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Phenolic screening and biological activities of *Chenopodium botrys* L. extracts

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Chenopodium botrys L. ekstraktlarının fenolik taraması ve biyolojik aktiviteleri

Abstract: The genus *Chenopodium* is a genus that includes over 200 species and contains annual or perennial herbaceous flowering plant species scattered almost worldwide. While species belonging to the *Chenopodium* genus are used to treat various diseases, modern pharmaceutical research has revealed their strong anticancer, antifungal and antibacterial potential. In this study, some biological effects of *Chenopodium botrys* (methanol and ethanol) extracts were evaluated by helping some analytical approaches. The antioxidant and cytotoxic potentials of the extracts were revealed. In this study, 4 phenolic components in the extracts were determined with the HPLC technique and to determine the antioxidant activity of the plant extracts TPC (total phenolic content), RPA (reducing power activity), and DPPH (2,2′-diphenyl-1-picrylhydrazyl) methods were used. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to determine the cytotoxic effect of the extracts on the HL60 cell line. The results showed that both extracts have important properties that should be evaluated, and especially the ethanolic extract is more effective than the methanolic extract in all aspects (TPC, antioxidant capacity, and cytotoxicity). As a conclusion, it is clear that this plant needs to be investigated further for the isolation and characterization of new and effective phytochemicals. We hope that this study could be a precursor for future studies.

Key words: Antioxidant, HPLC, HL60, MTT, phenolic

Özet: *Chenopodium* cinsi, 200'den fazla türe sahip ve hemen hemen tüm Dünya'da yayılış gösteren tek yıllık veya çok yıllık otsu çiçekli bitki türlerini içeren bir cinstir. *Chenopodium* cinsine ait türler çeşitli hastalıkların tedavisinde kullanılırken, modern farmasötik araştırmalar güçlü antikanser, antifungal ve antibakteriyel potansiyellerini ortaya çıkarmıştır. Bu çalışmada, *Chenopodium botrys* (metanol ve etanol) ekstraktlarının bazı biyolojik etkileri (antioksidan ve sitotoksik potansiyelleri), çeşitli analitik yaklaşımlar yardımıyla değerlendirilmiştir. Çalışmada ekstraktlarda bulunan 4 fenolik bileşenin tespiti HPLC tekniği ile yapılmış, bitkinin antioksidan aktivitesini belirlemek için TPC, RPA ve DPPH yöntemleri kullanılmıştır. Ekstraktların HL60 hücre hattı üzerindeki sitotoksik etkisini belirlemek için ise MTT testi kullanılmıştır. Sonuçlar, her iki ekstraktın da değerlendirilmesi gereken önemli özelliklere sahip olduğunu ve özellikle etanolik ekstraktın her yönden (TPC, antioksidan kapasite ve sitotoksisite) metanolik ekstrakttan daha etkili olduğunu göstermiştir. Sonuç olarak, yeni ve etkili fitokimyasalların izolasyonu ve karakterizasyonu için bu bitkinin daha fazla araştırılması gerektiği açıktır. Bu çalışmanın gelecekteki çalışmalar için bir öncü olabileceğini umuyoruz.

Anahtar Kelimeler: Antioksidan, fenolik, HPLC, HL60, MTT

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1. Introduction

Medicinal and aromatic plants are so productive that they have had many uses such as food, medicine, cosmetics, and spices for years, and they are known to have been used for similar purposes since the beginning of human history. Herbal therapy is preferred in many countries globally, especially in undeveloped countries, under different names such as traditional therapy, complementary therapy, natural treatment, and information and inferences are made every day (Acıbuca and Budak, 2018). According to the World Health Organization (WHO), three-quarters of the world's population resort to solutions (herbs) for public health. It is estimated that up to four billion people living in the world rely on herbal medicinal products as the primary source of health care, and traditional medical practice, which includes the use of herbs, is seen as an integral part (Bodeker et al., 2005; Bandaranayake, 2006; Ekor, 2014). In addition to herbal treatments' widespread and economic nature, many people turn to phytotherapy as it is regarded as an alternative to modern medicine. In Turkey, extracts obtained from various parts of plants (leaves, flowers, bark, or other parts) by different methods are widely used in the treatment and/or preventing many diseases, from cancer to diabetes (Bozyel et al., 2019).

Chenopodium L. is a genus that includes single or perennial herbaceous flowering plant species that spread almost anywhere in the world and has more than 200 species. There are 15 species belonging to the genus *Chenopodium* in Turkey (Güner et al., 2012). *Chenopodium botrys* L. species, known by the public, is widely distributed in Turkey and grows naturally in the West and Central Black Sea, Inner West Anatolia, Upper Sakarya, and Middle Kızılırmak and Adana sections (Güner et al., 2012). The herb has various uses in traditional medicine; there are studies on the use of the plant in the treatment of cold and asthma and the analgesic, anthelmintic, and headache-relieving effects of the leaves. Many *Chenopodium* species have been reported to be used in traditional therapy and have numerous medicinal properties. The species included in the genus are used in the treatment of various diseases, especially chest complaints, cough, abdominal pain, lung obstruction, and nervous disorders (Yadav et al., 2007). *Chenopodium botrys* L., which are spread in various regions of the world, have different usage areas for each

region. In Iranian traditional medicine, *C. botrys* is used as an expectorant, anticonvulsant/antiepileptic, and tonic, as well as in the treatment of asthma (Zargari, 1993). In France and Southern Europe, *C. botrys* is used in cold and humoral asthma (Yadav et al., 2007), in Serbian traditional medicine, its dried above-ground parts are sometimes consumed as a spice, sometimes as an infusion or liquid extract, and used for diuretic, antispasmodic, carminative and antidiarrheal (Maksimovic et al., 2005). In the Skardu valley of Pakistan, the whole herb infusion is used orally to treat stomach pain, liver complaints, and headache, and it is also known as a laxative and diuretic (Bano et al., 2014). Young leaves and branches of *C. botrys* are used in wound healing in the Kohistan valley of Pakistan (Hazrat et al., 2011). In India, *C. botrys* is used as a diuretic, antispasmodic, menstrual, pectoral in asthma and cold, and it has been reported to be good for stomach and liver diseases (Khare, 2007). In Lahul, the Indian state of Punjab, *C. botrys* is used as a popular flavoring for meat, cheese, and barley soup (Koelz, 1979; Kletter and Krichbaum, 2001). *Chenopodium botrys* grown in Germany since the 19th century is generally used against moths and as a medicinal plant (Hanelt, 2001). In Spain, it has been reported that *C. botrys*, known as "Valladolid tea" is used in the treatment of coughs and digestive system disorders and as an anthelmintic (Pardo de Santayana et al., 2005). Besides, according to previous pharmacological reports, *C. botrys* is thought to be an interesting new candidate plant for cancer treatment (Morteza-Semnani, 2015).

This study evaluates and compares various biological effects (such as antioxidant and cytotoxic) of extracts obtained from *C. botrys* species collected from the Konya region.

2. Materials and Method

2.1. Plant material and extraction

The plant material was collected from its natural habitat, and locality information of the plant specimen is Konya, Çayırbağı, meadow slopes, 1050-1100m. The plant was collected and determined by Dr. Tuna UYSAL, and the voucher specimen was deposited in the KNYA herbarium (TU-3749). Firstly, the material was dried under suitable conditions without direct sunlight and moisture. Afterward, the aerial parts of the material powdered with the grinding mill were extracted with different solvents (ethanol and methanol) for 6-8 hours, respectively, with a soxhlet device. The crude extract was evaporated by a rotary evaporator. The extracts obtained were coded as CB-Met and CB-Et and stored at + 4°C until use. The % efficiency calculations for extracts will be made according to the formula below;

% yield= (A1 * 100) / A2

A1 given in the formula shows the weight of the dried extract by removing the solvent, and A2 indicates the dry weight of the plant used.

2.2. HPLC analyses

After the extraction process, the extracts were dissolved in methanol and passed through a 0.22 µm sterile filter. The prepared extracts were taken to Selçuk University Research and Development Centre for HPLC analysis. Analyses were performed with the Shimadzu instrument using the INERTSIL ODS-3V column. Working conditions were determined as 30°C temperature, 1.0 ml/min flow rate, and 280-330 nm wavelength. 20 µl injection volume was used for each extract. In our study, four phenolic substances were scanned and quantified in our extracts. Standards were prepared by dissolving in HPLC grade methanol. Used standards; protocatechuic acid, catechin hydrate, trans-ferulic acid, and benzoic acid. The total analysis time is approximately 45 minutes.

2.3. Antioxidant assay

2.3.1. Total phenolic content

The total phenolic content (TPC) of each extract was evaluated according to the previous method (Slinkard and Singleton, 1977; Ahmed et al., 2015). Each extract was prepared by dissolving 1 mg per ml. The mixture was incubated in an ultrasonic bath for 5 minutes to obtain a well-mixed solution. To 300 µl of this solution taken in a tube, 3.16 ml distilled water, 1 ml methanol, and 200 µl Folin reagent were added. Then, after incubation at room temperature, 600 µl sodium carbonate solution (10 %) was added, and the tube was covered with aluminum foil and incubated in a water bath at 40°C for half an hour. A blank was prepared using the same procedure but replacing the plant extract with an equal volume of methanol. The absorbance of the extracts was determined at 765 nm. The standard curve of gallic acid was obtained using the same procedure. Total phenolic content was expressed as µg of gallic acid equivalents (GAE) per ml, which was calculated using the formula, y=0.0053x-0.0293 where y is the absorbance at 765 nm and x is the amount of gallic acid equivalent (µg ml^{-1}).

2.3.2. DPPH analysis

The radical scavenging activity of the extracts was measured using the DPPH test. DPPH analysis was carried out according to the Chu method but with minor modifications (Chu et al., 2000; Ahmed et al., 2015). The extracts were added to 0.01 % DPPH at various concentrations (0- 2.5 mg ml^{-1}) (100 µl) and was incubated at 37°C for 30 min. Absorbance was measured at 490 nm, and radical scavenging activity (RSA) was calculated as 50 % inhibition (IC_{50}) values for each sample. Also, ascorbic acid was used as the reference compound, and the IC_{50} value was determined.

DPPH RS activity (%) = (1 - A sample / A control) × 100

2.3.3. Reducing power activity (RPA)

To determine the reducing power of extracts, the Oyaizu method was used (Oyaizu, 1986; Ahmed et al., 2015). Five different dilutions were prepared from each plant extract using 50 % methanol (between 0-1 mg ml^{-1}). 2.5 ml of plant extract, 2.5 ml of sodium phosphate buffer (0.2 M, pH 6.6), and 2.5 ml of potassium ferricyanide (1 % w/v) were added to each tube and mixed. The mixture was incubated at 50°C for 20 minutes in a water bath, then trichloroacetic acid (10 % w/v) was added. 5 ml of the mixture was placed in a test tube, and 5ml of distilled water and 1ml of ferric chloride (0.1 % w/v) solution were added and mixed. A reducing power graph was generated by measuring the absorbance at 700 nm. As a control, the same volume of 50 % methanol was used instead of the extracts.

2.4. Cell culture and cytotoxic activity

According to the previous reports, different *Chenopodium* species such as *C. anthelminticum* L. and *C. bonus-henricus* (L.) Rchb. were studied on the HL60 cell line, and this is the first study in *C. botrys* (Efferth et al., 2002; Kokanova-Nedialkova et al., 2019). For this reason, human acute promyelocytic leukemia cell line HL60 was used for cytotoxicity assignment. Cells were maintained in RPMI-1640 medium (Sigma) supplemented with 10 % fetal calf serum at 37°C in a humidified atmosphere of 5 % CO_2. The prepared extracts were applied to the HL60 cell line at various concentrations (0-1 mg ml^{-1}) and time intervals (24-48h). After adding different extracts concentrations to the cell line, the cytotoxic potential of the extracts was evaluated using the MTT test. At the end of the incubation period, 5 mg ml^{-1} MTT solution was added to the cells treated with the extracts and left to incubate for 4 hours. At the end of the period, the contents of the wells were drained, and 100 µl of isopropanol was added to each well to dissolve the formazan crystals formed (Mosmann, 1983). Plates were read on an ELISA reader at 540 nm wavelength. The effect of the extracts on cell viability was calculated by comparing the absorbance values obtained from the control group (no treatment). Analyses were done in triplicate, at least two replicates per plate. Mean values for cell viability values were taken into account. Statistical analysis was performed using GraphPad Prism 9 for Windows (GraphPad Software, San Diego, CA, USA). Data were compared using one-way ANOVA and post hoc Dunnett's test (*$p < 0.03$, **$p < 0.002$, ***$p < 0.0002$ and ****$p < 0.0001$).

3. Results

3.1. HPLC results

The quantities of the standards and the four phenolic substances in the extracts used according to the HPLC analysis are given in Table 1 and their chromatograms in Figure 1, respectively.

When we evaluate the HPLC results, we can clearly say that the phenolic compounds scanned in the extracts are found in different amounts. The most common phenolic components in the ethanol and methanol extracts were found as trans-ferulic acid and catechin hydrate, respectively. It has been determined that other phenolic components (protocatechuic acid and benzoic acid) in the content of the extracts are present in close amounts. (Table 1).

3.2. Antioxidant properties of the extracts

The total phenolic contents of the extracts were evaluated via Folin-Ciocalteu assay (Figure 2). The results showed that the linear relationship was good at detection intervals. It was determined that *Chenopodium* extracts prepared with ethanol have higher phenolic content (78.21 ± 2.60 µg ml^{-1} GAE) compared to extract prepared with methanol. The DPPH assay results correlated with total phenolic content. Also, ethanolic extract shows much more free radical scavenging activity than methanol extracts. The IC_{50} doses of the extracts were given in Table 2, and the IC_{50} dose of ascorbic acid was calculated as 21.6 µg ml^{-1}. On the other hand, the reducing power capacities (or antioxidant potential) of the ethanolic and methanolic extracts of *C. botrys* was determined, and the results are shown in Figure 3. The ethanolic extract showed higher reducing power than the methanolic extract, similar to other experiments (TPC and DPPH). Also, the two extracts showed an almost similar tendency to decrease potency with an increase in extract concentration.

Table 1. The phenolics and amounts at the *C. botrys* extracts

CB-Met						
Phenolic	**Unit**	**I. Read**	**II. Read**	**III. Read**	**Mean**	**SD**
Protocatechuic Acid	**ppm (mg/l)**	2.14	2.15	1.98	2.09	0.095
Catechin Hydrate	**ppm (mg/l)**	8.22	8.29	8.08	8.20	0.107
Trans-Ferulic Acid	**ppm (mg/l)**	0.35	0.33	0.33	0.34	0.012
Benzoic Acid	**ppm (mg/l)**	1.83	2.26	2.04	2.04	0.215
CB-Et						
Protocatechuic Acid	**ppm (mg/l)**	1.66	1.73	1.70	1.70	0.035
Catechin Hydrate	**ppm (mg/l)**	4.66	4.46	4.58	4.57	0.101
Trans-Ferulic Acid	**ppm (mg/l)**	9.41	9.45	9.37	9.41	0.040
Benzoic Acid	**ppm (mg/l)**	2.83	2.83	2.7	2.79	0.075

	LOD	**LOQ**
Protocatechuic Acid	0.09	0.28
Catechin Hydrate	0.15	0.44
Trans-Ferulic Acid	0.03	0.08
Benzoic Acid	0.20	0.60

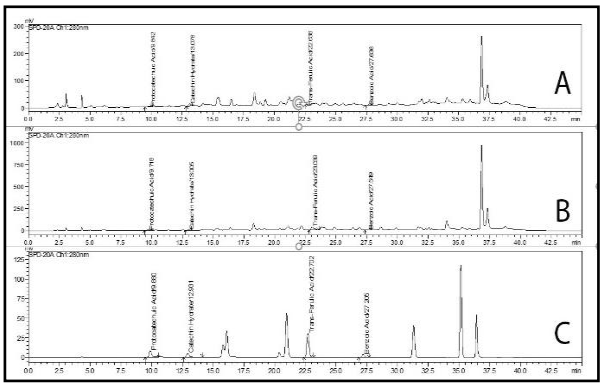

Figure 1. The chromatograms of the *C. botrys* extracts and analytic standards (A: CB-met; B: CB-et; C: standards)

3.3. Cytotoxic potential of the extracts

In this study, the cytotoxic activity of different *C. botrys* extracts were determined using the MTT assay on HL60 cell line exposed to 0-1 mg ml^{-1} extracts in two-time intervals (24 and 48 hours). According to the absorbance results compared with the control group, we can clearly say that both extracts show dose and time-dependent cytotoxic activity in the HL60 cell line. The % viability graphs and IC_{50} doses of the extracts were given in Figure 4 and Table 2, respectively. According to the cytotoxicity test results, it was determined that ethanolic extract was more effective on HL60 cells than methanolic extract after both 24 and 48 hours of application.

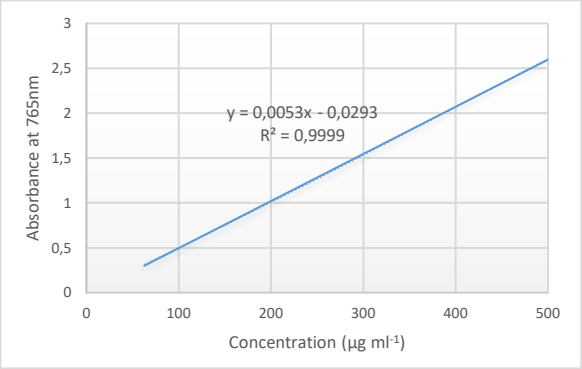

Figure 2. Gallic acid standard curve graph for Folin-Ciocalteu Assay.

4. Discussions

Medicinal plants have been used in traditional healthcare systems since prehistoric times and are still the most important source of healthcare for the vast majority of the world's population (Acıbuca and Budak, 2018). The contribution of ethnobotanical studies to modern medicine is increasing day by day, and new inferences are made on this subject. Since most of the newly developed drugs have an orientation to natural resources and especially to plants, studies have also focused on plants and phytochemicals in this context. It has been reported that many *Chenopodium* species are used in traditional therapy and have numerous medicinal properties. Species in the genus are used to treat various diseases, including chest complaints, cough, abdominal pain, lung obstruction, and nervous disorders

Table 2. Antioxidant capacity and cytotoxic activities of *C. botrys* extracts

Assay	CB-met	CB-et
the yield of the extracts %	14.15	9.95
TPC (µg ml^{-1} GAE)	69.11 ± 1.86	78.21 ± 2.60
DPPH IC_{50} values (mg ml^{-1})	1.44 ± 0.024	0.88 ± 0.019
MTT IC_{50} values (mg ml^{-1}) 24h	0.314 ± 0.045	0.173 ± 0.015
MTT IC_{50} values (mg ml^{-1}) 48h	0.210 ± 0.051	0.122 ± 0.004

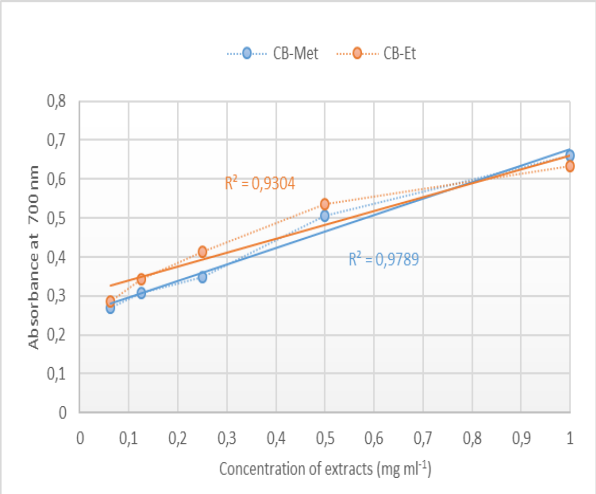

Figure 3. Reducing power of the *C.botrys* extracts expressed as absorbance at 700 nm (n = 3).

