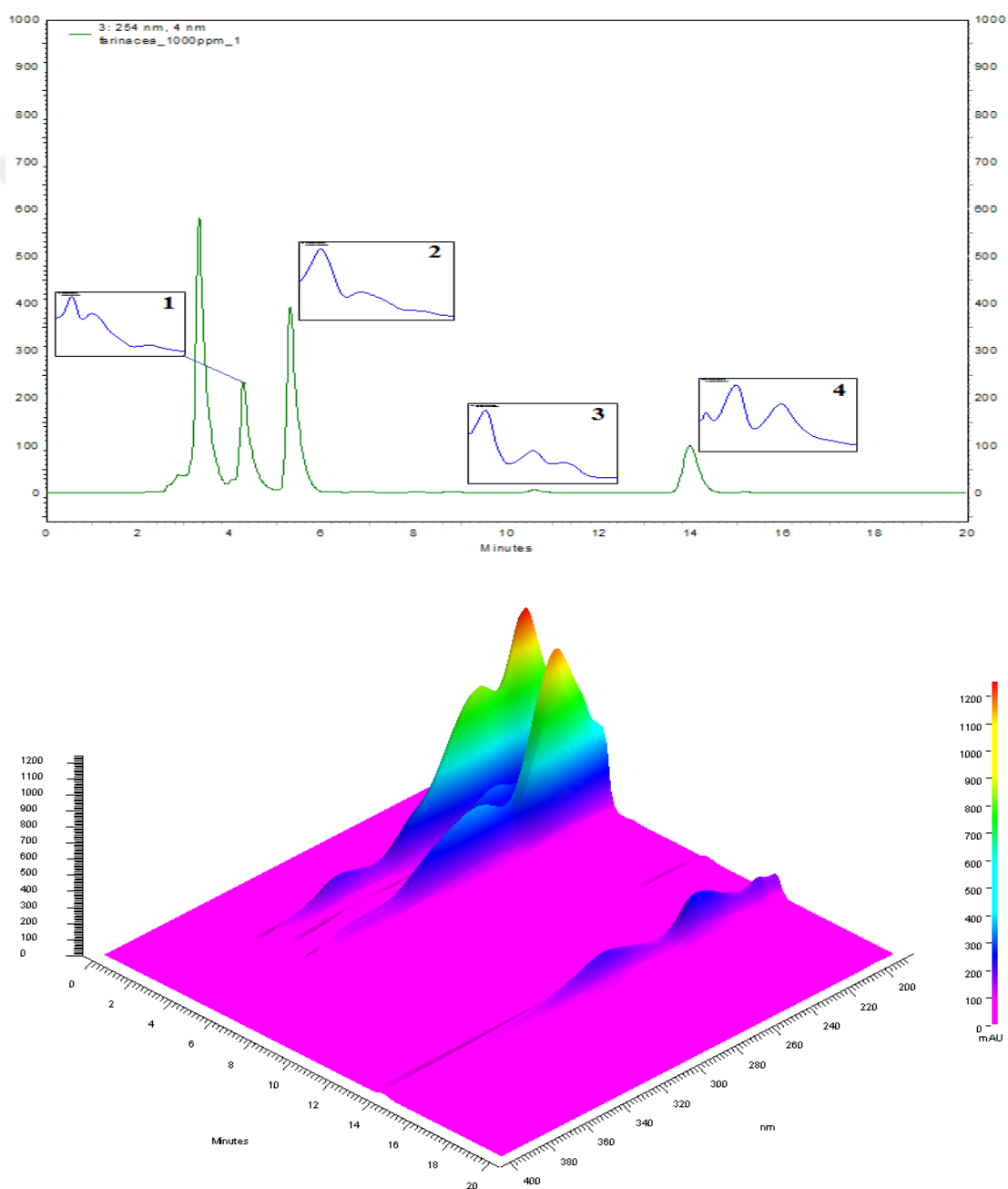


Figure 4.34. HPLC chromatogram of *R. farinacea* with UV spectrum and 3D view.