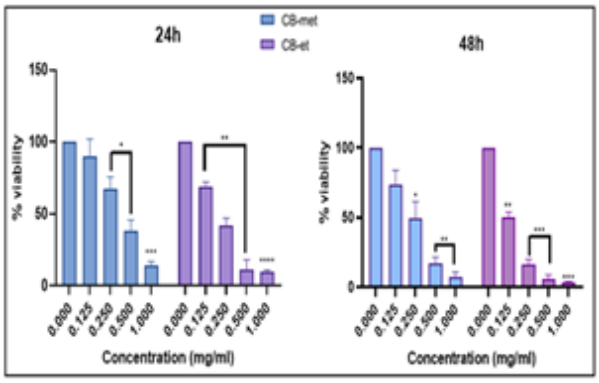

Figure 4. MTT assay graphs of methanolic and ethanolic extracts of *C. botrys* on HL60 cells (*p < 0.03, **p < 0.002, ***p < 0.0002 and ****p < 0.0001).

(Yadav et al., 2007). Modern pharmaceutical research has also revealed the potent antibacterial antifungal and anticancer activities of these herbs (Bhargava et al., 2009; Khoobchandani et al., 2009; Baldi and Choudhary, 2013; Gawlik-Dziki et al., 2013; Miranda et al., 2014). As far as we know, plants from Chenopodiaceae have extremely high polyphenol content, especially flavonoids (Repo-Carrasco-Valencia et al., 2010). As a result of our HPLC analysis, a high rate of catechin hydrate was detected in the methanolic extract. Catechin hydrate, a phenolic compound derived from plants, is a powerful antioxidant that scavenges free radicals and has anticancer potential. Previous studies have reported that catechin exerts anticancer effects by blocking the proliferation of MCF-7 and SiHa cells (Alshatwi, 2010; Al-Hazzani and Alshatwi, 2011). Moreover, it has been reported that the highest amount of trans ferulic acid in ethanolic extract content reduces the strength and anticancer properties of reactive oxygen species. It has been reported that trans ferulic acid exhibits antiproliferative effects in the lung (H1299) and colon cancer (Caco-2) cells and increases the radiosensitivity of cervical cancer cells (Janicke et al., 2011; Karthikeyan et al., 2011; Fong et al., 2016). However, the data on cytotoxic and anticancer activities of Chenopodiaceae species are limited. According to the literature, *C. ambrosioides* L. has been reported to have a potent anti-tumor effect and the potential to prevent cancer formation (Nascimento et al., 2006; Potawale et al., 2008). Still, another study conducted on certain *Chenopodium* species in Poland reported that they could suppress cancer cells growing in vitro (Nowak et al., 2016). Ascaridol, one of the major components of *Chenopodium* genus essential oil, is reported to have a cytotoxic effect on various cell lines (CCRFCEM, HL60, MDA-MB-231) (Efferth et al., 2002). The methanolic extract of *C. bonus-henricus* and some of its compounds have been reported to exhibit concentration-dependent cytotoxic effects in HL60, SKW-3, Jurkat E6-1, BV-173, and K-562 cell lines (IC_{50} doses ranging 124.5–258 µg/ml) (Kokanova-Nedialkova et al., 2019). Although the results obtained in our study suggest that the cytotoxic activity of the extracts on HL60 cell lines may be due to the phenolic content, it is also thought that might be a synergistic effect as well. We have noted that there are some studies in the literature about the biological potential of *C. botrys*. The most important point that distinguishes our study from other studies is the plant's extraction technique and habitat. As known, habitat has a significant effect on the content and diversity of secondary plant metabolites. Also, the results of the plant metabolites obtained may be affected by different extraction methods. In line with this idea, both our

extraction and plant locations are different, and the findings differ from previous reports. Andov et al. (2015) reported that the total phenolic contents of *C. botrys* species spread in Macedonia were 27.77-71.25 mg/g GAE and DPPH IC_{50} doses of 0.26-2.1 mg ml^{-1}. Özer et al. (2016) evaluated the antioxidant capacity and enzyme inhibitory effects of ethanolic and aqueous extracts prepared by the maceration method from *C. botrys* samples collected from the Isparta region. As a result, they reported that *C. botrys* could potentially prevent various diseases, especially those associated with oxidative stress (4.38 - 21.77 mg/g TE DPPH scavenging activity). In a study conducted in Pakistan, it was reported that the chloroform fraction of the methanolic extract obtained from *C. botrys* has the highest antioxidant capacity (65.9 ± 0.41 DPPH scavenging activity at a concentration of 1 mg ml^{-1}), and the plant is quite rich in bioactive compounds responsible for its antioxidant potential (Ullah et al., 2017). As far as we know, phenolic substances protocatechuic acid and catechin hydrate, which were not detected in previous studies, were detected in different amounts in our extracts. In line with the data obtained in our study, we found that both extracts have antioxidant and cytotoxic potential, and the ethanolic extract was superior to methanolic extract in terms of total phenolic, antioxidant, and reducing power, and this result was consistent with the cytotoxic effect.

As a conclusion, *C. botrys* extracts were found to contain important phenolic compounds that play an essential regulatory role in oxidation, and this study revealed that the extracts exhibit multiple biological activities such as radical scavenging and cytotoxic abilities. It is clear that this plant needs to be investigated for the isolation and characterization of new and effective phytochemicals, and we hope this study could be a precursor for future studies.

Conflict of Interest

Authors have declared no conflict of interest.

Authors' Contributions

The authors contributed equally.

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Comparative vascular anatomies of some orchid species

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Bazı orkide türlerinin karşılaştırmalı damar anatomileri

Abstract: In this study, we examined the vascular anatomy of leaves with different morphological features of 11 orchid species. Plant samples were collected from various localities in the Black Sea Region. Fresh leaves were dried and stocked, and their vascular structures were analyzed by clearing and staining. Significant differences were determined in the leaves of taxa in terms of characters such as total leaf perimeter and area, number of veins and nodes, total vein length, total vein area, average vein length, average vein width, average vein surface area, average vein volume, and average areolar area. According to the findings, the topological and morphometric features of the veining can reflect the systematic and phylogenetic relationships of orchids.

Key words: Anatomy, venation network, morphometry, *Orchidaceae*

Özet: Bu çalışmada 11 orkide türüne ait farklı morfolojik özellikleri olan yaprakların damar anatomileri incelenmiştir. Bitki örnekleri Karadeniz Bölgesi'ndeki çeşitli lokalitelerden toplanmıştır. Taze yapraklar kurutularak stoklanmış, saydamlaştırma ve boyama işlemi uygulanarak damar yapıları analiz edilmiştir. Taksonların yapraklarında toplam yaprak çevresi ve alanı, damar ve boğum sayısı, toplam damar uzunluğu, toplam damar alanı, ortalama damar uzunluğu, ortalama damar genişliği, ortalama damar yüzey alanları, ortalama damar hacmi ve ortalama areol alanı gibi karakterler bakımından önemli farklılıklar tespit edilmiştir. Bulgulara göre damarlanmanın topolojik ve morfometrik özellikleri, orkidelerin sistematik ve filogenetik ilişkilerini yansıtabilir.

Anahtar Kelimeler: Anatomi, damar ağı, morfometri, *Orchidaceae*

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1. Introduction

One of the largest families in the angiosperms of *Orchidaceae* display natural distribution with over 200 taxa in Turkey (Güner and Aslan, 2012). Additionally, there are many rare and/or endemic orchid species in our country. In addition to consumption of orchids as drinks (sahlep), use in ice cream production and cultivation as decorative plants, they have economic value as an effective treatment for diseases like tuberculosis, chest pain and asthma (Sezik, 1984; Hossain, 2011). However, due to species displaying distribution in a very large area, excessive intraspecies variation of flowers and leaves especially and high hybridization abilities, there are many systematic problems with orchids (Arditti, 1977; Dressler, 1993). Additionally, mistaken plant identification and different taxonomic opinions further complicate these problems.

The leaves of land plants display diversity in terms of vein architecture (Roth-Nebelsick et al., 2001). Monocot plants have main veins organized parallel to the midrib and anastomosis at the distal and basal tips of the leaf blade. Secondary veins have transverse pattern, and develop between main veins to form short interconnections (Conklin et al., 2019). The architecture of the vascular bundles is affected by the phylogenetic or genetic character of the plant in addition to the ecological conditions it exists in (Sack et al., 2012; Blonder et al., 2020). Additionally, led by carbohydrate and water-carrying capacity, they ensure the occurrence of many basic physiological functions like photosynthesis and perspiration, resistance to injury, mechanical support or lignin-derived costs (Vincent, 1982; Niklas, 1999; Givnish et al., 2005; Brobbrib et al., 2007; Agrawal and Konno, 2009; Katifori et al., 2010; Brodribb et al., 2016; John et al., 2017; Blonder et al., 2011, 2018; Ohtsuka et al., 2018). For this reason, the vein shapes represent the basis for studies in many disciplines. However, there are very limited numbers of studies about the vein anatomy and vein patterns in orchids, especially (Cameron and Dickison, 1998; Mani et al., 2021). This research comparatively investigates the vein shapes and structure of 11 genera among representative species included in the *Orchidaceae* family (*Cephalanthera* Rich., *Coeloglossum* Lindl., *Dactylorhiza* Neck. Ex Nevski, *Gymnadenia* R.Br., *Hymantoglossum* Spreng., *Limodorum* L., *Orchis* L., *Platanthera* Rich., *Serapias* L., *Spiranthes* Rich., *Steveniella* Schltr.) distributed in the Black Sea Region to test the usefulness of vein architecture as a characteristic showing systematic relationships between genera. This study was also applied for the first time on the leaf vein structures of orchids in Turkey.

2. Materials and Method

Plant samples were collected from a variety of localities in the Black Sea region (Table 1). For each species, mean 10 leaf samples were pressed and dried for several days at 60 °C. Leaf samples were taken from at least three individuals. Three different leaves for each individuals were studied, with leaf sizes being small, medium and large. ANOVA test was performed on the obtained data to

determine the importance of leaf-anatomical data for taxa. Tests were completed with the aid of SPSS 20 program.

2.1. Clearing

The transparency method was applied by taking Vasco et al. (2014) as reference. Accordingly, dry leaf samples were left for 1-3 days at 40-54 ℃ in 5% NaOH solution.

Samples were washed with distilled water and treated for durations lasting from 20 s to 10 min with 4.5-5.5% sodium hypochlorite. Leaf samples were passed through a graded ethanol series (50%, 70%, 95%) and stained with safranin by applying the standard protocol.

2.2. Photograph Metadata and Analysis

Leaves with scale added were photographed from the same distance using a NIKON D700 brand camera and analyzed with the aid of the LEAF GUI (Price et al., 2011) program. After a series of cropping and cleaning procedures, RGB photographs were converted to grayscale and vein, node and areole features were calculated. While determining the threshold value, global and local adaptive thresholding approaches were used to obtain binary images representing vein regions with one and nonvein regions with zero, and vein segmentation was achieved. In the vein network, nodes and vein tips were determined with representative single pixel width. Metric calculations for veins and the full leaf were completed using software.

3. Results

When the vein topology of leaves from 11 species belonging to the *Orchidaceae* family are investigated, the taxa displayed clear differentiation based on secondary vein pattern. In *Limodorum abortivum* (L.) Sw., the parallel vein pattern disappears at the leaf tips. For *Cephalanthera kotchyana* Renz & Taubenheim, *Coeloglossum viride* Hartm., *Dactylorhiza urvilleana* (Steud.) H.Baumann & Künkele, and *Gymnadenia conopsea* (L.) R.Br. secondary connections between parallel veins are notable (Fig. 1). These connections intensify toward the leaf tip.

Table 1. List of *Orchidaceae* taxa, localities and collection date

Taxa	Subfamily	Locality	Collection date	Voucher	Habitats	Elevation
Cephalanthera kotschyana	E	Kavak, Samsun	April, 2015	Ss, 29	Deciduous forests	750
Cephalanthera kotschyana	E	Kurupelit, Samsun	April, 2015	Omuhb, 7711	Open forests	225
Coeloglossum viride	O	Köprübaşı, Trabzon	June, 2015	Omub, 8253	Meadows	1820
Dactylorhiza urvilleana	O	Köprübaşı, Trabzon	June, 2015	Omub, 7787	Open forests, meadows	850
Dactylorhiza urvilleana	O	Kavron, Rize	June, 2015	Omub, 7818	Alpine to subalpine meadows	2000
Dactylorhiza urvilleana	O	Santa, Gümüşhane	June, 2015	Omub, 8066	Alpine to subalpine meadows	1600
Gymnadenia conopsea	O	Köprübaşı, Trabzon	July, 2015	Mka, 27	Alpine to subalpine meadows	1670
Himantoglossum caprinum	O	Kurupelit, Samsun	June, 2015	Omuhb, 7739	Coniferous forests, open forests	245
Himantoglossum caprinum	O	Boyabat, Sinop	June, 2015	Mka, 28	Coniferous forests,	300
Limodorum abortivum	E	Kavak, Samsun	June, 2015	Mka, 30	Deciduous forests	450
Limodorum abortivum	E	Kurupelit, Samsun	June, 2015	Omuhb, 3036	*Quercus* forests	150
Orchis mascula subsp. *pinetorum*	O	Abant, Bolu	May, 2015	Ss, 16	Edges of coniferous forests	985
Orchis mascula subsp. *pinetorum*	O	Çambaşı, Ordu	May, 2015	Omuhb, 7829	Edges of coniferous forests	1515
Orchis mascula subsp. *pinetorum*	O	Kurupelit, Samsun	May, 2015	Omuhb, 7712	*Quercus* forests	*163*
Orchis mascula subsp. *pinetorum*	O	Köprübaşı, Trabzon	June, 2015	Omuhb, 7725	Open areas, meadows	2300
Platanthera chlorantha	O	Kavak, Samsun	June, 2015	Mka, 33	Deciduous forests	870
Platanthera chlorantha	O	Kurupelit, Samsun	June, 2015	Omuhb, 4123	Deciduous forests, *Quercus* forests	157
Serapias bergonii	O	Ünye, Ordu	May, 2015	Mka, 36	Meadows	60
Serapias bergonii	O	Kale, Giresun	May, 2015	Mka, 34	Meadows	90
Spiranthes spiralis	O	Köprübaşı, Trabzon	Sep, 2015	Mka, 49	Open forests, meadows	860
Spiranthes spiralis	O	Kurupelit, Samsun	Sep, 2015	Mka, 38	Meadows	205
Steveniella satyrioides	O	Ünye, Ordu	May, 2015	Mka, 50	Edges of deciduous forests	90
Steveniella satyrioides	O	Bafra, Samsun	May, 2015	Mka, 40	Edges of deciduous forests	120
Steveniella satyrioides	O	Kurupelit, Samsun	May, 2015	Omuhb, 3041	Deciduous forests, *Quercus* forests	210

E: *Epidendroideae,* O: *Orchidoideae*

In *Orchis mascula* subsp. *pinetorum* (Boiss. & Kotschy) G.Camus, *Serapias bergonii* E.G.Camus and *Steveniella satryoides* (Spreng,) Schltr the secondary vein network is distributed homogeneously throughout the full leaf blade, different to other species. In *Himantoglossum caprinum* (M.Bieb.) Spreng and *Platanthera clorantha* (Cruster) Rchb. the secondary vein network between the primary parallel veins displays net-like pattern. Additionally, the free tipped secondary veins are distributed between areoles in these taxa. In *Spiranthes spiralis* (L.) Chevall, the secondary veins have wave-like pattern and as a result of this pattern, *Spiranthe spiralis* has irregular areoles compared to the rectangular shape of areoles in other species (Fig. 1).

For vein morphometric properties, metric features like total leaf circumference and area; total vein network length and area; vein and node numbers; mean vein length, width, 2D, 3D area and volume; and mean areole equivalent diameter, circumference, area and convex area were investigated. Leaves from photosynthetic or saprophyte species cultivated in different habitats were identified to have significant differences in terms of general leaf properties, vein and areole features. Total leaf circumference and area, vein and node numbers, total vein network length and total area covered by the vein network were higher for *Dactylorhiza urvilleana*, *Himantoglossum caprinum* and *Platanthera chlorantha* compared to other species. In terms of total leaf features, the lowest values were for *Coeloglossum viride* and *S. spiralis*, while lowest vein and node numbers were observed in *Cephalanthera kotchyana* and *Limodorum abortivum* (Table 2).