Table 4.20. All detected lichen compounds by HPLC-DAD analysis.

Lichen Compounds	Class	Retention Time	UV Spectrum	Lichen Species
Constictic acid	Depsidone	2,95	213, 310	<i>L. pulmonaria</i>
Stictic acid	Depsidone	3,47	212, 306	<i>L. pulmonaria</i>
Norsitictic acid	Depsidone	4,38	212, 238, 310	<i>B. capillaris</i> , <i>P. glauca</i> , <i>L. pulmonaria</i> , <i>R. farinacea</i>
Protocetraric acid	Depsidone	4,58	212, 242, 320	<i>C. islandica</i>
Hypoprotocetraric acid	Depsidone	5,36	216, 258	<i>R. farinacea</i>
Fumarprotocetraric acid	Depsidone	5,42	212, 240, 318	<i>C. islandica</i>
Psoromic	Depsidone	6,48	208, 240	<i>B. capillaris</i>
Vulpinic acid	Pulvinic acid derivatives	6,7	204, 234, 276, 354	<i>L. vulpina</i> , <i>U. florida</i>
Evernic acid	Depside	6,77	212, 270, 304	<i>E. Prunastri</i> , <i>R. fastigiata</i>
Physodic acid	Depsidone	6,99	212, 264, 314	<i>P. furfuracea</i>
Sekikaic acid	Depside	8,02	218, 264, 304	<i>A. ciliaris</i> , <i>R. fastigiata</i> , <i>E. divaricata</i>
Obtusatic acid	Depside	9,63	212, 278, 312	<i>R. fastigiata</i>
Gyrophoric acid	Depside	10,65	212, 270, 304	<i>A. ciliaris</i> , <i>L. vulpina</i> , <i>U. florida</i> , <i>R. farinacea</i>
Divaricatic acid	Depside	10,78	212, 270, 304	<i>E. divaricata</i>
Homosekikaic acid	Depside	12,16	216, 264, 304	<i>R. fastigiata</i>
Usnic acid	Dibenzofuran	13,7	234, 282	<i>B. capillaris</i> , <i>R. fraxinea</i> , <i>E. prunastri</i> , <i>C. Islandica</i> , <i>A. ciliaris</i> , <i>R. fastigiata</i> , <i>E. divaricata</i> , <i>U. florida</i> , <i>R. farinacea</i>
Atranorin	Depside	14,87	210, 252, 320	<i>E. prunastri</i> , <i>A. ciliaris</i> , <i>P. furfuracea</i> , <i>L. vulpina</i> , <i>P. glauca</i>
Chloroatranorin	Depside	18,77	212, 250, 314, 350	<i>E. prunastri</i> , <i>P. furfuracea</i> , <i>P. glauca</i>
Orsellinic acid	Hydroxy benzoic acid derivatives	3,10	212, 262, 300	<i>E. prunastri</i>

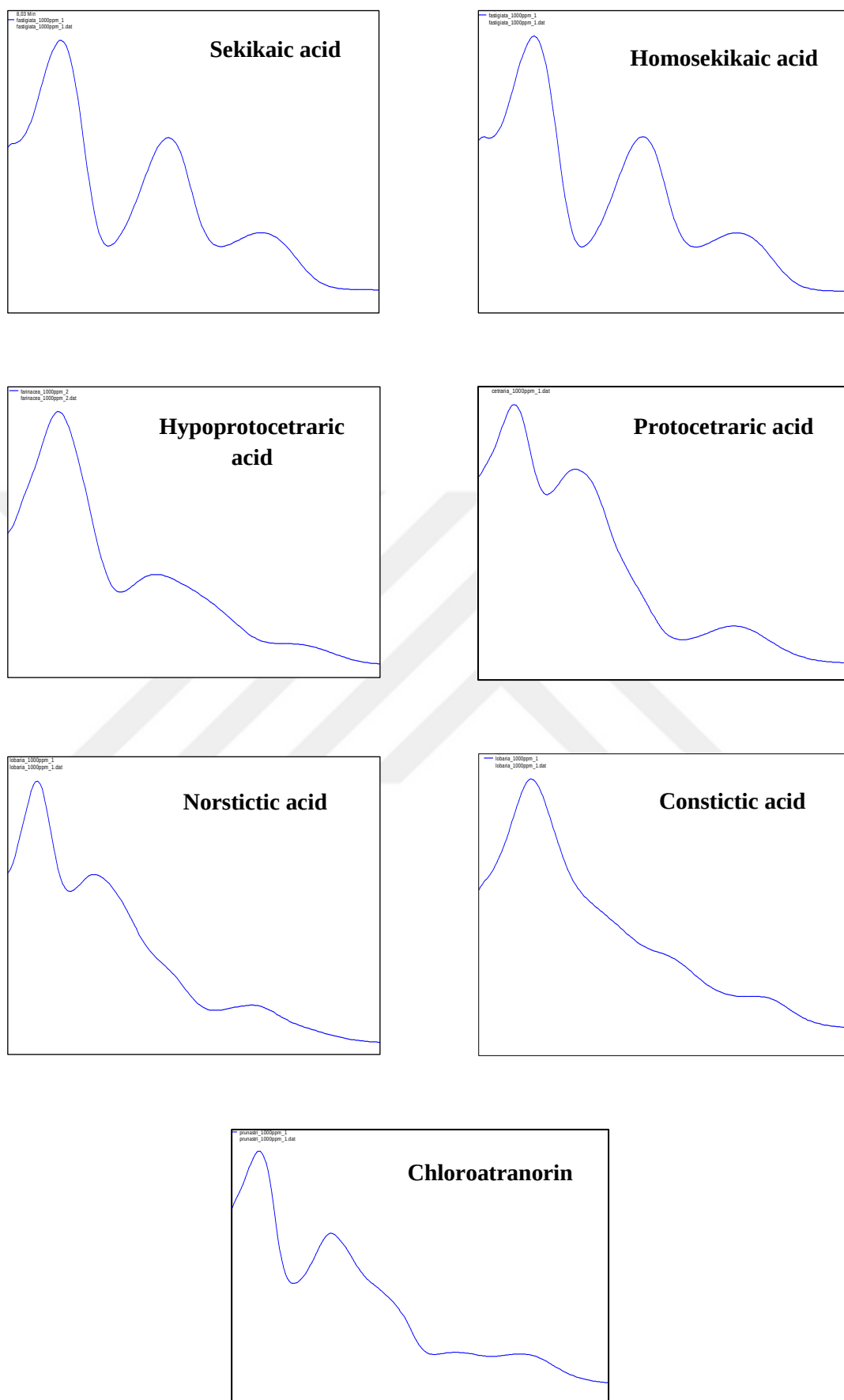


Figure 4.35. Identified UV spectra of lichen compounds by reference.