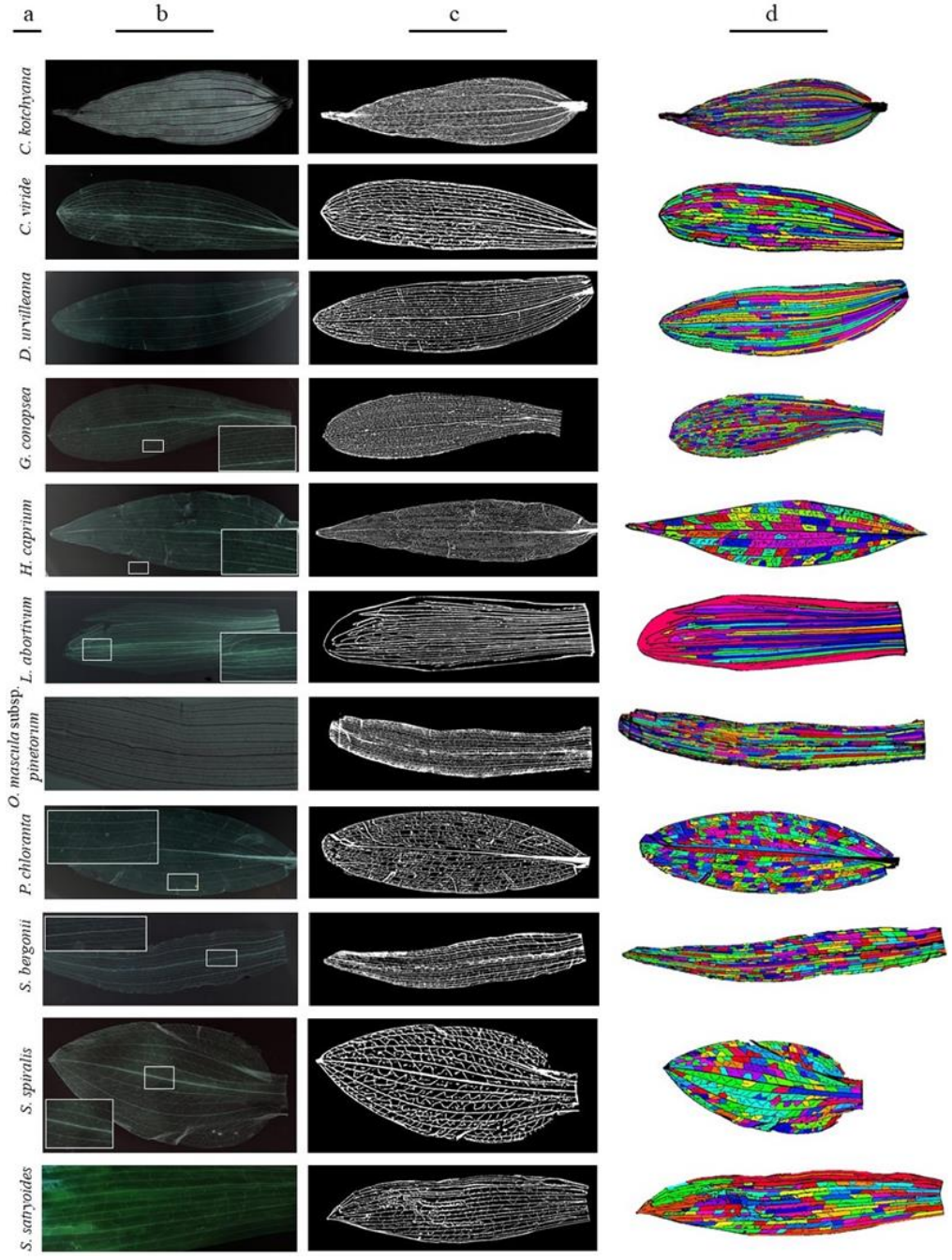

Figure 1. Vein segmentation of taxa. a: taxon b: RGB photographic samples, c: binary images showing veins white and non-vein regions in black, d: image of color labeled areoles (each color represents different areoles)

Table 2. Average morphometric data of the leaf vein characters of taxa

	Leaf						Vein					Areole				
Taxa/ Character	Area (mm^2)	Perimeter (mm)	Total Length of Network (mm)	Total Network Area (mm^2)	Number of Veins	Number of Nodes	Length (mm)	Width (mm)	2D Area (mm^2)	3D Area (mm^2)	Volume (mm^3)	Area (mm^2)	Convex Area (mm^2)	Perimeter (mm)	Equivalent Diameter (mm)	Average Distance (mm)
Cep kot	560.0	146.1	2159.1	210.0	1241	1285	1.69	0.06	0.09	0.29	0.01	1.8	7.3	17.0	0.85	0.04
Coe vir	284.8	88.7	1009.0	110.9	3317	2888	0.23	0.05	0.01	0.05	0.00	0.3	0.5	2.9	0.37	0.04
Dac urv	2867.4	309.1	5230.9	869.0	14033	13106	0.29	0.08	0.03	0.09	0.01	1.5	3.0	5.9	0.49	0.05
Gim con	795.7	187.2	2779.5	280.4	7792	7078	0.30	0.05	0.02	0.05	0.00	0.4	0.6	3.7	0.53	0.05
Him cap	2390.1	256.5	5682.6	717.3	18005	17926	0.23	0.06	0.02	0.05	0.00	1.0	2.2	3.2	0.20	0.03
Lim abo	599.7	110.7	1166.1	178.7	2133	1981	0.48	0.07	0.04	0.13	0.01	1.8	4.1	8.4	0.56	0.04
Orc mas	782.8	234.9	2699.3	307.6	6857	5842	0.33	0.07	0.02	0.07	0.00	0.4	0.5	3.0	0.40	0.04
Pla chl	2562.8	232.5	5200.6	783.9	16740	15361	0.23	0.08	0.02	0.07	0.01	1.1	1.4	4.6	0.63	0.04
Ser ber	351.8	130.9	1106.7	115.3	2417	2140	0.39	0.06	0.02	0.07	0.00	0.4	0.6	3.3	0.47	0.07
Spi spi	327.4	87.3	1201.0	100.9	5370	4735	0.15	0.05	0.01	0.02	0.00	0.2	0.3	1.8	0.28	0.05
Ste sat	842.2	166.7	1818.8	231.4	4454	4077	0.34	0.06	0.02	0.08	0.00	1.1	1.7	5.4	0.63	0.04

The situation is different for features like mean vein dimensions, vein surface area and volume. *Cephalanthera kotchyana* and *Limodorum abortivum* had much higher vein length values compared to other species. Additionally, these species had higher values for 2D and 3D vein area compared to other species. *Spiranthes spiralis* had the lowest values for mean vein dimensions (Table 2,3).

For areole features, clear differences were not observed for criteria like the mean distance (mean Euclidean distance between each areole pixel and vein pixel) and equivalent diameter (diameter of the region with the same normalized secondary axis moment). However, there were variability for criteria like areole area, areole convex area (area of the convex shell covering the areole) and areole circumference in many species. These values were high for species like *Cephalanthera kotchyana* and *Limodorum abortivum* and lowest for *Coeloglossum viride* and *Spiranthes spiralis* (Fig. 2, Table 2).

Table 3. Descriptive statistic for vein and areole of taxa

		Vein					Areole				
Taxa		Length (mm)	Width (mm)	2D Area (mm^2)	3D Area (mm^2)	Volume (mm^3)	Area (mm^2)	Convex Area (mm^2)	Perimeter (mm)	Equivalent Diameter (mm)	Average Distance (mm)
Cep kot	Mean	1.686	0.060	0.092	0.288	0.011	1.848	7.294	17.050	0.846	0.039
	Std. Deviation	4.180	0.063	0.199	0.625	0.029	5.001	21.300	39.912	1.283	0.014
Coe vir	Mean	0.234	0.054	0.015	0.047	0.002	0.310	0.451	2.942	0.372	0.041
	Std. Deviation	0.368	0.029	0.031	0.098	0.006	0.698	1.165	5.828	0.506	0.024
Dac urv	Mean	0.286	0.082	0.029	0.091	0.006	1.488	2.985	5.934	0.490	0.048
	Std. Deviation	0.550	0.055	0.080	0.253	0.036	7.974	22.780	26.640	1.287	0.043
Gim con	Mean	0.298	0.051	0.017	0.053	0.002	0.412	0.583	3.667	0.529	0.055
	Std. Deviation	0.430	0.022	0.031	0.098	0.005	1.071	2.067	7.697	0.495	0.025
Him cap	Mean	0.232	0.060	0.017	0.055	0.003	1.010	2.249	3.196	0.204	0.034
	Std. Deviation	0.407	0.033	0.057	0.180	0.046	12.545	30.203	35.144	1.116	0.015
Lim abo	Mean	0.478	0.066	0.041	0.128	0.006	1.763	4.125	8.369	0.558	0.042
	Std. Deviation	1.066	0.035	0.103	0.323	0.018	11.368	37.811	41.429	1.393	0.037
Orc mas	Mean	0.327	0.066	0.022	0.068	0.003	9.579	10.085	6.310	0.482	0.041
	Std. Deviation	0.499	0.042	0.033	0.103	0.006	77.204	81.280	49.989	3.486	0.114
Pla chl	Mean	0.227	0.081	0.022	0.069	0.006	1.077	1.438	4.595	0.628	0.070
	Std. Deviation	0.278	0.059	0.058	0.182	0.070	3.547	5.437	12.131	0.989	0.064
Ser ber	Mean	0.388	0.060	0.023	0.072	0.003	0.393	0.594	3.283	0.471	0.052
	Std. Deviation	0.617	0.034	0.033	0.104	0.004	1.317	3.302	9.572	0.528	0.028
Spi spi	Mean	0.154	0.047	0.008	0.025	0.001	0.218	0.297	1.787	0.277	0.040
	Std. Deviation	0.170	0.024	0.012	0.037	0.002	0.766	1.068	4.585	0.449	0.030
Ste sat	Mean	1.686	0.060	0.092	0.288	0.011	1.114	1.666	5.406	0.634	0.063
	Std. Deviation	4.180	0.063	0.199	0.625	0.029	3.287	6.403	16.438	1.010	0.058

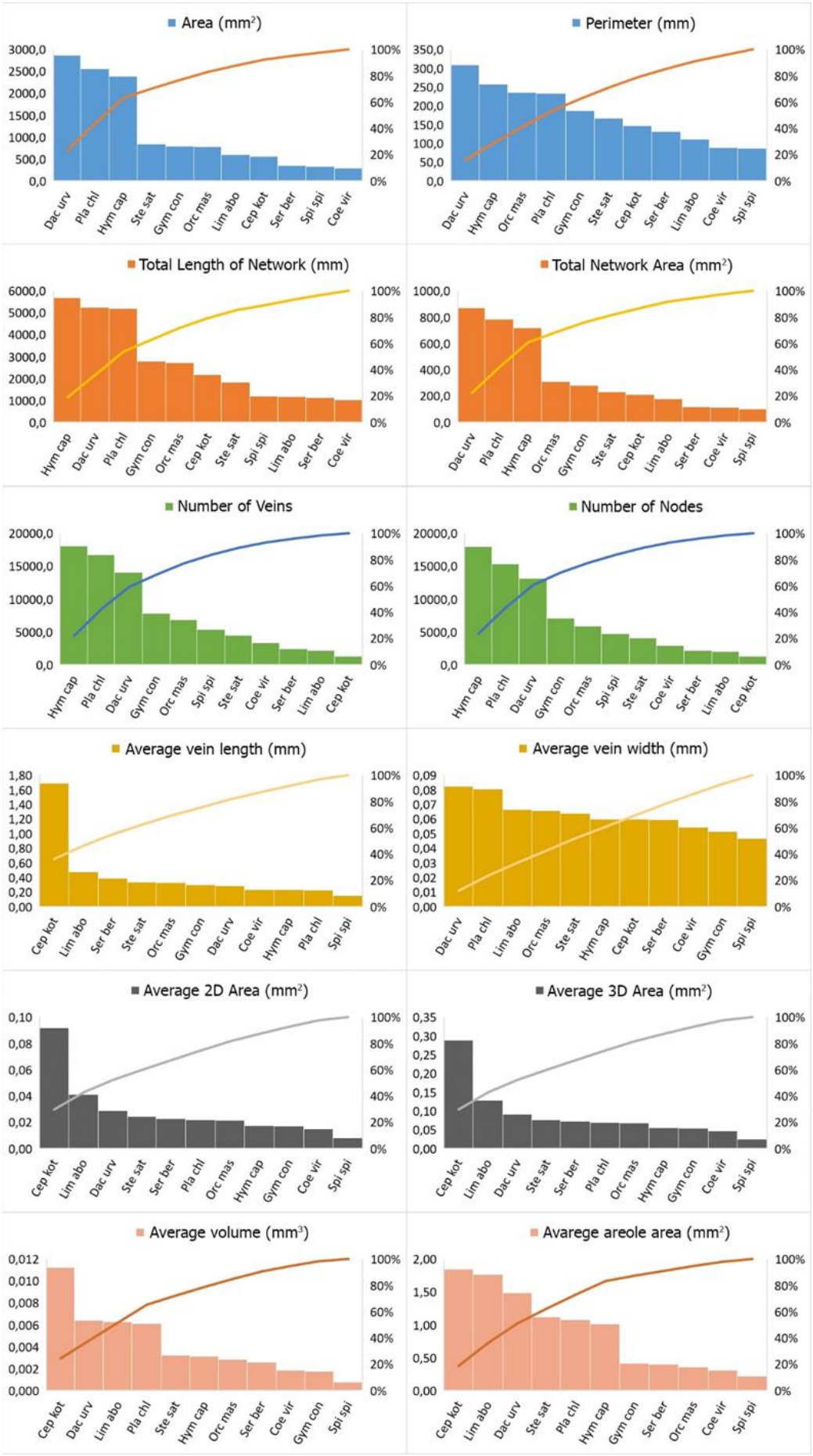

Figure 2. Pareto graphs of vein morphometric data

It was revealed that the characters using ANOVA test were important for at least one taxa (Table 4).

4. Discussions

Orchidaceae is taxonomically divided into five subfamilies (*Epidendroideae, Orchidoideae, Vanilloideae, Cyprepedioideae* and *Apostasioideae*). The species common in Turkey are from the *Epidendroideae* and *Orchidoideae* subfamilies. The members of this family are common in the whole country (Sezik, 1984). The leaf vein topographic and morphometric features of some representative orchid species growing in Turkey were comparatively assessed between genera. These features clearly separated the epidendroid and orchidoid taxa. Software defined the junction point of two or more vein elements as a node and defined the vein segments between the two nodes as veins (Larese et al., 2014). The vein and node numbers in epidendroid species were fewer than for orchidoid species. In other words, sparse location of secondary connecting veins between parallel veins causes higher mean vein and areole dimensions. For this reason, these characteristics reflect the phylogenetic differentiation between two subfamilies (epidendroid and orchidoid) of the investigated species (Cameron et al., 1999).

Among *Orchideae* tribes members which were the topic of the study, *Spiranthes spiralis* was classified within the *Cranichideae* tribes, different to other orchidoid species. This is different to other orchidoid taxa due to leaves characterized by wavy secondary veins and irregular areole shapes. In this group, *Coeloglossum viride*, *Dactylorhiza urvilleana*, and *Gymnadenia conopsea* taxa are closely related on dendrograms obtained with molecular markers (Pridgeon, 1997; Batemann et al., 2003). In these three taxa, secondary vein connections intensify toward leaf tips, and the topology of these veins may indicate characteristics shared at the genera level.

Table 4. ANOVA analysis for vein and areole characters

	Charachters		Sum of Squares	df	Mean Square	F	Sig.
Vein	Length	Between Groups	2967.46	10.00	269.77	534.40	0.00
	Width		12.24	10.00	1.11	580.45	0.00
	2D Area		10.82	10.00	0.98	244.20	0.00
	3D Area		106.76	10.00	9.71	244.20	0.00
	Volume		0.40	10.00	0.04	20.94	0.00
Areole	Area		7098.31	10.00	645.30	8.10	0.00
	Convex Area		19606.59	10.00	1782.42	5.84	0.00
	Perimeter		44539.87	10.00	4049.08	9.39	0.00
	Equivalent Diameter		229.52	10.00	20.87	23.38	0.00
	Average Distance		1.36	10.00	0.12	79.67	0.00

Many species may be characterized in terms of criteria evaluating vein and areole structure. The results show that the topology of secondary veins between parallel veins in orchids are preserved during evolution. For this reason, it is considered that when preliminary preparation procedures are completed correctly and carefully, this method will provide easy, rapid and reliable data which will be beneficial to assess relationships between genera in problematic groups like orchids or to identify possible intergeneric hybrid taxa.

Conflict of Interest

Authors have declared no conflict of interest.

Authors' Contributions

The authors contributed equally.

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In vitro germination and vegetative growth characteristics of *Gypsophila pilulifera* (*Caryophyllaceae*) seeds grown under abiotic stress conditions

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Abiyotik stres koşullarında yetiştirilen *Gypsophila pilulifera* (*Caryophyllaceae*) tohumlarının in vitro çimlenme ve vejetatif büyüme özellikleri

Abstract: *Gypsophila pilulifera* Boiss. & Heldr. is an economically important critically endangered (CR) endemic species of Turkey. This species is a Mediterranean element and type sample is located in square C3 (Antalya). Plants are much more sensitive to abiotic stress factors, especially during germination and seedling development stages. In this study, *in vitro* germination and seedling growth properties of *G. pilulifera* Boiss. & Heldr. (*Caryophyllaceae*) were investigated under salt and drought stress. The seeds were cultured in hormone-free MS media containing increasing doses of NaCl (50, 100, 150, 200 mM) and PEG 6000 (5%, 10%, 15% and 20%). Germinated seeds were counted every three days for the first week and every day for the second week. The root and hypocotyl length, number of leaves, fresh weight and vigor index of the plants were determined at the end of 21 days. A negative correlation was determined between increased salt and drought stress and all vegetative growth parameters. Although germination was obtained on MS medium with the highest level of drought (20%), there was no germination on MS medium containing 200 mM NaCl. As a result, it was determined that the plant was more sensitive to salinity stress than drought and high salt concentrations inhibited the germination by 100%.

Keywords: Drought, *Gypsophila pilulifera*, salinity, vegetative growth, vigor index.

Özet: *Gypsophila pilulifera* Boiss. & Heldr. Türkiye'de ekonomik açıdan önemli, nesli tükenmekte olan (CR) endemik bir türdür. Bu tür bir Akdeniz elementidir ve tip örneği C3 karesinde (Antalya) bulunmaktadır. Bitkiler, özellikle çimlenme ve fide gelişme aşamalarında abiyotik stres faktörlerine çok daha duyarlıdır. Bu çalışmada *in vitro* koşullarda *G. pilulifera* Boiss. & Heldr. (*Caryophyllaceae*) 'in tuz ve kuraklık stresi altında çimlenme ve fide büyüme özellikleri incelendi. Tohumlar, artan NaCl (50, 100, 150, 200 mM) ve PEG 6000 (%5, %10, %15 ve %20) dozlarını içeren hormonsuz MS ortamında kültüre alındı. Çimlenen tohumlar ilk hafta üç günde bir ve ikinci hafta için her gün sayıldı. Bitkilerin kök ve hipokotil uzunluğu, yaprak sayısı, taze ağırlık ve canlılık indeksi 21 gün sonunda belirlendi. Artan tuz ve kuraklık stresi ile incelenen tüm bitkisel büyüme parametreleri arasında negatif bir korelasyon belirlendi. Ayrıca kuraklık oranı en yüksek olan MS besiyerinde (%20) çimlenme elde edilmesine rağmen 200 mM NaCl içeren MS besiyerinde çimlenme elde edilemedi. Sonuç olarak bitkinin tuzluluk stresine kuraklığa göre daha duyarlı olduğu ve yüksek tuz konsantrasyonlarının çimlenmeyi %100 engellediği belirlenmiştir.

Anahtar Kelimeler: *Gypsophila pilulifera*, kuraklık, tuzluluk, vejetatif büyüme, vigor indeks.