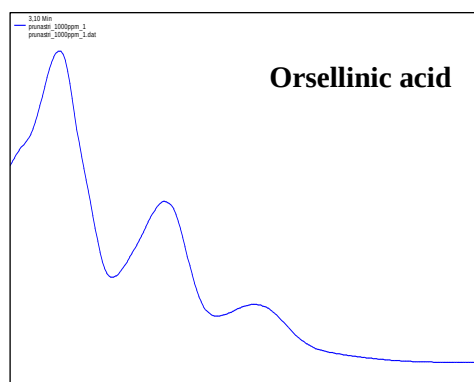
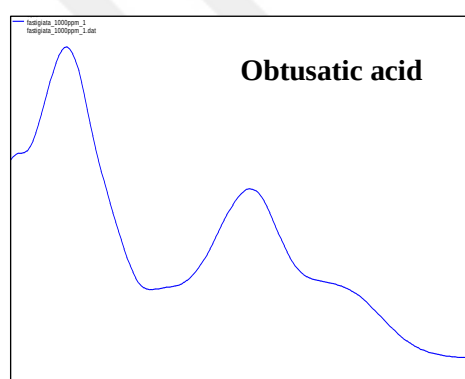
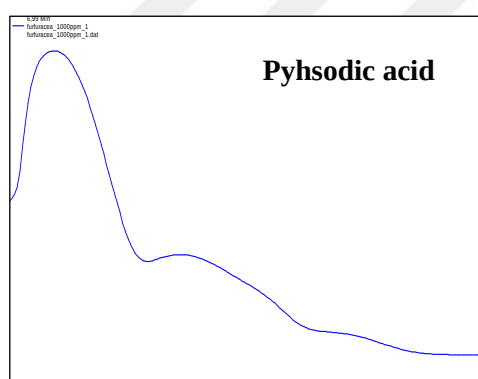
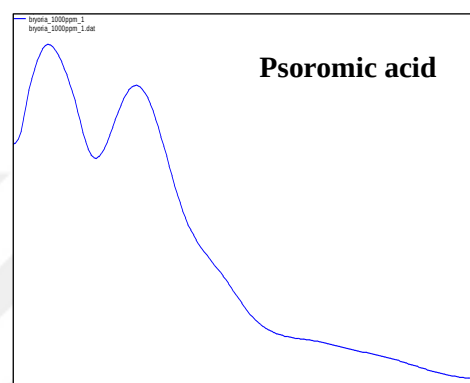
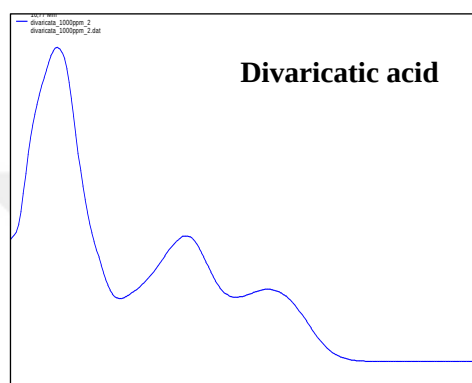
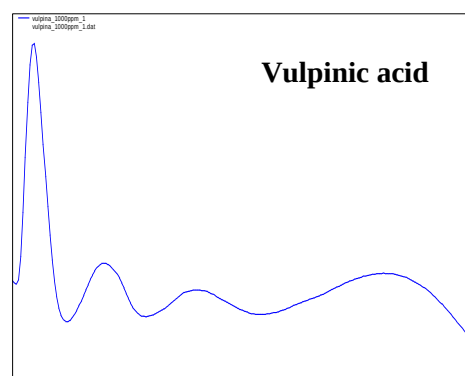
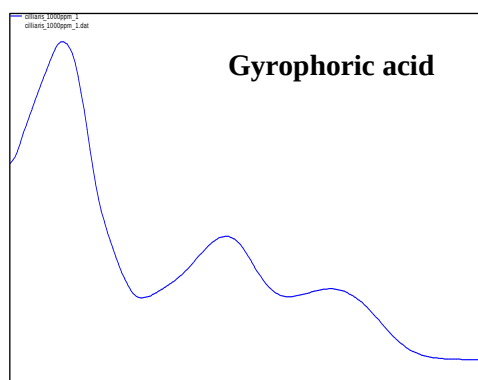


Figure 4.35 continued. Identified UV spectra of lichen compounds by reference.

5. CONCLUSION

Lichens are symbiotic systems consisting of a filamentous fungus and a photosynthetic partner (an eukaryotic alga and/or cyanobacterium). They have been used as ingredients in folk medicines for centuries and many cultures have used lichens to treat a variety of ailments as part of their traditional medicines (Dayan & Romagni, 2001). Demonstrated biological roles based on lichen chemistry include: antibiotics, antiproliferative, antioxidants, antiinflammatory, anti-HIV, anti-cancer, immunomodulation, and anti-protozoans. Although lichens are one of the more promising reservoirs of secondary compounds demonstrating some level of biological activity; a very limited number of compounds have been studied (Boustie & Grube, 2005).

Antibacterial activities of 39 extracts obtained from 13 lichen species were evaluated against 10 bacterial strains. Among the all lichen extracts, methanol extracts had better antibacterial activity than acetone and aqueous extracts. The best inhibitory activity was obtained with methanol extracts of *Ramalina fraxinea* (2M) against eight out of ten tested bacterial strains in the range of 9.5 ± 0.3 – 30.5 ± 1.3 mm in diameter. The weakest antibacterial activity was showed by methanol extract of *Anaptychia ciliaris* (5M) against only two strains; *S.epidermidis* (10.8 ± 0.5 mm) and *S.pyogenes* (12.3 ± 0.6 mm) (Table 4.1). *B. capillaris*, *R. fraxinea*, *P. furfuracea*, *R. fastigiata*, *L. vulpina*, *P. glauca* and *U. florida* showed antibacterial activity against some tested bacterial strain for the first time in this study. Moreover, MeOH extract of *R. fraxinea* (2M) exhibited the best antibacterial activity against *P. aeruginosa* with the inhibition zones of 18.8 ± 0.8 mm. This result was even better than all reference antibiotics. *K. pneumonia* was only inhibited by methanol extract of *R. fastigiata* (7M) as compared to all the lichen samples. It also exhibited better activity than some reference antibiotics. Among the all lichens tested, acetone extract of *L. vulpina* (9A) exhibited the strongest antibacterial activity against *S. aureus* with the inhibition zone of 31.5 ± 0.5 mm. Antibacterial activity of methanol extract of *U. florida* (11M) against *S.epidermidis* was the best activity when compared the all other lichen species. When compared with all lichen species, the acetone extract of *L.*

pulmonaria (12A) showed the best activity against *S. pyogenes*. This result was better than some reference antibiotics.

Antioxidant capacity of lichen species was determined by screening DPPH radical scavenging activity, total phenolic contents and total flavonoid contents. At 50 µg/ml (minimum active concentration), methanol extract of *Pseudoevernia furfuracea* (6M) exhibited the strongest DPPH radical scavenging activity (81.1 %). Except *Bryoria capillaris* (1M) and *Lobaria pulmonaria* (12M), all extracts of lichens indicated gradually antioxidant activities at 100 µg/ml and 200 µg/ml. Among the all extracts, *Bryoria capillaris* (1M) included the most phenolic content with 163.3 ± 0.00 mg Gallic acid equivalent/ g dried extract and the highest flavonoid contents with 154.5 ± 0.001 mg catechol equivalent/ g dried extract. Antioxidant capacity of *L. vulpine* and total flavonoid content of *R. fraxinea*, *P. furfuracea*, *R. fastigiata*, *E. divaricate*, *U. florida* and *L. pulmonaria* were determined for the first time with this study.

The cytotoxic effects of 18 crude extracts (acetone, methanol and water) of *R. fraxinea*, *C. islandica*, *L. vulpina*, *P. glauca*, *U. florida* and *L. pulmonaria* at various concentrations were evaluated with *in vitro* cytotoxicity assay against MCF-7 (human breast cancer) and HepG2/C3A (human hepatocellular carcinoma) cell lines. Except *C. islandica*, acetone extract of all tested species revealed the antiproliferative activity. while methanol or water extract of *R. fraxinea* didn't show any cytotoxic activity, acetone extract exhibited antiproliferative activity against HepG2/c3A cell line (148.3 ± 2.2 µg/ml). This is the first report for antiproliferative activity of *R. fraxinea*. Antiproliferative activity of acetone extract of *L. vulpina* were evaluated for the first time on HepG2/C3A cell line (175.7 ± 1.4 µg/ml) in our study. Acetone extract of *U. florida* showed antiproliferative activity against HepG2/c3A cell line (181.9 ± 2.1 µg/mL) for the first time. *L. pulmonaria* exhibited the highest cytotoxic activity on HepG2/C3A (90.2 ± 1.2 µg/mL) among the all tested lichen species. There has been no study about antiproliferative activity of *L. pulmonaria* up to now.