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1. Introduction

In plants faced with abiotic and biotic environmental stresses, especially abiotic stresses such as drought, salinity, and extreme temperature affect all stages of growth and development. The stages where plants are most susceptible to abiotic stress are seed germination, seedling development, and flowering stages (Patade et al., 2011; Partheeban et al., 2017). Abiotic stress, which reduces the average crop yield in cultivated plants by more than 50%, is the primary cause of crop yield loss worldwide (Wang et al., 2004). As a result of stress, biological tension which is explained as a change in plant metabolism and morphology and a decrease in growth is occurred (Salisbury and Ross, 1992). Plants react to this tension with some physiological and metabolic changes and in this way they try to overcome stress conditions with the least damage (Kalefetoğlu and Ekmekçi, 2005). Understanding the response of plants to drought and salinity stress, which is a common problem in many parts of the world, has therefore become an important issue (Jamil et al., 2011; Korkmaz and Durmaz, 2017).

Gypsophila species, which are found as annual, biennial or perennial, belong to the *Caryophyllaceae* family of the order Caryophyllales. The name 'Gypsophila' has been given to a group of plants that adapt to gypsum environments (Williams, 1989). Species belonging to this genus distribute in arid areas around Anatolia in Turkey. (Davis et al., 1988). The roots of species belonging to the genus *Gypsophila* are rich in triterpenoid saponins. Saponins are commercially important substance used commercially in the detergent, adjuvant and cosmetic industry because of their emulsifying and foaming properties. In addition, phytochemicals, including saponins, have the potential to be used against various diseases such as cancer. Therefore, it is also used for medical purposes (Mujeeb et al., 2014; Arslan et al., 2012; Gevrenova et al., 2010).

In Turkey, sixty three *Gypsophila* species are known and 41 of these species are endemic (Özçelik and Özgökçe, 2021) and according to IUCN, *G. pilulifera* Boiss. & Heldr.

appears to be in the Critically Endangered (CR) category (Ekim et al., 2000).

In addition, the species is very rich in saponins (Arslan et al., 2012) and has great commercial importance. In the study, it was aimed to investigate the *in vitro* germination and vegetative growth tolerance of *G. pilulifera*, a Mediterranean element is endemic to Turkey and is in the CR category under drought and salinity stress. In this way, besides contributing to the protection of the natural populations of the species, it would also be contributed to determine suitable conditions for the production of this species, which also has economic and medicinal value. As a result of climatic and ecological changes, the survival of the species, which is already potentially endangered, will be severely restricted due to possible drought and salinity stress in the future. In this context, by researching the production potential of this species against possible stresses and ensuring its production by creating the determined optimum conditions, will be prevented from extinction from nature, and its use in landscaping and medicine will be increased.

2. Materials and Method

2.1. Material

In the study, the seeds of *G. pilulifera* which grown in Lara locality of Antalya province were collected in October 2019. The experiments were carried out in the Organic Agriculture Laboratory of Vocational School of Technical Sciences of Akdeniz University, in June 2020.

2.2. Sterilization of Seeds

The seeds dried on blotting papers were packed and stored at + 4 ° C in the dark. The seeds were washed with detergent before surface sterilization and kept under running tap water for 15 minutes. For surface sterilization, the seeds were kept in 20% sodium hypochlorite (NaOCl) solution for 20 minutes and then kept in 70% ethyl alcohol for 2 minutes. Then, sterilization was completed by rinsing in sterile distilled water 3 times for 5 minutes.

2.3. Germination and Vegetative Development Parameters

In order to determine the effects of salt and drought on germination and vegetative growth parameters, hormone-free MS medium containing NaCl (50, 100, 150, 200 mM) and PEG 6000 (5%, 10%, 15% and 20%) at different concentrations were used. MS medium (Murashige and Skoog, 1962) without NaCl and PEG 6000 was used as the control medium. After adding 30 g/L sucrose to the medium as a carbon source, the pH was adjusted to 5.7 and it was autoclaved after adding 7 g/L agar. PEG 6000 was added to sterilized media after sterile filtering.

The trials were set up in three duplications with 10 seeds per replicate according to the randomized plot design. Germination percentages of the seeds was determined at the end of the first week and at the end of the second week. Radicle and hypocotyl lengths, fresh weights was measured and, survival times and vigor index were determined at the end of the third week. In accordance with the International Seed Testing Association (ISTA) rules, the trials for germination tests were completed on the 14th day (ISTA, 2007). The number of germinated seeds was determined by counting at approximately the same hour every day, based on the emerge of the radicle from the testa starting from the day the seeds were cultured. Percentage of germination and vigor index were determined according to Gosh et al. (2014) and Hu et al. (2005) respectively.

Germination percentage (%) = Number of seeds germinated / Total number of seeds placed in jars x 100 (Gosh et al., 2014)

Vigor index = [Germination percentage x (radicle length + hypocotyl length)] (Hu et al., 2005)

2.4. Analysis of Data

In both trials, the data were subjected to statistical evaluation with ANOVA test after testing their suitability to a normal distribution, and the differences between vegetative growth parameters were statistically determined by multiple comparison tests (TUKEY, DUNCAN). Pearson Correlation was used to evaluate the relationships between growth parameters, germination, and survival times.

3. Results

In the study, as a result of the statistical analysis, it was determined that there was a significant difference between drought and salt doses in terms of germination ($p < 0.001$). When the effects of salt and drought doses on germination rate were examined, it was found that stress increased with each dose increase compared to control. Germination occurred in MS medium containing 20% PEG, the highest dose used in drought stress trials, but the germination percentage decreased from 93% (in control group) to 43%. In salt stress trials, the germination percentage decreased significantly at each dose, and germination was not observed in seeds at a dose of 200 mM Nacl (Figure 1,2).

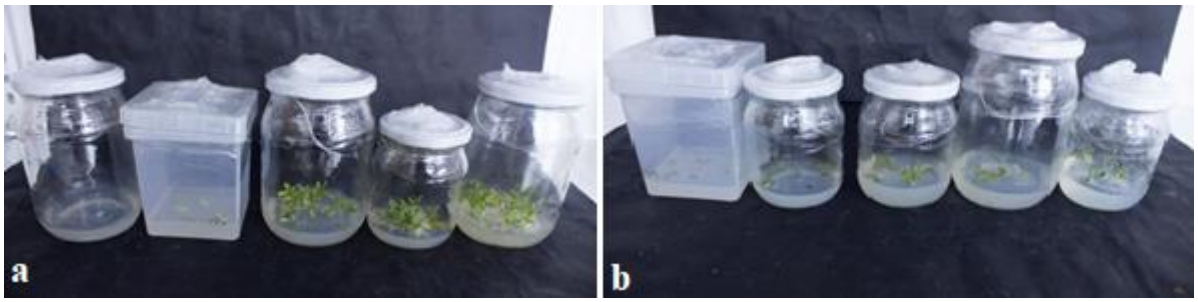

Figure 1. The appearance of the seeds at the end of the 2nd week in MS mediums containing different doses of drought and salinity (a. Drought: From right to left 0, 5%, 10%, 15%, 20% PEG 6000; b. Salinity: From right to left 0, 50, 100, 150, 200 mM NaCl

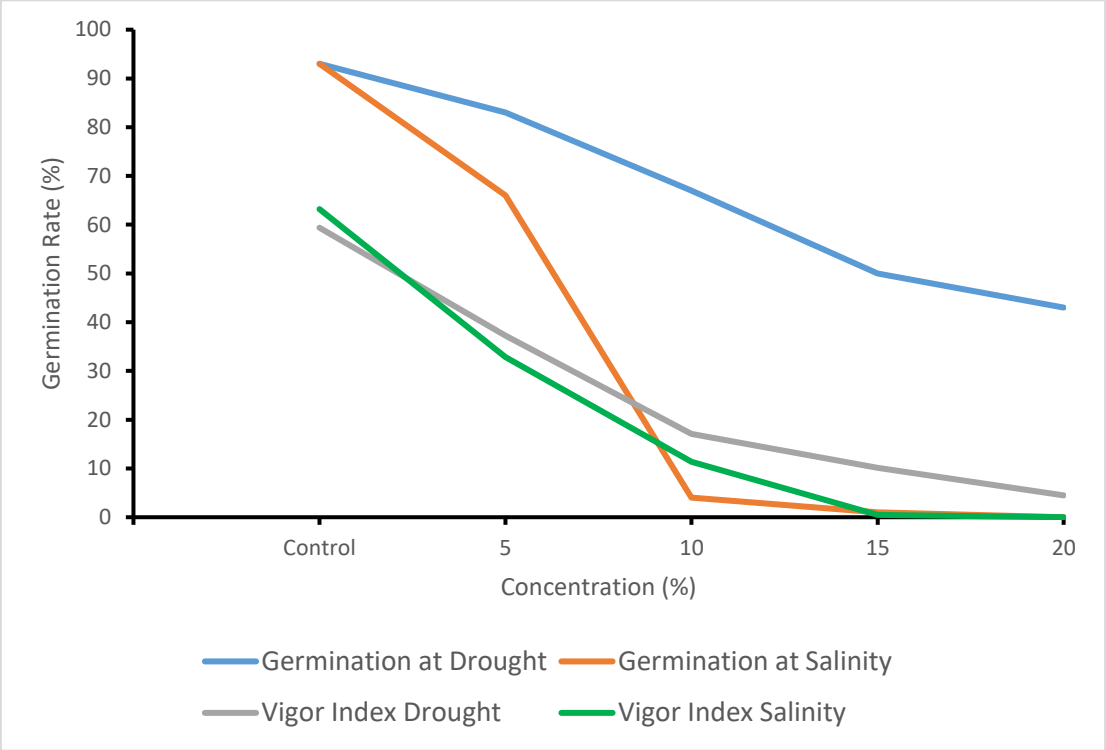

Figure 2. The germination percentages of seeds at the end of the 2nd week and vigor index at the end of the 3rd week in MS medium containing different doses of drought and salinity

A strong negative correlation was determined between increased salt and drought stress and radicle length, hypocotyl length, leaf number, plant fresh weight and survival time (Figure 3). On the other hand, there was a negative correlation between increased salt stress and germination time, while a positive correlation was found between increased drought stress and germination time.

Increasing drought stress did not make a statistically significant difference in terms of germination times. On the other hand, the length of the radicle, the length of the hypocotyl, the number of leaves, the fresh weight of the plant and the survival time decreased with the increase of drought ($r_{\text{Radicle Length}} = -0.802$; $p < 0.001$, $r_{\text{Hypocotyl Length}} = -0.816$; $p < 0.001$, $r_{\text{Number of Leaves}} = -0.786$; $p < 0.001$, $r_{\text{Fresh Weight}} = -0.793$; $p < 0.001$, $r_{\text{Germination Time}} = 0.168$; $p > 0.05$,

$r_{\text{Survival Time}} = -0.350$; $p < 0.001$). Besides, increased salt stress decreased the length of the radicle, hypocotyl length, number of leaves, fresh weight of the plant and the survival time, however, it caused a prolongation of the germination period ($r_{\text{Radicle Length}} = -0.801$; $p < 0.001$, $r_{\text{Hypocotyl Length}} = -0.799$; $p < 0.001$, $r_{\text{Number of Leaves}} = -0.793$; $p < 0.001$, $r_{\text{Fresh Weight}} = -0.783$; $p < 0.001$, $r_{\text{Germination Time}} = -0.553$; $p < 0.001$, $r_{\text{Survival Time}} = -0.693$; $p < 0.0001$).

Also, with increasing drought stress, the differences in terms of radicle length ($F = 83.286$; $df_1 = 4$; $df_2 = 145$;

$p < 0.001$), hypocotyl length ($F = 94.099$; $df_1 = 4$; $df_2 = 145$; $p < 0.001$), number of leaves ($F = 77.913$; $df_1 = 4$; $df_2 = 145$; $p < 0.001$), plant fresh weight ($F = 83.417$; $df_1 = 4$; $df_2 = 145$; $p < 0.001$) and survival time ($F = 5.213$; $df_1 = 4$; $df_2 = 145$; $p < 0.001$) were significant in all concentrations. There was no statistical difference between increased drought stress and germination time (Table 1). On the other hand, with increasing salinity stress, the differences in terms of radicle length ($F = 97.618$; $df_1 = 4$; $df_2 = 145$; $p < 0.001$), hypocotyl length ($F = 94.765$; $df_1 = 4$; $df_2 = 145$; $p < 0.001$), number of leaves ($F = 109.154$; $df_1 = 4$; $df_2 = 145$; $p < 0.001$), plant fresh weight ($F = 83.417$; $df_1 = 4$; $df_2 = 145$; $p < 0.001$), survival time ($F = 35.074$; $df_1 = 4$; $df_2 = 145$; $p < 0.001$) and germination time ($F = 18.239$; $df_1 = 4$; $df_2 = 145$; $p < 0.001$) were significant (Table 2).

Vigor indexes of *G. pilulifera* seeds showed a negative correlation at all concentrations of increased salt and drought stress. In drought stress, the vigor index, which was 59.38 in the control group, decreased to 4.49 at 20% PEG concentration. A sharp decline was seen in salt stress conditions. The vigor index, which was 63.19 in the control group, almost halved at 50 mM NaCl concentration and reached 32.89. It decreased to 0 at 200 mM NaCl concentration (Fig. 2).

4. Discussions

Turkey is the gene center of the *Gypsophila* genus, which has high economic value due to the saponins they contain (Özçelik and Muca, 2010). The use of most of the species belonging to this genus from nature without culturing causes to be extinct of these species (Özçelik and Yıldırım, 2011). *G. pilulifera* Boiss. & Heldr. (Caryophyllaceae) is also not cultivated and the areas where it is spread are open to anthropogenic effect. This situation may cause to decrease the population density of the species and eventually to extinction. Researching the growing conditions and tolerance to abiotic factors of this endemic species, which also has medical-economic value, is important for its culture and production. In addition, increasing the agricultural production by selecting plants tolerant to stress factors has become an inevitable necessity in today's world where abiotic stress factors are showing more and more effects.

Table1. Vegetative growth values of *G. pilulifera* at different drought concentrations

	Doses (mM)	Mean±SE			N
			Tukey HSD[a]	Duncan[a]	
Radicle Length	0	31.833±1.616	d	d	30
	5	20.633±1.732	c	c	30
	10	9.533±1.265	b	b	30
	15	5.567±1.042	ab	a	30
	20	2.833±0.612	a	a	30
	Total	14.080±1.055			150
	ANOVA	**F=83.286; df_1=4; df_2=145; p<0.0001**			
Hypocotyl Length	0	27.767±1.407	d	d	30
	5	17.400±1.470	c	c	30
	10	7.700±1.028	b	b	30
	15	4.567±0.859	ab	ab	30
	20	1.700±0.369	a	a	30
	Total	11.827±0.922			150
	ANOVA	**F=94.099; df_1=4; df_2=145; p<0.0001**			
Number of Leaves	0	11.400±0.622	d	d	30
	5	7.567±0.657	c	c	30
	10	3.067±0.437	b	b	30
	15	1.933±0.365	ab	ab	30
	20	1.133±0.266	a	a	30
	Total	5.020±0.385			150
	ANOVA	**F=77.913; df_1=4; df_2=145; p<0.0001**			
Fresh Weight	0	0.265±0.014	d	d	30
	5	0.170±0.015	c	c	30
	10	0.086±0.012	b	b	30
	15	0.030±0.006	a	a	30
	20	0.030±0.006	a	a	30
	Total	0.116±0.008			150
	ANOVA	**F=83.417; df_1=4; df_2=145; p<0.0001**			
Germination Time	0	6.400±0.361	a	a	30
	5	6.767±0.615	a	a	30
	10	6.600±0.739	a	a	30
	15	5.667±0.920	a	a	30
	20	5.333±0.926	a	a	30
	Total	6.153±0.330			150
	ANOVA	**F=0.700; df_1=4; df_2=145; p>0.593**			
Survival Time	0	13.067±0.648	c	b	30
	5	11.667±0.969	bc	b	30
	10	10.200±1.143	abc	ab	30
	15	7.800±1.270	ab	a	30
	20	7.267±1.267	a	a	30
	Total	10.000±0.511			150
	ANOVA	**F=5.213; df_1=4; df_2=145; p<0.0001**			

Previous studies also enlighten the negative impact of salt stress on plant growth and development. Kumar (2013) reported that the germination percentage, germination rate and normal seedling percentage decreased in plants under salt stress. Due to the decrease in photosynthesis rate in drought stress, there is a decrease in vegetative growth, stem development and especially leaf development are more sensitive to water deficiency than root development (Çırak and Esendal, 2006). In the first periods of drought conditions, root growth was triggered and stem elongation slowed down in order to reach more water (Öztürk, 2015).

Karakaş et al. (2015) researched the tolerance of seeds of *Salsola soda* L. and *Portulaca oleracea* L., which are salt stress tolerant halophyte plants to increased NaCl doses under *in vitro* germination conditions. At the end of the second week, they found that there was a negative relationship increased salt concentration with the germination percentage, radicle and hypocotyl length, fresh weight, germination and vigor index of germinating seeds.

Şimşek et al. (2018) found that citrus rootstocks continued to survive and reproduce at increasing PEG doses under *in vitro* conditions, but their performance deteriorated. Ertekin et al. (2017) found that with the increasing salt concentration in 4 different common vetch varieties, germination rates, germination indices, root lengths, stem lengths and shoot fresh weights decreased significantly, and average germination times increased. Similar to these researches, radicle length, hypocotyl length, leaf number, plant fresh weight and survival time of *G. pilulifera* were significantly decreased under increasing salt and drought conditions compared to control application.