In this study , quantification of chosen five standards (usnic acid, atranorin, stictic acid, evernic acid and fumarprotocetraric acid) in 13 lichen species was performed by HPLC. Also, 14 different lichen compounds in the lichen species were only identified qualitatively by comparing of the absorbance spectral data in the

literatures (Yoshimura et al., 1994). Generally, acetone extracts had higher lichen compound content than methanol extracts. While acetone extract of *Usnea florida* was detected as usnic acid source significantly, four species (*P. furfuracea*, *L. vulpina*, *P. glauca* and *L. pulmonaria*) didn't possess any amount of usnic acid. Stictic acid was only found in methanol and acetone extracts of *L. pulmonaria*. Atranorin was detected in five different species; *E. prunastri*, *A. ciliaris*, *P. furfuracea*, *L. vulpina* and *P. glauca*. The highest amount of atranorin was found in acetone extract of *P. furfuracea*. Evernic acid was determined in *Evernia prunastri* and *Ramalina fastigiata*. Acetone extract of *E. prunastri* is very important source of evernic acid. Fumarprotocetraric acid was only found in *Cetraria islandica* (Iceland moss). Moreover, norstictic acid, psoromic acid and usnic acid were identified in *B. capillaris* and these compounds were reported for the first time. *R. fastigiata* included evernic acid, sekikaic acid, obtusatic acid, homosekikaic acid and usnic acid. Sekikaic acid, homosekikaic acid and obtusatic acid were identified for the first time in *R. fastigiata*. While sekikaic acid was detected for the first time in *Evernia divaricata*, Gyrophoric acid was also identified for the first time in *L. vulpina*. we identified norstictic acid, atranorin and chloroatranorin in *P. glauca*. This study was the first identification of chloroatranorin and norstictic acid in *P. glauca*. We revealed that usnic acid and vulpinic acid were found in *Usnea florida* by comparison of retention time and UV spectra in HPLC. Vulpinic acid was reported firstly in this study. We detected hypoprotocetraric acid, norsitictic acid and usnic acid in *R. farinacea* by comparing the retention times and UV spectra (200-400 nm) from HPLC-DAD. Hypoprotocetraric acid and gyrophoric acid were identified in *R. farinacea* for the first time.

Herein, the results confirmed the antibacterial and antioxidant activities of 13 lichen species and antiproliferative activities of 6 lichen species. Definitive clinical studies are needed to fully understand different medicinal uses lichens and their compounds, but some properties of lichens still need consideration. Their slow growth, both in nature and in axenic cultures, may be regarded as a major problem for the production of metabolites (Shukla et al. 2010). In recent years, methods for cultivating lichen mycobionts and lichen tissue have been developed, and these have given rise to hopes for the production of metabolites which are otherwise difficult to obtain. Another promising technique may prove to be the transfer of genes which are

responsible for the synthesis of lichen substances into other rapidly growing organisms. After solving the problems, most of tested lichen compounds may be a suitable source for drugs that could improve the treatment of infections caused by Gram positive and Gram negative bacteria and also it can be a source of food additives as antioxidant due to preventing the liposomal oxidation and prevention the cancer in diet.



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7. CURRICULUM VITAE

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Education:

September 2008 - June 2013 *Undergraduate* – Abant İzzet Baysal University,
Faculty of Science, Biology Department Bolu,
TURKEY

February - June 2013 *Undergraduate via Erasmus Programme* –
Jagiellonian University, Pharmacognosy
Department, Krakow, **POLAND**

July 2013 – November 2015 *M.Sc.*– Abant İzzet Baysal University, Graduate
School of Natural and Applied Sciences,
Department of Biology, Bolu, **TURKEY**

Work Experience

2011 - 2014 **Volunteer President** - University Society of
The TEMA Foundation (The Turkish
Foundation for Combating Soil Erosion, for
Reforestation and the Protection of Natural
Habitats)

2011-... **Volunteer Trainer** - Educational Volunteers
Foundation of Turkey (TEGV)

2014 - ...

Volunteer Trainer - TEMA Foundation's Project "Step to Save the Earth".

Skills and abilities

Computer

Knowledge of MS Project, Word, Excel, Powerpoint

Language

English

Analytical skill

HPLC Analysis
TLC Analysis

Laboratory skill

Antibacterial activity-Disc Diffusion Method
Antioxidant Activities-Free Radical Scavenging
DPPH Method, Total Phenol and Flavonoid
Assays

Fields of Research:

Lichenology

Research Projects

Determination of antiproliferative activities of some lichens collected from Bolu, Turkey, Abant İzzet Baysal University Scientific Research Project, Project number: 2013.03.01.591, **Researcher**, 2013.

Phytochemical analysis of the selected lichen metabolites, Jagiellonian University Scientific Research Project, **Researcher**, 2013.

Determination of biological activities (antibacterial and antioxidant) of some lichen species collected from Bolu and analyses of some lichen metabolites, Abant İzzet Baysal University Scientific Research Project, Project number: 2014.03.01.702, **Researcher**, 2014- continuing.

In vitro regeneration of *Astragalus gymolobus* Fischer, an endemic plant, and antioxidant capacities and comparison of phenolic constituents of plant materials obtained from two different sources (in vitro-grown and field-grown), The Scientific and Technological Research Council of Turkey (TUBİTAK) Project, Project Number: 114Z098, **Scholarship Holder** 2014-2015.

Scientific Publications and Presentations:

Tas I, Yıldırım AB, Ozyigitoglu G, Turker AU (2015). Biological Activity and HPLC Analysis of Some lichens collected from Bolu, I. National Plant Biology Congress, Poster Presentation, Bolu, Turkey

Tas I, Yıldırım AB, Ozyigitoglu G, Turker AU (2015). HPLC Analysis and Determination of Biological Activity of Three Different *Ramalina* Species, I. National Plant Biology Congress, Poster Presentation, Bolu, Turkey

Tas I, Yıldırım AB, Ozyigitoglu G, Turker AU (2015). Antibacterial Activity and HPLC Analysis of Two Different Lichen Species, II. National Mycology Symposium, Poster Presentation, Istanbul, Turkey

Yıldırım AB, Tas I, Özkan E, Turker AU (2015). Antibacterial Activity and Phenolic Analysis of Some Medical Plants Growing in Bolu, I. National Plant Biology Congress, Poster Presentation, Bolu, Turkey

Özkan E, Karakas FP, Yıldırım AB, Tas I, Eker I, Yavuz MZ, Turker AU (2015). Antibacterial, antioxidant, antiproliferative effects of *Inula viscosa* and Determination of Phenolic Content, I. National Plant Biology Congress, Poster Presentation, Bolu, Turkey

Yıldırım AB, Tas I, Uyar E, Turker AU (2015). Tissue Culture and HPLC Studies of an Endemic Plant, *Astragalus gymnolobus*, I. National Plant Biology Congress, Poster Presentation, Bolu, Turkey

Congress, Symposium and Workshop Attended

Workshop Lichens Culture Methods and Practises

Workshop General Characteristics of Macrofungi and Basic Identification Techniques

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