Germination percentage decreased under salt stress conditions in *G. oblanceolata* Barkoudah, which is an endemic and endangered halophyte species in Turkey. Only

Table 2. Vegetative Growth Values of *G. pilulifera* at Different Salt Concentrations

	Doses (mM)	Mean±SE			N
Radicle Length	0	34.633±1.776	d	d	30
	50	17.900±2.376	c	c	30
	100	6.533±1.500	b	b	30
	150	0.100±0.0557	a	a	30
	200	0,000±0.000	a	a	30
	Total	11.833±1.260			150
	ANOVA	**F=97.618; df_1=4; df_2=145; p<0.0001**			
Hypocotyl Length	0	28.800±1.465	d	d	30
	50	15.300±2.042	c	c	30
	100	5.333±1.224	b	b	30
	150	0.367±0.212	a	a	30
	200	0.000±0.000	a	a	30
	Total	9.960±12.881			150
	ANOVA	**F=94.765; df_1=4; df_2=145; p<0.0001**			
Number of Leaves	0	12.800±0.688	d	d	30
	50	5.400±0.737	c	c	30
	100	2.133±0.498	b	b	30
	150	0.267±0.158	ab	a	30
	200	0.000±0.000	a	a	30
	Total	4.120±0.450			150
	ANOVA	**F=109.154; df_1=4; df_2=145; p<0.0001**			
Fresh Weight	0	0.260±0.015	c	d	30
	50	0.115±0.015	b	c	30
	100	0.041±0.009	a	b	30
	150	0.008±0.004	a	a	30
	200	0.000±0.000	a	a	30
	Total	0.085±0.009			150
	ANOVA	**F=96.772; df_1=4; df_2=145; p<0.0001**			
Germination Time	0	6.067±0.325	b	c	30
	50	5.200±0.737	b	c	30
	10	4.867±0.846	b	c	30
	150	1.800±0.668	a	b	30
	200	0.000±0.000	a	a	30
	Total	3.587±0.326			150
	ANOVA	**F=18.239; df_1=4; df_2=145; p<0.0001**			
Survival Time	0	13.067±0.648	c	c	30
	50	9.333±1.225	b	b	30
	100	7.267±1.266	b	b	30
	150	1.767±0.784	a	a	30
	200	0.000±0.000	a	a	30
	Total	6.287±0.563			150
	ANOVA	**F=35.074; df_1=4; df_2=145; p<0.0001**			

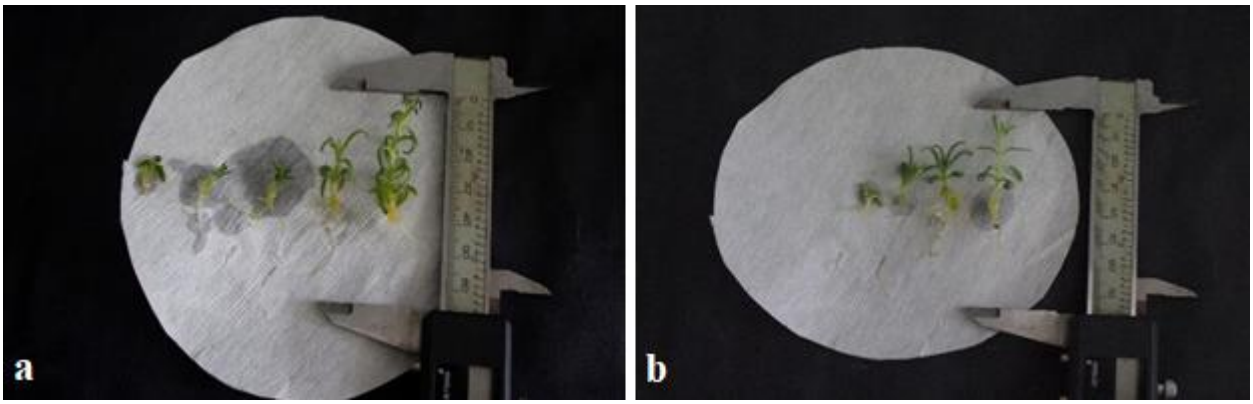

Figure 3. Radicle-hypocotyl length and number of leaves of plants at the end of the third week at different drought and salinity doses (a. Drought: 5%,10%, 15%, 20% PEG 6000 from right to left; b. Salinity: 0, 50, 100, 150 mM NaCl from right to left.)

a few seeds were able to germinate at 100 mM salt concentration (Sekmen et al. 2012). Consistent with this result, *G. pilulifera* was also more sensitive to salt stress than drought stress, and germination rate and vigor index were reduced by half in saline conditions compared to control plants. Even no germination was obtained in the presence of 200 mM NaCl.

While the germination time showed a homogeneous distribution under increasing drought stress, germination

times were also delayed in increasing salinity stress. In addition, at 200 mM NaCl concentration, the vigor index decreased to zero. These results showed that *G. pilulifera* was more sensitive to salinity stress and germination tolerance was higher in drought stress than salinity stress. *G. aucheri* Boiss., a xerophytic plant, was also found to be more tolerant of drought conditions, similar *G. pilulifera* (Esen et al., 2012). The Red List Index, which tracks the average extinction of the species over time, shows that the generations of the endangered groups become more at risk over time (Kurt, 2017). As with many species that have spread in narrow areas and evolved under difficult conditions, the rare compounds found in this species are used in many sectors and their economic importance increases due to these rarity. Crude saponin extract obtained from *G. pilulifera* species, which is in the critically endangered (CR) category and spread in an area open to andropogenic effects, is effective on *Bacillus subtilis* (Özbek Yazıcı and Özmen, 2018). Also, stem extracts and their fractions have free radical scavenging effects (Chima et al., 2014) the species whose roots are very rich in triterpenoid saponins (Arslan et al., 2012) has the potential to be used for both cut flower and landscaping (Kaya et al., 2012) is a very important species in terms of economics. In this study, germination and growth responses of *G. pilulifera* species against drought and salt stress were determined. It has been detected that the species can be grown under increasing drought conditions, but it is important to determine the salinity of the soil, especially by soil analysis. It is thought that the data obtained at the end of this study will contribute to the production and sustainability of the species under salt and drought stress conditions, which are predicted to increase even more in the coming years.

Conflict of Interest

Authors have declared no conflict of interest.

Authors' Contributions

The authors contributed equally.

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Clavulinopsis fusiformis, a new record for Turkish mycobiota

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Clavulinopsis fusiformis, Türkiye mikobiyotası için yeni bir kayıt

Abstract: *Clavulinopsis fusiformis* (Sowerby) Corner. is reported for the first time for the mycobiota of Turkey. This species is the fourth member of the genus *Clavulinopsis* Overeem in Turkey and characterized by its yellow and fasciculated fruit bodies, ellipsoid to subglobose or globose, one to multi-guttulate basidiospores with a distinct apiculus, and the presence of clamp connection at the base of basidia. The collected sample is described briefly and the photographs related to its macro and micromorphologies were provided.

Key words: *Agaricales, Basidiomycota,* biodiversity, new record, taxonomy

Özet: *Clavulinopsis fusiformis* (Sowerby) Corner. Türkiye mikobiyotası için ilk kez rapor edilmiştir. Bu tür *Clavulinopsis* Overeem cinsinin Türkiye'deki dördüncü üyesidir, ve sarı ve fasikül halindeki gövdesi, elipsoit, küremsiye yakın veya küremsi, tek veya çok damlacıklı, belirgin apikulusa sahip bazidiyosporlar ve bazidiyumların tabanında kulplu bağlantı ile karakterizedir. Toplanan örnek kısaca betimlenmiş ve makro ve mikromorfolojilerine ilişkin fotoğrafları verilmiştir.

Anahtar Kelimeler: *Agaricales, Basidiomycota,* biyoçeşitlilik, yeni kayıt, taksonomi

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1. Introduction

Clavulinopsis Overeem is a genus of coral fungi in the family *Clavariaceae*. It was first established by Casper van Overeem (1923). Members of the genus are widespread (Kirk et al., 2008) and characterized by yellow, orange or beige coloured, simple or regularly dichotomously branched, cylindric or fusoid stem with obtuse, inflated or cylindric, thin-walled hypha with clamps, truncate or attenuated apex, 2-4 spored basidia with clamps at the base, and smooth or echinulate spores often with big oil drop (Knudsen and Vesterholt, 2018; Petersen, 1969).

Kirk et al. (2008) reports the existence of 33 species, but Index Fungorum (2021) lists 68 conformed *Clavulinopsis* species, three of which, *C. corniculata* (Schaeff.: Fr.) Corner, *C. helvola* (Pers.) Corner and *C. umbrinella* (Sacc.) Corner, were also reported from Turkey (Vlaev, 1915; Pekşen and Karaca, 2003; Doğan et al., 2005; Yağız et al., 2005; Solak et al., 2015).

But the current checklist (Sesli et al., 2020) and the latest contributions (Allı et al., 2019; Yakar et al., 2019; Yıldız et al., 2019; Akçay, 2020; Yeşil et al., 2020; Acar et al., 2021; Doğan, 2021; Kaygusuz et al., 2021; Sesli, 2021; Uzun, 2021) on Turkish mycobiota indicate that, *C. fusiformis* hasn't been reported from Turkey before.

The study aims to make a contribution to the mycobiota of Turkey.

2. Materials and Method

The basidiocarps of *C. fusiformis* were collected during a routine field study in İyidere district of Rize province. Color photographs of the fruit bodies were taken in the field and ecological properties were recorded. Then the samples were collected and transferred to the fungarium within paper boxes. The samples were dried in an air conditioned room and prepared as fungarium material. Investigations were performed under a Leica trinocular microscope by preparing slides from dry specimens. Photographs related to micromorphology were obtained through a Leica ICC50HD digital camera. Identification was performed with the help of

Breitenbach and Kränzlin (1986), Ellis and Ellis (1990), Jordan (1995), Bessette et al. (2007), Kuo and Methven (2014). Furtado et al. (2016) and Acharya et al (2017).

The specimens are kept at Van Yüzüncü Yıl University Herbarium (VANF).

3. Results

Fungi R.T. Moore

Basidiomycota R.T. Moore

Agaricales Underw.

Clavariaceae Chevall

Clavulinopsis fusiformis (Sowerby) Corner, Monograph of Clavaria and allied Genera, (Annals of Botany Memoirs No. 1): 367 (1950)

Syn: [*Cavlaria ceranoides* Pers., *Clavaria compressa* Schwein., *Clavaria fusiformis* Sowerby, *Clavaria fusiformis* f. a*urantiaca* S. Imai, *Clavaria fusiformis* var. *ceranoides* W.G. Sm., *Clavaria fusiformis* var. *congoensis* Beeli, *Clavaria inaequalis* var. *fusiformis* (Sowerby) Fr., *Clavaria platyclada* Peck, Bull. *Clavulinopsis fusiformis* var. *bispora* K.S. Thind & Sharda, *Ramaria ceranoides* (Pers.) Gray, *Ramariopsis fusiformis* (Sowerby) R.H. Petersen]

Macroscopic and microscopic features: Basidiocarps 65-130 × 4-9 mm, cylindrical to slenderly fusiform, usually

twisted and pointed at the apex, some flattening or compressed with longitudinal grooves, surface smooth, bright to pale yellow, fading with age, somewhat darker toward the base and brownish at the tips at maturity (Fig 1). Flesh yellow, fragile. Taste somewhat bitter, odor not distinctive. Basidia 45-58 × 6.5-8 µm, slenderly clavate to subclavate, almost hyaline, with 2-4 sterigmata of 5.5-7 µm long (Fig 2a), clamps present. Basidiospores 5.5-8.2 × 5-7.7 µm, broadly ellipsoid, subglobose to almost subspherical, smooth, with drops or a large guttule and a prominent apiculus (Fig 2b).

Clavulinopsis fusiformis was reported to grow gregariously or in dense clusters with fused bases, on soil among grass or mosses, in poor meadows, grassland or in woods under hardwoods, conifers or shrubs (Breitenbach and Kränzlin, 1986; Buczacki, 1989; Ellis and Ellis, 1990; Jordan, 1995; Bessette et al., 2007, Acharya et al., 2017; Knudsen and Vesterholt, 2018).

Figure 1. Basidiocarps of *Clavulinopsis fusiformis*

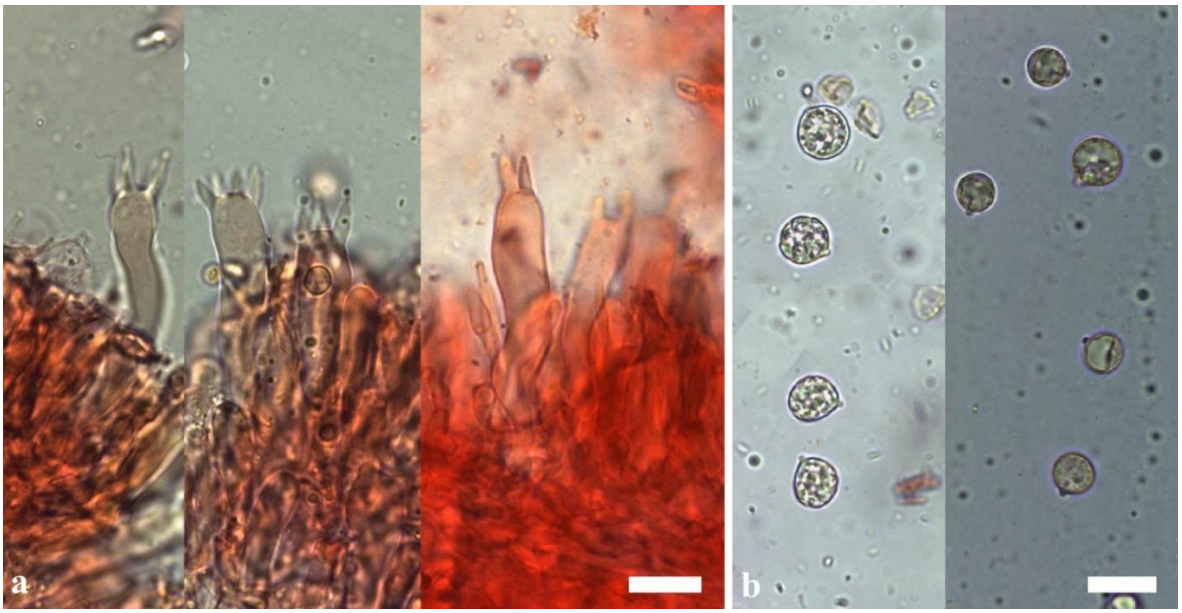

Figure 2. Basidia (a), and basidospores (b) of *Clavulinopsis fusiformis* (bars: 10 µm) (a: kongo red; b: water)

Specimens examined: Rize, İyidere, Denizgören village, Cami cemetery, on soil among mosses in coniferous forest., 40°58′408N, 40°22′351E, 118 m, 14.11.2015, AK. 2998.

4. Discussions

Clavulinopsis fusiformis was given as new record for Turkish mycobiota as the fourth member of the genus *Clavulinopsis*. In general, macro and micromorphology are in agreement with those given in literature (Breitenbach and Kränzlin, 1986; Buczacki, 1989; Ellis and Ellis, 1990; Jordan, 1995; Bessette et al., 2007, Acharya et al., 2017; Knudsen and Vesterholt, 2018).

Though Breitenbach and Kränzlin (1986) and Jordan (1995) give the spore size of *C. fusiformis* up to 6.5 × 6 µm, but we measured them up to 8.2 × 7.7 µm. Bessete et al. (2007) and Knudsen and Vesterhold (2018) also give the spore size up to 9 × 9 µm.

Clavulinopsis fusiformis is often confused with *C. laeticolor* (Berk. & M.A. Curtis) R.H. Petersen. But some macro and micromorphological characters differentiate these two species. *Clavulinopsis fusiformis* is typically yellow while *C. laeticolor* is usually some shade of orange. *Clavulinopsis laeticolor* is usually mild in taste, while *C. fusiformisis* typically bitter. Spores of *C. laeticolor* are subglobose to pyriforme while those of *C. fusiformis* spores are subglobose to almost spherical (Desjardin et al., 2014).

Lincoff (1981), Russel (2006) and Bessette et al. (2007) reports *C. fusiformis* as an edible species. But local people know it as an inedible fungus.

Conflict of Interest

Authors have declared no conflict of interest.

Authors' Contributions

The authors contributed equally.

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Comparative analysis of GC-MS of *Isolona* Engl. (*Annonaceae*) in Nigeria and the Cameroons

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Nijerya ve Kamerun'daki *Isolona* Engl. (*Annonaceae*) bitkisinin karşılaştırmalı GC-MS analizi

Abstract: The comparative assessment of six (6) *Isolona* species occurring in Nigeria and the Cameroons was undertaking using GC-MS analysis. The analysis was carried out with methanol extract and one hundred and seventy-six (176) phyto-constituents identified and scored at different retention times ranges from 3.22min in *I. hexaloba* (Pierre) Engl. & Diels. to 34.56min in *I. campanulata* Engl. & Diels.. The prominent compounds were scored in carboxylic and its derivates while the least compound was identified in alkyne. Highest M.wt. was 741.5 in *I. congolana* (De Wild.&T.Durand) Engl. & Diels. at retention time of 34.52 min and the lowest M.wt. was 84.15 in *I. thonneri* (De Wild.&T.Durand) Engl. & Diels. at retention time 3.44 min. Hexadecanoic acid, methyl ester formula $C_{17}H_{34}O_2$ and M.wt of 270.4 was scored in all the six species suggested a diagnostic characters for the genus *Isolona*. Quantitatively, the amount of the analytes isolated in all the species ranges from 0.1% in *I. hexaloba* to 59.36% in *I. thonneri.* Two UPGMA distant trees were constructed with RMSD and Euclidean index to illustrate the relationships among the *Isolona* species based on the difference chemical constituents. The distance tree revealed the separation of *I. thonneri* from all other species, also a sub-cluster tree of *I. hexaloba, I. campanulata* and *I. zenkeri* (Engl.) Dyer. was also constructed. The highest distance value level is 9.576 scored between *I. thonneri* and *I. congolana* while the highest similarity level was 2.911 scored in *I. hexaloba* and *I. campanulata*. The overlapping phyto-constituents characters revealed the closeness of the taxa studied, this invariably will update the existing data in the genus and ultimately in the family *Annonaceae*.

Key words: Comparative analysis, GC-MS, *Isolona*, Phyto-constituents

Özet: Nijerya ve Kamerun'da bulunan altı (6) *Isolona* türünün GC-MS analizi kullanılarak karşılaştırmalı değerlendirmeleri yapılmıştır. Analiz metanol özütü ile gerçekleştirildi ve yüz yetmiş altı (176) fito-bileşen tanımlandı ve *I heksaloba*'da 3.22 dakika ile *I. campanulata*'da 34.56 dakika arasında değişen farklı alıkonma sürelerinde tanımlanmaları gerçekleştirildi. Öne çıkan bileşikler karboksilik ve türevleri olur iken, en az tepit edilen bileşikler alkinlerde tanımlandı. En yüksek M.wt. 34.52 dakikalık alıkonma zamanında *I. congolana*'da 741.5 olarak ölçülürken, düşük M.wt. değeri 84.15 ile 3.44 dk alıkonma süresinde *I. thonneri'*de tespit edildi. Heksadekanoik asit, metil ester formülü $C_{17}H_{34}O_2$ ve 270.4 M.wt olarak değerlendirilen bileşik, altı türün tamamında tespit edildi ve *Isolona* cinsi için bir tanısal karakter önerildi. Kantitatif olarak, tüm türlerde izole edilen analit miktarı, *I. hexaloba*'da %0.1 ile *I. thonneri'*de %59.36'ya kadar değişim gösterdi. Farklı kimyasal bileşenlere dayalı olarak *Isolona* türleri arasındaki ilişkileri göstermek için RMSD ve Öklid indeksi ile iki UPGMA ağacı oluşturuldu. Oluşturulan ağaç, *I. thonneri*'nin diğer tüm türlerden ayrıldığını ortaya çıkarmış, ayrıca *I. hexaloba, I. campanulata* ve *I. zenkeri*'nin bir alt ağaç oluşturduğu görüldü. En yüksek uzaklık değeri *I. thonneri* ve *I. congolana* arasında 9.576 olarak puanır iken, en yüksek benzerlik düzeyi *I. hexaloba* ve *I. campanulata*'da 2.911 puan almıştır. Örtüşen fito-bileşen karakterleri, incelenen taksonların yakınlığını ortaya çıkardı, bu durum her cinsteki ve nihayetinde Annonaceae familyasındaki mevcut verileri güncellenmesine neden olacaktır.

Anahtar Kelimeler: Karşılaştırmalı analiz, GC-MS, *Isolona*, Fito-bileşenler

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1. Introduction

Annonaceae Juss. are a pantropical family of trees, shrubs, and lianas belonging to the order Magnoliales (APGII, 2003) with *c.* 130 genera and *c.* 2500 species (Chatrou et al., 2004). In Africa, the family comprises *ca.* 42 genera and 500 species (Bremer et al., 2009; Smith et al., 2010; Couvreur et al., 2012; Zeng et al., 2014). The African genera were treated as a whole for the last time over 100 years ago by Engler and Diels (1901). Since then, regional accounts have been published in the second half of the last century such as those for Flore du Gabon (Le Thomas, 1969), Flora of West Tropical Africa (Hutchinson et al., 1954) and Flora of Tropical East Africa (Verdcourt, 1971). African *Annonaceae* have been largely understudied in recent years when compared to Neotropical and South-East Asian taxa (Maas et al., 2003; Couvreur et al., 2006; Couvreur et al., 2008). One of these genera is *Isolona* Engl., a sizeable genus with *ca.* 21 species distributed across the tropical zone. *Isolona* Engl. flowers are hermaphroditic and are unusual as all six petals are basally connate, forming a single whorl (Couvreur, 2009). Relatively little is known about the reproductive biology, although the flowers of *I. campanulata* Engl. & Diels have been shown to be protogynous, with the reproductive phases extending over a 2-day (or possibly 3-day) period, with diurnal receptivity (Gottsberger et al., 2011). The species are mainly trees and there are accounts on exomorphology (Maas et al., 2003; Couvreur, 2009).

According to Panichpol and Waterman (1978), the Annonaceae is perhaps one of the least chemically known families'. A comprehensive review article published by" Leboeuf et al. (1982) updates all the important phytochemical research on Annonaceae members. Moreover, many earlier studies are only fragmentary and systematic reinvestigations are necessary in many cases. In order to draw valid conclusions on chemotaxonomic features of the Annonaceae, more chemical investigations are necessary. However, there is paucity of information on the structures of these chemicals and it has not been entrenched into taxonomic studies. Therefore, in search for additional identification criteria and pharmacological properties of the genus, the phyto-constituents characters of 6 African *Isolona* species occurring in Nigeria and Cameroons were investigated with a view to update the existing data within the family.

2. Materials and Methods

Dried herbarium samples of the six (6) species of *Isolona* from West and Central African obtained from the National Herbarium Yaounde, Cameroon YA(IH) were used for the study. The herbarium abbreviations follow Holmgren and Holmgren (2003) while the specific samples of the studied species and their herbarium information were *Isolona campanulata*: P.T. Francis, Dec. 1945; *I. congolana*: Westphal, 15/5/78; *I. dewevrei*: R. Letouvzey, 8/7/75; *I. hexaloba*: R. Letouvzey, 16/5/63; *I. thonneri*: R. Letouvzey, 23/3/70 and *I. zenkeri*: Endengle Elais, 1955-1956; showing species name, collector and date of collection respectively.

Two to 5 g of the plant sample was cut into small sizeable pieces and weighed then mixed with absolute methanol at a 1:20 ratio (100 g in 1 L solvent) and shaked thoroughly for 2-3 days. The extract was filtered by Whatman filter paper then the filtrate concentrated using the BUCHI Switzerland rotary evaporator to remove the methanol and obtain pure extract. 30 ml of the solvent was repeated twice in the process and the extract was concentrated using nitrogen concentration and later treated with silica gel and anhydrous sodium sulphate. The GC-MS used for analysis is Agilent technologies 7890GC system coupled with mass spectrometer of model Agilent technologies 5975. The mobile phase is helium gas and the stationery phase is the column with length 30 m, internal diameter 0.320 mm, thickness 0.25 µm. The oven temperature program, initial temperature is 80 °C to hold for 2 min at 10 °C per min to final temperature of 240 °C to hold for 6 min, the volume injected 1 µl. Method follows Cunha et al. (2004) and Phrompittayarat et al. (2008).

The phytocomponents of the extracts from the different species were identified based on direct comparison with the database of National Institute Standard and Technology (NIST) and Royal Society of Chemistry and National Library of Medicine.

2.1. Data analysis

The separated compounds by GC-MS analysis were scored in binary matrices, where 0 stands for the absence and 1 stands for the presence of a compound for all studied *Isolona* species; these codes were detailed in Table 1. Statistical analysis of the data of the compounds identified was carried out by using an online program Dendro UPGMA (A dendrogram Construction Utility) using RMSD coefficient and a software Paleontological Statistics software used for constructing Euclidean similarity index, scatter plot, and PCA using used for the constructing. This method follows the approaches of Gamal et al. (2017).

3. Results

The GC-MS comparative analysis of six (6) species of *Isolona* namely: *I. congolana, I. dewevrei, I. hexaloba, I. thonneri, I. zenkeri* and *I. campanulata* isolated and identified one hundred seventy six (176) compounds at different retention times between 3.26 min and 34.56 min (Table 1). The highest number of compounds separated and recorded was in *I. thonneri* at retention time between 3.44 and 33.86 min, while the lowest number of compounds was 29 at a retention time between 3.69 and 34.52 min in *I. congolona*. The highest M.wt. was 741.5 with a compound named Cyclodecasiloxane, eicosamethyl- and scored in *I. congolana* at retention time of 34.52 min and the lowest M.wt. was 78.13 (Mercaptoethanol, 2TMS derivatives) in *I. thonneri* at retention time 3.44 min. The first compound dissociated in all the species was between 3.26 min and 3.69 min retention time except *I. zenkeri* at about 8.24 min. The last compounds separated lies between retention time of 33.78 and 34.56 min but *I. dewevrei* last compound was dissociated at retention time of 20.39 min. The amount of analytes isolated in all the species ranges from 0.1% with decided name: 1H-Indole-2-carboxylic acid, 6-(4-ethoxyphenyl)-3-methyl-4-oxo-4,5,6,7-tetrahydro-, isopropyl ester and hexasiloxane, 1,1,3,3,5,5,7,7,9,9, 11,11-dodecamethyl- in *I. hexaloba* to 59.36% with decided name: 1-Butanol, 2-methyl- ; cyclobutane, ethyl- and decane, 2,2,3-trimethyl- in *I. thonneri.* A compound named cyclononasiloxane, octadecamethyl was scored in *I. congolana, I. zenkeri* and *I. campanulata.* Also compounds with decided name octasiloxane, 1,1,3,3, 5,5,7,7,9,9,11,11,13,13, 15,15-hexadecamethyl- and heptasiloxane, 1,1,3,3, 5,5,7,7,9,9,11,11,13,13-tetradecamethyl- were scored in *I. hexaloba, I. thonneri, I. zenkeri* and *I. campanulata.* Likewise, hexadecanoic acid, methyl ester with the formula $C_{17}H_{34}O_2$ and molecular weight of 270.4 was scored in all the six species (Table 1).

3.1. *Isolona congolana*

There were twenty-nine phyto-compounds identified in *I. congolana* broadly grouped into alkanes, alcohol, organo-compound, alkaloid, carboxylic acids and its derivatives. A compound with decided name cyclodecasiloxane, eicosamethyl-, formula $C_{20}H_{60}O_{10}Si_{10}$ scored with the highest molecular weight of about 741.54 was found in *I. congolana*. Most prominent compounds in *I. congolana* were the carboxylic acids with fourteen (14) compounds and the least compound was an alkaloid with decided name benzene, nitroso- formula C_6H_5NO (Table 1). The retention time for the dissociation of the compounds was between 3.69 and 34.52min with M.wt ranged from 107.11 in Benzene, nitroso- to 741.53 in cyclodecasiloxane, eicosamethyl- (Fig. 1).

3.2. *Isolona dewevrei*

The M.wt of thirty-two (32) compounds separated between 3.36 and 20.39 min retention times was between 92.19 in a compound with decided name trimethylsilyl fluoride formula C_3H_9FSi and 270.45 in a compound with

Table 1. The GC-MS analysis of six (6) species of *Isolona* based on the phyto-chemical constituents

S/N	COMPOUNDS	R.T	Area%	M.F	M.W	A	B	C	D	E	F
	ALCOHOL										
1.	1,2-Benzenediol, 3,5-bis(1,1-dimethylethyl)-	32.86	4.13	$C_{14}H_{22}O_2$	222.32	0	0	0	0	0	1
2.	1-Butanol, 2-methyl-	3.44	59.36	$C_7H_{14}O_2$	130.19	0	0	0	1	0	0
3.	2-Naphthalenemethanol, decahydro-. alpha.,.alpha.,4a-trimethyl-8-methylene-, [2R-(2.alpha.,4a.alpha.,8a.beta.)]-	16.45	8.3	$C_{15}H_{26}O$	222.37	0	0	0	0	1	1
4.	Benzenemethanol, 4-methyl-	3.68	1	$C_8H_{10}O$	122.16	0	0	0	0	0	1
5.	Benzyl alcohol	7.384	7.81	C_7H_8O	108.14	0	1	0	0	0	0
6.	Bicyclo[2.2.1]heptan-2-ol, 1,7,7-trimethyl-, acetate, (1S-endo)-	11.258	5.88	$C_{12}H_{20}O_2$	196.29	1	0	0	0	0	0
7	Heptaethylene glycol, TBDMS	17.21	1.62	$C_{20}H_{44}O_8Si$	440.41	0	0	0	1	0	0
8.	Mercaptoethanol, 2TMS derivative	32.82	9.99	C_2H_6OS	78.13	0	0	0	0	1	0
9.	Pentaethylene glycol, TBDMS	17.21	1.62	$C_{16}H_{36}O_6Si$	352.54	0	0	0	1	0	0
10.	Phenol, 3,4-dimethyl-	3.693	2.55	$C_8H_{10}O$	122.16	1	0	0	0	0	0
11.	1H-1,2,3-benzotriazole, 5,6-dichloro	15.91	2.14	$C_6H_3Cl_2N_3$	188.01	0	1	0	0	0	0
	ALKALOID										
12.	Benzene, nitroso-	3.693	2.55	C_6H_5NO	107.11	1	0	0	0	0	0
13.	Benzo[h]quinoline, 2,4-dimethyl-	33.07	1.09	$C_{15}H_{13}N$	207.27	0	0	1	1	0	1
14.	Etiron	3.67	0.28	$C_3H_9BrN_2S$	184.9	0	1	0	0	0	0
15.	Hexestrol, 2TMS derivative	33.86	0.24	$C_{24}H_{38}O_2Si_2$	414.73	0	0	0	1	0	0
16.	Oxime-, methoxy-phenyl-_	5.376	1.29	$C_8H_9NO_2$	151.16	0	0	1	0	0	0
17.	1-Methyl-2-aminomethylimidazole	23.36	1.64	$C_5H_9N_3$	111.15	0	0	0	1	0	0
18.	4-Benzamido-4-dichloromethyl-2-phenyl-2-imidazolin-5-one	3.26	7.5	$C_9H_{10}N_2$	146.19	0	0	0	0	0	1
19.	Methenamine	10.69	1.03	$C_6H_{12}N_4$	140.19	0	0	1	0	0	0
20	5-Methyl-2-phenylindolizine	33.19	1.92	$C_{15}H_{13}$	207.27	0	0	1	0	0	0
21.	1H-Benzo[4,5]furo[3,2-f]indole	33.4	4.35	$C_{14}H_9NO$	207.23	0	0	0	1	0	0
22.	1H-Indole, 1-methyl-2-phenyl-	32.78	0.8	$C_{15}H_{13}N$	207.27	0	0	0	1	0	0
23.	1H-Indole, 5-methyl-2-phenyl-	33.51	1.04	$C_{15}H_{13}N$	207.27	0	0	0	1	0	0
24.	1H-Indole-2,3-dione, 5,7-dichloro-	15.91	2.14	$C_8H_3Cl_2NO_2$	216.02	0	1	0	0	0	0
25.	1H-Indole-2-carboxylic acid, 6-(4-ethoxyphenyl)-3-methyl-4-oxo-4,5,6,7-tetrahydro-, isopropyl ester	32.58	0.1	$C_{21}H_{25}$	355.43	0	0	1	0	0	1
26.	3H-indole, 2-methyl-3-phenyl-	33.76	1.83	$C_{16}H_{15}N$	221.3	0	0	0	1	0	1
27.	3-Amino-6-oxo-7a-phenyl-4aH,5H,7H-pyrrolo[2,3-c]pyridazine-4,5-dicarbonitrile	3.68	1	$C_{12}H_{10}N_4O_2$	242.23	0	0	0	0	0	1
28.	2-Ethylacridine	31.83	1.3	$C_{15}H_{13}N$	207.27	0	0	1	1	0	1
29.	n(sup1),n(sup4)-dibenzoylpiperazin	3.362	19.97	$C_{18}H_{18}N_2O_2$	294.3	0	0	1	0	0	0
30.	N,N-Dimethyl-4-nitroso-3-(trimethylsilyl)aniline	34.05	1.2	$C_{11}H_{18}N_2OSi$	222.36	0	0	0	0	0	1
31.	Pyrido[2,3-d]pyrimidine, 4-phenyl-	33.55	0.34	$C_{13}H_9N_3$	207.23	0	0	1	0	0	0
32.	1-(4-Nitrophenyl)piperazine	4.81	1.08	$C_{10}H_{13}N_3O_2$	207.23	0	0	0	1	0	0
33.	.gamma.-Muurolene	13.92	1.52	$C_{15}H_{24}$	204.35	0	0	0	0	1	0
	ALKANE										
34.	2,3,5-Trioxabicyclo[2.1.0]pentane, 1,4-bis(phenylmethyl)-	11.67	0.35	$C_{16}H_{14}O_3$	254.28	0	1	0	0	0	0
35.	1-Bromoeicosane	8.359	2.29	$C_{20}H_{41}Br$	361.44	1	0	0	0	0	0
36.	2-Methylhexacosane	8.519	2.98	$C_{27}H_{56}$	380.7	1	0	0	0	0	0
37.	Bicyclo[3.1.1]heptane, 2,6,6-trimethyl-, (1.alpha.,2.beta.,5.alpha.)	19.05	2.27	$C_{10}H_{18}$	138.25	0	0	1	0	0	0
38.	Cyclobutane, ethyl-	3.44	59.36	C_6H_{12}	84.16	0	0	0	1	0	0
39.	Decane, 2,2,3-trimethyl-	3.44	59.36	$C_{13}H_{28}$	184.36	0	0	0	1	0	0
40.	Decane, 2-methyl-	16.923	7.18	$C_{11}H_{24}$	156.31	1	0	0	0	0	0
41.	Dotriacontane	16.831	2.24	$C_{32}H_{66}$	450.87	1	0	0	0	0	0
42.	Eicosane	30.24	16.77	$C_{20}H_{42}$	282.55	0	0	0	0	1	0
43.	Eicosane, 9-octyl-	32.49	11.79	$C_{28}H_{58}$	394.76	0	0	0	0	1	0
44.	Hentriacontane	8.519	2.98	$C_{31}H_{64}$	436.84	1	0	0	0	0	0
45.	Heptacosane, 1-chloro-	30.24	16.77	$C_{27}H_{55}Cl$	415.18	0	0	0	0	1	0
46.	Hexadecane	15.395	2.24	$C_{16}H_{34}$	226.44	1	0	0	0	0	0
47.	Hexadecane, 2,6,11,15-tetramethyl-	16.923	7.18	$C_{20}H_{42}$	282.5	1	0	0	0	0	0
48.	Hexane, 2,2,3-trimethyl-	3.361	0.98	C_9H_{20}	128.25	0	1	0	0	0	0
49.	Methylene chloride	3.608	3.56	CH_2Cl_2	84.93	0	0	1	0	0	0
50.	Nonadecane	32.49	11.79	$C_{18}H_{38}$	254.49	0	0	0	0	1	0
51.	Octacosane	30.24	16.77	$C_{28}H_{58}$	394.77	0	0	0	0	1	0
52.	Pentane, 3-ethyl-2,2-dimethyl-	3.361	0.98	C_9H_{20}	128.25	0	1	0	0	0	0
53.	Tetracontane, 3,5,24-trimethyl-	15.395	2.24	$C_{43}H_{88}$	605.2	1	0	0	0	0	0
54.	Tridecane	16.093	2.87	$C_{13}H_{28}$	184.36	1	0	0	0	0	0
	ALKENE										
55.	5,7-Dimethylenebicyclo[2.2.2]oct-2 -ene	11.67	0.35	$C_{10}H_{12}$	132.2	0	1	0	0	0	0
56.	Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-,[1R-(1R*,4 Z,9S*)]-	13.84	1.86	$C_{15}H_{24}$	204.35	0	0	0	0	1	0
57.	Neophytadiene	19.05	2.27	$C_{20}H_{38}$	278.51	0	0	1	0	0	0
58.	Tricyclo[4.2.1.0(2,5)]non-7-ene, 3,4-	32.69	0.64	$C_{27}H_{64}O_6Si_8$	709.48	0	0	1	0	0	1

	di(tris(trimethylsilyloxy)silyl)-										
	ALKYNE										
59.	3-Octen-5-yne, (Z)-	9.078	7.55	C_8H_{12}	108.18	0	1	0	0	0	0
	BENZENE										
60.	1,3-Benzodioxole, 4-methoxy-6-(2-nitro-1-propenyl)-	14.89	1.1	$C_{11}H_{12}O_3$	192.21	0	0	0	1	0	0
61.	Benzene, 3-butenyl-	11.67	0.35	$C_{10}H_{12}$	132.20	0	1	0	0	0	0
	CARBOXYLIC AND ITS DERIVATIVES										
62.	2'-Hydroxy-5'-methylacetophenone, TMS derivative	33.57	0.4	$C_9H_{10}O_2$	150.17	0	0	1	0	0	0
63.	N-Benzenesulfonylazetidin-3-one	3.26	7.5	$C_9H_9NO_3S$	211.24	0	0	0	0	0	1
64.	Propenone, 3-(2-benzoxazolylthio)-1-phenyl-	12.67	0.93	$C_{16}H_{11}NO_2S$	280.99	0	0	0	1	0	0
65.	1,4-benzenedicarboxylic acid, mono(1-methylethyl) ester	29.28	2.74	$C_{11}H_{12}O_4$	208.21	0	0	0	0	1	0
66.	10-Octadecenoic acid, methyl ester	23.46	2.54	$C_{19}H_{36}O_2$	296.5	0	0	1	0	1	0
67.	12-Octadecenoic acid, methyl ester	23.35	7	$C_{19}H_{36}O_2$	294.47	0	0	0	0	1	0
68.	13-Octadecenoic acid, methyl ester	23.46	2.54	$C_{19}H_{36}O_2$	296.49	0	0	1	0	0	0
69.	14-Octadecenoic acid, methyl ester	23.35	7	$C_{19}H_{36}O_2$	296.5	0	0	0	0	1	0
70.	1-Benzazirene-1-carboxylic acid, 2,2,5a-trimethyl-1a-[3-oxo-1-butenyl] perhydro-, methyl ester	33.39	0.64	$C_{15}H_{23}NO_3$	265.35	0	0	1	1	0	0
71.	1-Butanol, 3-methyl-, acetate	4.7	0.93	$C_7H_{14}O_2$	130.19	0	1	0	0	0	0
72.	1H-Cyclopenta[1,3]cyclopropa[1,2]benzene, octahydro-7-methyl-3-methylene-4-(1-methylethyl)-, [3aS-(3a.alpha.,3b.beta.,4.beta.,7.alpha.,7aS*)]-	13.92	1.52	$C_{15}H_{24}$	204.35	0	0	0	0	1	0
73.	2-(5-Phenyl-2-oxazolyl)benzoicaci	3.68	1	$C_{16}H_{11}NO_3$	265.26	0	0	0	0	0	1
74.	2-(Acetoxymethyl)-3-(methoxycarbonyl)biphenylene	33.04	0.56	$C_{17}H_{14}O_4$	281.99	0	0	1	1	0	1
75.	2-(Isobutoxycarbonyl)benzoic acid	29.36	3.06	$C_{12}H_{14}O_4$	222.24	0	0	1	0	0	0
76.	2,4,6-Trimethylbenzoic acid, TMS derivatives	31.909	30.76	$C_{13}H_{20}O_2Si$	236.38	1	0	0	0	0	0
77.	2-Amino-5-methylbenzoic acid	5.376	1.29	$C_8H_9NO_2$	151.16	0	0	1	0	0	0
78.	2-Amino-6-methylbenzoic acid	5.376	1.29	$C_8H_9NO_2$	151.16	0	0	1	0	0	0
79.	3-(4-Hydroxyphenyl)propionitrile	3.693	2.55	C_9H_9NO	147.17	1	0	0	0	0	0
80.	3-Chloropropionic acid, benzyl ester	9.198	2.65	$C_{10}H_{11}ClO_2$	198.64	0	1	0	0	0	0
81.	3-Methyl-hexanoic acid	3.361	0.98	$C_7H_{14}O_2$	130.19	0	1	0	0	0	0
82.	3-Quinolinecarboxylic acid, 6,8-difluoro-4-hydroxy-, ethyl ester	32.74	0.19	$C_{12}H_9F_2NO_3$	253.2	0	0	1	1	0	1
83.	3-Trimethylsilyloxystearic acid, trimethylsilyl ester	30.03	10.04	$C_{24}H_{52}O_3Si_2$	444.8	0	0	0	0	1	0
84.	4-Benzyloxybenzoic acid	19.772	0.58	$C_{14}H_{12}O_3$	228.24	0	1	0	0	0	0
85.	4-pyridinecarboxylic acid, 3-cyano-2-hydroxy-6-methyl-5-nitro-, methyl ester	14.89	1.1	$C_8H_6N_2O_2$	162.15	0	0	0	1	0	0
86.	7-Hexadecenoic acid, methyl ester (Z)-	23.36	1.64	$C_{17}H_{32}O_2$	268.4	0	0	0	1	0	0
87.	9,12-Octadecadiynoic acid, methylester	13.84	1.86	$C_{19}H_{34}O_2$	294.47	0	0	0	0	1	0
88.	Acetic acid, [4-(1,1-dimethylethyl)phenoxy]-, methyl ester	32.59	2.11	$C_{13}H_{18}O_3$	221.99	0	0	0	0	0	1
89.	Acetic acid, 1,7,7-trimethyl-bicyclo[2.2.1]hept-2-yl ester	11.258	5.88	$C_{12}H_{20}O_2$	196.29	1	0	0	0	0	0
90.	acetic acid, 2-[bis(methylthio)methylene]-1-phenylhydrazide	32.74	0.19	$C_6H_6N_2S_2$	170.26	0	0	1	0	0	0
91.	Acetic acid, phenylmethyl ester	9.347	45.54	$C_9H_{10}O_2$	150.18	0	1	0	0	0	0
92.	Acridine-9-carbaldehyde	33.86	0.24	$C_{14}H_9NO$	207.23	0	0	0	1	0	0
93.	Allyl 2-ethyl butyrate	7.899	0.45	$C_9H_{16}O_2$	156.22	0	1	0	0	0	0
94.	Benzoic acid, 2-hydroxy-, phenylmethyl ester	19.772	0.58	$C_{17}H_{18}O_3$	270.32	0	1	0	0	0	0
95.	Benzoic acid, 2-methoxy-, methyl ester	4.81	1.08	$C_9H_{10}O_3$	166.17	0	0	0	1	0	0
96.	Benzoic acid, hydrazide	8.25	2.22	$C_7H_8N_2O$	136.15	0	0	0	0	0	1
97.	Benzoic acid, methyl ester	8.27	0.33	$C_8H_8O_2$	136.15	0	0	0	1	1	1
98.	Bis(2-ethylhexyl) phthalate	29.36	3.06	$C_{24}H_{38}O_4$	390.56	0	0	1	1	1	0
99.	Butanoic acid, ethyl ester	3.459	3.21	$C_6H_{12}O_2$	116.16	0	1	0	0	0	0
100.	Carbonic acid, prop-1-en-2-yl tridecyl ester	16.093	2.87	$C_{17}H_{32}O_3$	284.43	1	0	0	0	0	0
101.	Carbonic acid, undecyl vinyl ester	16.831	2.24	$C_{14}H_{26}O_3$	242.35	1	0	0	0	0	0
102.	cis-13-Octadecenoic acid	23.46	2.54	$C_{19}H_{36}O_2$	296.5	0	0	1	0	0	0
103.	Diethyl Phthalate	15.52	17.62	$C_{12}H_{14}O_4$	222.24	0	1	0	0	0	0
104.	Diisooctyl phthalate	29.28	2.74	$C_{24}H_{38}O_4$	390.6	0	0	0	0	1	0
105.	Dodecanoic acid, methyl ester	14.44	1.02	$C_{13}H_{26}O_2$	214.34	0	0	1	0	0	0
106.	Ethyl (2-hydroxyphenyl)acetate, TMS derivative	19.73	1.88	$C_{10}H_{12}O_3$	180.2	0	0	0	1	0	0
107.	Formic acid, 1-(4,7-dihydro-2-methyl-7-oxopyrazolo[1,5-a]pyrimidin-5-yl)-, methyl ester	2.33	34.33	$C_5H_{10}O_2$	102.13	0	0	0	0	0	1
108.	Heptadecanoic acid, 16-methyl-, methyl ester	23.77	4.87	$C_{19}H_{38}O_2$	298.50	0	0	0	0	1	0
109.	Heptanoic acid, methyl ester	20.39	1.92	$C_8H_{16}O_2$	144.21	0	0	0	1	0	0
110.	Hexadecanoic acid, 2-methyl-	20.42	8.23	$C_{20}H_{40}O_2$	312.53	0	0	0	0	0	1
111.	Hexadecanoic acid, methyl ester	20.39	0.38	$C_{17}H_{34}O_2$	270.45	1	1	1	1	1	1
112.	Hexanoic acid, 2-propenyl ester	7.899	0.45	$C_9H_{16}O_2$	156.22	0	1	0	0	0	0
113.	Isobornyl acetate	11.258	5.88	$C_{12}H_{20}O_2$	196.29	1	0	0	0	0	0
114.	Methoxyacetic acid, 2-pentadecyl ester	8.359	2.29	$C_{18}H_{36}O_3$	300.5	1	0	0	0	0	0
115.	Methoxyacetic acid, 2-tetradecyl ester	8.359	2.29	$C_{17}H_{34}O_3$	286.4	1	0	0	0	0	0
116.	Methoxyacetic acid, 3-tridecyl ester	16.093	2.87	$C_{16}H_{32}O_3$	272.42	1	0	0	0	0	0
117.	Methyl anthranilate	12.05	3.05	$C_8H_9NO_2$	151.16	0	1	0	0	0	0
118.	Methyl salicylate	9.827	3.61	$C_8H_8O_3$	152.15	0	1	0	0	0	0

119.	Methyl stearate	23.86	2.53	$C_{19}H_{38}O_2$	298.50	0	0	1	0	1	1
120.	Methyl tetradecanoate	17.29	1.3	$C_{15}H_{30}O_2$	242.39	0	0	1	0	0	0
121.	Methyl tropate, (.alpha.)-, TMS derivative	19.73	1.88	$C_{10}H_{12}O_3$	180.20	0	0	0	1	0	0
122.	N-Methyl-1-adamantaneacetamide	32.54	0.45	$C_{13}H_{21}NO$	207.31	0	0	1	1	1	1
123.	Octacosylheptafluorobutyrate	16.831	2.24	$C_{32}H_{57}F_7O_2$	606.78	1	0	0	0	0	0
124.	Pentadecanoic acid, 14-methyl-, methyl ester	20.39	14.1	$C_{17}H_{34}O_2$	270.5	0	0	0	0	1	1
125.	Pentadecanoic acid, methyl ester	20.39	1.92	$C_{16}H_{32}O_2$	256.42	0	0	0	1	0	0
126.	Perfluoropropionic acid, TBDMS derivate	3.67	0.28	$C_3HF_5O_2$	164.03	0	1	0	0	0	1
127.	Phthalic acid, heptyl oct-3-yl ester	29.36	3.06	$C_{23}H_{36}O_4$	376.53	0	0	1	0	0	0
128.	Prop-2-enoic acid, 2-cyano-3-(3-methyl-2-thienyl)-, methyl ester	29.28	2.06	$C_{10}H_9NO_2S$	206.98	0	0	0	1	0	0
129.	Silicic acid, diethyl bis(trimethylsilyl) ester	33.1	1.91	$C_{10}H_{28}O_4Si_3$	296.58	0	0	0	1	0	1
130.	Sulfurous acid, butyl heptadecyl ester	8.519	2.98	$C_{21}H_{44}O_3S$	376.6	1	0	0	0	0	0
131.	Swep	15.91	2.14	$C_8H_7Cl_2NO_2$	219.98	0	1	0	0	0	0
132.	Tetradecanoic acid, 12-methyl-, methyl ester	20.436	4.71	$C_{16}H_{32}O_2$	256.42	1	0	0	0	0	1
133.	Tridecanoic acid, 12-methyl-, methyl ester	17.29	1.3	$C_{15}H_{30}O_2$	242.39	0	0	1	0	0	0
134.	Trimethylsilyl 2-(trimethylsilyloxy)propaneperoxoate	12.67	0.93	$C_{13}H_{33}NO_3Si_3$	335.66	0	0	0	1	0	0
135.	Undecanoic acid, 10-methyl-, methyl ester	14.44	1.02	$C_{13}H_{26}O_2$	214.34	0	0	1	0	0	0
136.	Benzylcarbamate	9.347	45.54	$C_8H_9NO_2$	151.16	0	1	0	0	0	0
137.	benzeneacetaldehyde, .alpha.-(methoxymethylene)-4-nitro-	32.78	0.8	$C_{10}H_9NO_4$	207.18	0	0	0	1	0	0
138.	Octanal, 2-(phenylmethylene)-	17.74	0.92	$C_{15}H_{20}O$	216.32	0	1	0	0	0	0
138.	(2E,4E)-N-Isobutyltetradeca-2,4-dienamide	33.24	0.32	$C_{18}H_{33}NO$	279.46	0	0	1	0	0	0
139.	Benzamide, N-[6-(2-furyl)-2-oxo-2H-pyran-3-yl]-	8.22	0.41	$C_{16}H_{11}NO_4$	256.99	0	0	0	1	0	0
140.	Salicylamide	9.827	3.61	$C_7H_7NO_2$	137.14	0	1	0	0	0	0
141.	Silane, ethenyldiethylmethyl-	23.67	1.79	$C_7H_{16}Si$	128.29	0	0	1	0	0	0
142.	1-(2-Thienyl)ethanonesemicarbazoneditms	17.21	1.62	C_6H_6OS	126.18	0	0	0	1	0	0
143.	1-(4-Methoxy-phenyl)-5,5-dioxo-hexahydro-5.lambda.(6)-thieno[3,4-b]pyrrol-2-one	29.28	2.06	$C_{13}H_{15}NO_4S$	281.33	0	0	0	1	0	0
144.	1,2,4-Triazine-3,5(2H,4H)-dione, 6-benzoylthio-	8.22	0.41	$C_7H_{11}N_3O_2$	169.18	0	0	0	1	0	0
145.	2-(Ethyl)oxybenzylideneacetophenone	33.61	0.58	$C_{17}H_{16}O_2$	252.31	0	0	0	0	0	1
146.	2-(n-Propyl)oxybenzylideneacetophenone	33.55	0.34	$C_5H_{12}O_2$	104.15	0	0	1	1	0	1
147.	1H-Cycloprop[e]azulene, decahydro-1,1,7-trimethyl-4-methylene-	13.84	1.86	$C_{15}H_{24}$	204.35	0	0	0	0	1	0
148.	Cryptomeridiol	16.48	2.1	$C_{15}H_{28}O_2$	240.38	0	0	0	0	0	1
149.	Phytol	23.67	1.79	$C_{20}H_{40}O$	296.53	0	0	1	0	0	0
150.	2(3H)-Furanone, 5-hexyldihydro-	15.18	1.3	$C_{11}H_{20}O_2$	184.28	0	1	0	0	0	0
151.	.delta.-Dodecalactone	7.899	0.45	$C_{10}H_{18}O_2$	170.25	0	1	0	0	0	0
	NAPHTALENE										
152.	2-[4-Acetamidophenylsulfonyl]-1,4-naphthoquinone	19.73	1.88	$C_{18}H_{13}NO_5S$	355.4	0	0	0	1	0	0
153.	Anthracene, 9-ethyl-9,10-dihydro-9,10-dimethyl-	33.46	0.84	$C_{18}H_{20}$	236.4	0	0	0	1	0	0
154.	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-m ethylethyl)-, (1.alpha.,4a.beta.,8a.alpha.)-	13.92	1.52	$C_{15}H_{24}$	204.35	0	0	0	0	1	0
155.	Anthracene, 9,10-dihydro-9,9,10-trimethyl-	32.73	0.29	$C_{17}H_{18}$	222.32	0	0	1	0	0	0
	ORGANO-COMPOUNDS										
156.	1,1,1,3,5,5,5-Heptamethyltrisiloxane	32.39	3.89	$C_7H_{22}O_2Si_3$	222.51	0	0	0	0	0	1
157.	1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy)tetrasiloxane	34.523	36.3	$C_{13}H_{39}O_5Si_6$	443.96	1	0	0	0	0	0
158.	3,3-Diisopropoxy-1,1,1,5,5,5-hexamethyltrisiloxane	32.73	0.29	$C_{12}H_{32}O_4Si_3$	324.63	0	0	1	1	0	1
159.	Cyclodecasiloxane, eicosamethyl-	34.523	36.3	$C_{20}H_{60}O_{10}Si_{10}$	741.54	1	0	0	0	0	0
160.	Cyclononasiloxane, octadecamethyl-	32.82	9.99	$C_{18}H_{54}O_9Si_9$	667.39	1	0	0	0	1	1
161.	Heptasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethyl-	33.86	0.91	$C_{14}H_{42}O_6Si_7$	503.07	0	0	1	1	1	1
162.	Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl-	32.58	0.1	$C_{12}H_{38}O_5Si_6$	430.94	0	0	1	1	0	0
163.	Hexasiloxane, tetradecamethyl-	31.909	30.76	$C_{14}H_{42}O_5Si_6$	458.99	1	0	0	0	0	1
164.	Methyltris(trimethylsiloxy)silane	32.11	3.32	$C_{10}H_{30}O_3Si_4$	310.69	0	0	1	0	0	1
165.	Octasiloxane, 1,1,3,3,5,5,7,7,9,9, 11,11,13,13,15,15-hexadecamethyl-	32.54	0.72	$C_{16}H_{48}O_7Si_8$	577.2	0	0	1	1	1	1
166.	Silane, [[5,5-dimethyl-4-methylene-2-(trimethylsilyl)-1-cyclopenten-1-yl]methoxy]trimethyl-	33.76	1.83	$C_{15}H_{30}OSi_2$	282.57	0	0	0	1	0	0
167.	tert-Butyldimethylfluorosilane	3.362	19.97	$C_6H_{15}FSi$	134.27	0	0	1	0	0	0
168.	Tetrasiloxane, decamethyl-	33.03	1.49	$C_{10}H_{30}O_3Si_4$	310.69	0	0	0	1	0	0
169.	Trimethylsilyl fluoride	3.67	0.28	C_3H_9FSi	92.187	0	1	0	0	0	0
170.	Trimethylsilyl-di(timethylsiloxy)-silane	32.54	0.72	$C_9H_{27}O_2Si_4$	279.65	0	0	0	1	0	0
171.	Tris(tert-butyldimethylsilyloxy)arsane	33.27	0.68	$C_{18}H_{45}AsO_3Si_3$	468.7	0	0	1	0	0	0
172	1-methyl-4-phenyl-5-thioxo-1,2,4-triazolidin-3-one	34.27	2.7	$C_9H_9N_3OS$	207.25	0	0	0	0	0	1
173.	4-Methylphenol, n-propyl ether	9.198	2.65	$C_4H_{10}OC_{10}H_{14}O$	150.22	0	1	0	0	0	0
174.	4-n-Hexylthiane, S,S-dioxide	23.36	1.64	$C_{11}H_{22}O_2S$	218.36	0	0	0	1	0	0
175.	Benzenecarbothioic acid	8.22	0.41	C_7H_6OS	138.19	0	0	0	1	0	0
176.	Benzoyl bromide	8.24	3.89	C_7H_5BrO	185.02	0	0	0	0	1	0

R.T- Retention time, P.A- Peak Area %, M.F- Molecular formula, M.W- Molecular weight, A- *Isolona congolana*, B- *I. dewevrei*, C- *I. hexaloba*, D- *I. thonneri*, E- *I. zenkeri*, F- *I.campanulata*

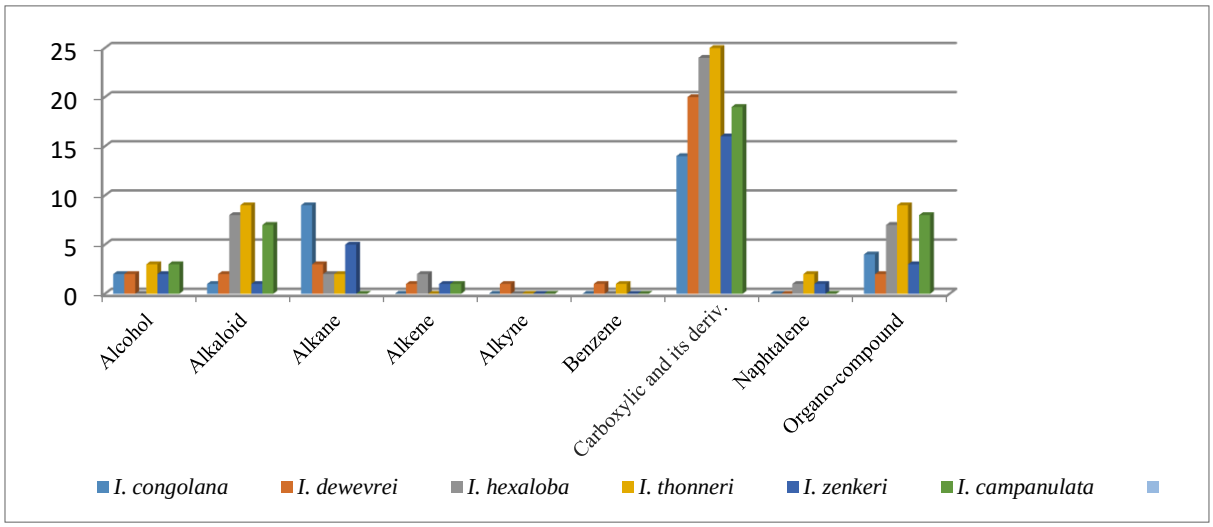

Figure 1: Showing the distribution of phyto-chemical compounds present in the Six (6) *Isolona* species

decided name hexadecanoic acid, methyl ester formula $C_{17}H_{34}O_2$. The prominent group of compounds in these species were scored in carboxylic and its derivatives with about twenty (20) while the least compounds were scored in alkene, alkyne and benzen with the compounds named: 5,7-dimethylenebicyclo [2.2.2]oct-2 –ene; 3-octen-5-yne, (Z)- and benzene, 3-butenyl- respectively (Table 1). In this species, the amount of the analytes isolated ranges from 2.28% with etiron; perfluoropropionic acid, TBDMS derivates and trimethylsilyl fluoride to 45.54% with acetic acid, phenylmethyl ester and decided name: 1-butanol, 2-methyl-; cyclobutane, ethyl- and benzylcarbamate (Table 1, Fig. 2).

3.3. *Isolona hexaloba*

In *I. hexaloba*, the retention time was between 3.36 and 33.78 min separated forty-four (44) compounds grouped into alkaloid, alkanes, alkenes, naphtalene, organo-compounds, carboxylic acid and its derivatives with M.wt ranging from 84.9 in methylene chloride formula CH_2Cl_2 to 709.48 in Tricyclo[4.2.1.0 (2,5)]non-7-ene,3, 4-di(tris(trimethylsilyloxy)silyl)- formula $C_{27}H_{64}O_6Si_8$. The following compounds: N-Methyl-1-adamantane-acetamide; Heptasiloxane,1,1, 3,3,5,5,7,7,9,9,11,11,13, 13-tetradecamethyl- and Octasiloxane,1,1,3,3,5,5,7,7,9, 9,11,11,13,13,15,15- hexadecamethyl- were replicated in *I. thonneri, I. zenkeri* and *I. Campanulata.*

A compound with a decided named Methyl stearate formula $C_{19}H_{38}O_2$ and M.wt of 298.50 was replicated in *I. zenkeri and I. campanulata* while Bis(2-ethylhexyl) phthalate formula $C_{24}H_{38}O_4$ with M.wt 390.56 was scored in *I. thonneri* and *I . zenkeri.* The most prominent were Carboxylic and its derivatives with about twenty-four (24) compounds (Table 1, Fig. 1).

3.4. *Isolona thonneri*

There are fifty-one (51) compounds identified in *I. thonneri* ranges from alcohol, alkaloid, alkanes, benzene, naphtalene, organo-compounds, carboxylic acids and its derivatives. The retention time for the dissociation of the compounds was between 3.44 min with 1-Butanol, 2-methyl-; cyclobutane, ethyl-; and decane, 2,2,3-trimethyl- to 33.86 min with hexestrol, 2TMS derivative; acridine-9-carbaldehyde and heptasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethyl-. Most prominent compounds were the carboxylic acid and its derivatives with twenty-five and least compound was scored in benzene with decided name 1,3-benzodioxole, 4-methoxy-6-(2-nitro-1-propenyl)- formula $C_{11}H_{12}O_3$ and W.mt 192.21. The following compounds namely: 2-ethylacridine ($C_{15}H_{13}N$); benzo[h]quinoline, 2,4-dimethyl- ($C_{15}H_{13}N$); 2-(Acetoxymethyl)-3-(methoxycarbonyl) biphenylene ($C_{17}H_{14}O_4$); 3-quinoline carboxylic acid, 6,8-difluoro-4-hydroxy-, ethyl ester ($C_{12}H_9F_2NO_3$); 1,2,4-Triazine-3,5(2H,4H)-dione, 6-benzoylthio- ($C_7H_{11}N_3O_2$) and 3,3-Diisopropoxy-1,1,1,5,5,5-hexamethyl trisiloxane ($C_{12}H_{32}O_4Si_3$) were scored and duplicated in *I. hexaloba* and *I. campanulata* (Table 1, Fig. 1).

3.5. *Isolona zenkeri*

At the retention time 34.14min, thirty different (30) compounds were separated with major compounds in carboxylic and its derivative. The M.wt for these compounds ranged from 78.13 to 667.39 having sixteen (16) compounds of carboxylic and its derivatives, five (5) alkanes, two (2) alcohols and a compound each of alkaloid and alkene. A compound with M.wt136.15, formula $C_8H_8O_2$ and decided name benzoic acid, methyl ester was scored in *I. campanulata* and *I. thonneri*. Also in this species, a compound with M.wt 667.39, formula $C_{18}H_{54}O_9Si_9$ and decided name cyclononasiloxane, octadecamethyl- was scored only in *I. congolana* and *I. campanulata* (Table 1, Fig. 1).

3.6. *Isolona campanualata*

At a retention time of 34.56min thirty-eight (38) different compounds were separated and about 27 duplicate compound with decided name octasiloxane,1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-, formula $C_{16}H_{48}O_7Si_8$ and W.mt 577.2 at different quantity and retention time. The molecular weights for these compounds ranged from 104.15to 709.48 including fourteen (14) unique compounds (Table 1). Compound with M.wt667.39, formula $C_{18}H_{54}O_9Si_9$ and decided name cyclononasiloxane, octadecamethyl-was scored in *I. congolana* and *I. zenkeri.* A compound with decided name Methyl stearate, formula $C_{19}H_{38}O_2$ and M.wt

298.50 was scored with *I. hexaloba* and *I. zenkeri*. Two compounds name pentadecanoic acid, 14-methyl-, methyl ester and 2-naphthalenemethanol, decahydro- alpha., alpha., 4a-trimethyl-8-methylene-, [2R-(2.alpha., 4a.alpha., 8a.beta.)]- were both scored in *I. zenkeri* (Table 1, Fig. 2).

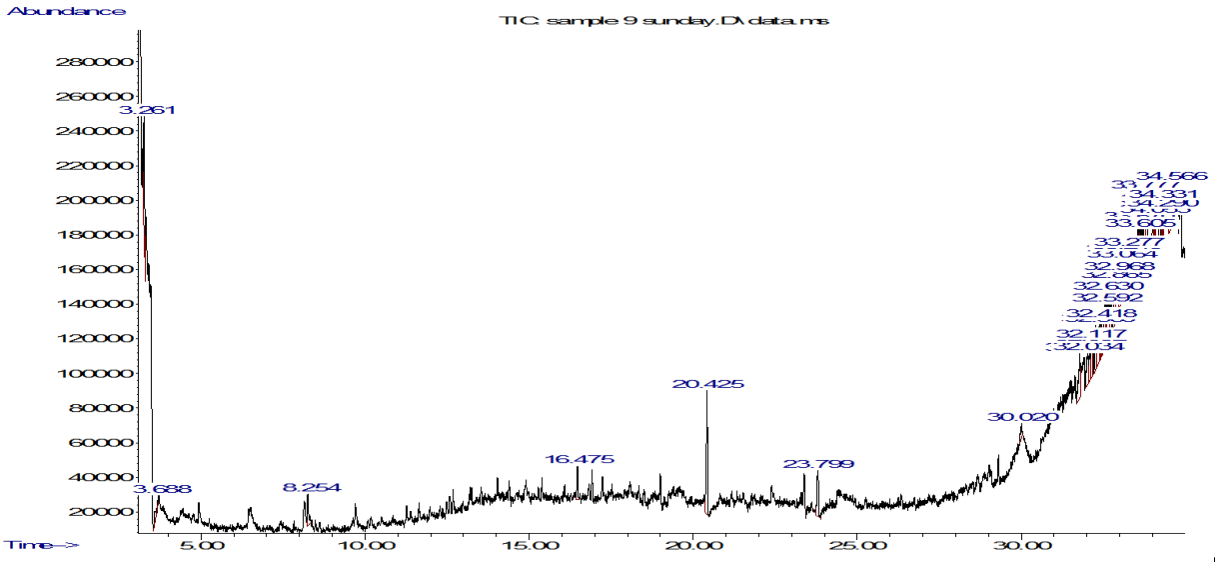

Figure 2. GC-MS Chromatogram of *I. campanualata*

3.7. Systematic implications of GC-MS analysis on *Isolona* species relationship

The distance values among the six (6) examined species showed evidence of similarity and differences using both the quantitative and qualitative phytochemical data. In general, the species varies from higher distance values to higher similarity (lower distance) values characterized by the species of the same genus indicated by the matrices (Figs. 4,5).

In a UPGMA distance tree based on the chemical constituents revealed by GC-MS between the six (6) *Isolona* species was presented. The distance matrix computed with Euclidean similarity index based on the quantitative characters of the constituents in each species (Fig. 4). The tree matrix produces clusters species and identified them to the specific. *I. thonneri* was well separated from all other species while the others were delineated within the sub-clusters. In figure 3, it was also clear in the scatter plot that *I. thonneri* has the highest distance from the centre. To corroborate the tree, the highest distance value level (9.576) was scored between *I. thonneri* and *I. congolana*. This was represented in the clustering tree. On the other hands, the highest similarity level was 2.911 scored in *I. hexaloba* and *I. campanulata.* The sub-cluster of *I. zenkeri*, *I. hexaloba* and *I. campanulata* was clearly grouped together in the scatter plot and closer to the centre. The distance between *I. zenkeri* and *I. hexaloba* was 4.168, whereas the similarity level scored in *I. campanulata* and *I. zenkeri* was 3.615 (Fig. 3).

The relationship between the examined species based on the analysis qualitative characters of chemical constituents as indicated by the RMSD coefficient tree constructed was illustrated in Figure 4. As observed in the quantitative analysis (Fig. 4). *I. thonneri* was also efficiently separated from the other *Isolona* species (Figure 5). A sub-cluster of the tree produced three species namely *I. hexaloba*, *I. campanulata* and *I. zenkeri* from the remaining taxa. Likewise in a cluster of *I. dewevrei* and I. *congolana* with genetic distance 5.367. The distance value between *I. zenkeri* and *I. thonneri* was 8.418 (Table 2).

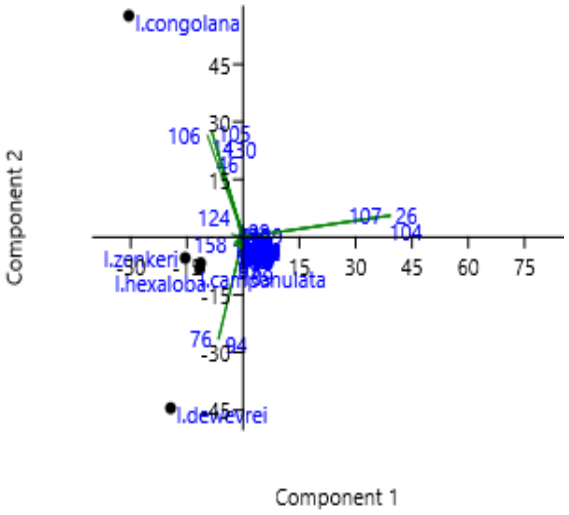

Figure 3. Scatter diagram based on the phyto-constituents of the six *Isolona* species

4. Discussion

Tetenyi (1987); Young et al. (1996); Akilan et al. (2014) and Gamal et al. (2017); have reported the systematics significance of phyto-constituents as an aid toward establishing the relationships among taxa below and above the species level. The GC-MS analysis separated all of the components in the examined samples and produced a specific spectral peak outputs. The total retention time to isolate the first compound and the last compound varies among the studied species. The differences in time taken for each chemical constituent to be dissociated are specific to the compounds and of great help in species identification.

Table 2. Matrix computed based on the analysis of variation in chemical constituents by GC-MS analysis for the *Isolona* species

	I. congolana	***I. dewevrei***	***I. hexaloba***	***I. thonneri***	***I. zenkeri***	***I. campanulata***
I. congolana	0	7.684	6.273	9.576	6.509	5.992
I. dewevrei		0	5.601	9.131	6.208	5.367
I. hexaloba			0	7.990	4.168	2.911
I.thonneri				0	8.418	7.815
I. zenkeri					0	3.615
I. campanulata						0

Cardoso et al. (2008) opined on the important of secondary metabolite in taxonomic questions. The size of the analytes determines the amount of the specific constituent isolated which can be a diagnostic character. The clear differences in chemical formula, Molecular weight, time taken to dissociate all the compounds, amount of compound produced at different time, number and shapes of the compounds in each species were all considered to delineate the six (6) species of *Isolona*. Faustino et al. (2017) profiled four species of *Calendula* L. by categorizing the phyto-chemicals into major compounds. In this study, the hundred and seventy-six (176) compounds were grouped into alcohol, alkaloid, alkenes, alkynes, benzenes, carboxylic and its derivatives, naphthalene and organo-compounds for additional identification. The absence or presence of these phyto-constituents among the studied *Isolona* species were profiled and used in grading and scoring each species, this established the interrelationship between them. The abundance of these compounds in number and amount of analytes were important in chemotaxonomy delimitation of *Isolona*. In all the species, carboxylic and its derivative were the most profound among the hundred and seventy-six compounds. It is noteworthy to mention that the present of Hexadecanoic acid, methyl ester was diagnostic for the genus *Isolona* having present in all the studied species.

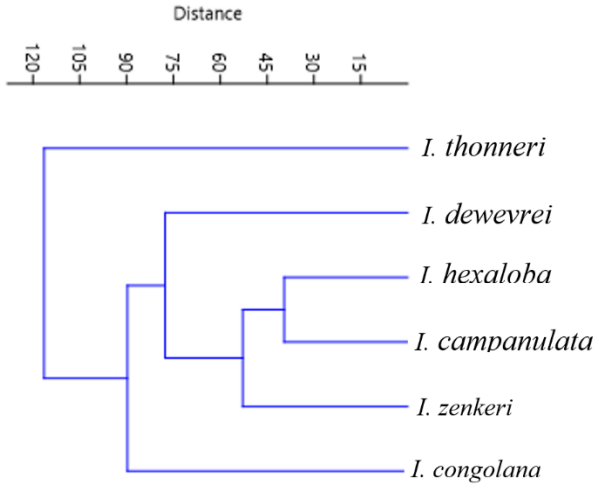

Figure 4. GC-MS analysis of UPGMA distant tree based on Euclidean similarity index showing the relationships among chemical constituents of *Isolona* species

Cardoso et al. (2008) and Mohy-UD-Din et al. (2010) investigated the phytoconstituents of the family Rubiaceae to analysis the taxonomic intricacies. The number of compounds produced was not the same in all the species studied. Graphical spectral peaks representation of each species revealed different phyto-compound at various retention times during dissociation. These were specific to the compound produced and scored. Gamal et al. (2017) categorized the compounds into six different retention times which was the basis for his work. In this 176 different compounds belonging to six (6) *Isolona* species were scored between 3.15 to 34.56 min. All the species except *I. zenkeri* has average of about 3.35 min to produce the first compound. The number of phytoconstituents in different species contributed to the determination of the rank of each species. Likewise, the profound majority of the compounds to be an extraction of carboxylic and its derivatives of the compounds suggest the important of such compounds in the chemotaxonomy of the genus.

In the cluster analysis of plant chemical using the RMSD and Euclidean index showed *I. thonneri* was efficiently separated from the remaining species. The response of scatter plot is in support of the trees where it clustered the trio of *I. zenkeri, I. hexaloba* and *I. campanulata.* This suggests the closeness of the three species because they shared more phyto-constituents than the rest of the species. The highest and lowest distance values by the matrix affirmed the relatedness among the six (6) species studied in this research.

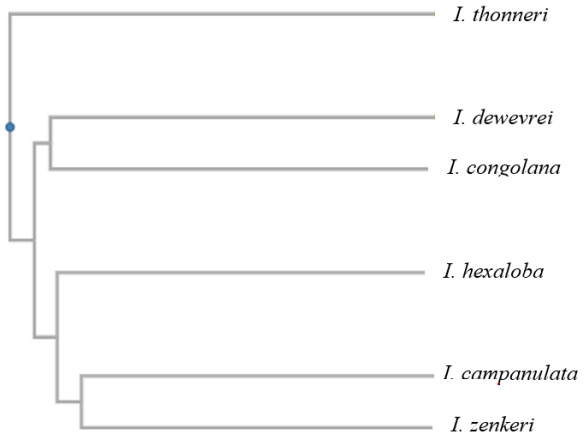

Figure 5. UPGMA distant tree constructed with RMSD coefficient showing the relationships among the *Isolona* species based on the qualitative analysis of chemical constituents

In conclusion, GC-MS analysis of methanol extract of six (6) *Isolona* species revealed one hundred and seventy-six (176) phytochemical constituents detected between 3.26 min to 34.56 min. Twenty-nine (29), thirty-two (32), forty-four (44), fifty-one (51), thirty (30) and thirty-eight (38) phyto-constituents were dissociated from *I. congolana, I. dewevrei, I. hexaloba, I. thonneri, I. zenkeri* and *I. campanulata*. The highest M.wt. was 741.5 scored in *I. congolana* at retention time of 34.52 min. and the lowest M.wt. was 84.15 in *I. thonneri* at retention 3.44

min. Hexadecanoic acid, methyl ester formula $C_{17}H_{34}O_2$ and M.wt of 270.4 was scored in all the six species studied. Similarity and distance matrices were calculated and two UPGMA distant trees constructed with RMSD and Euclidean similarity index to illustrate the relationships among the species based on the differences of chemical constituents. The overlapping phyto-constituents characters revealed the closeness of the taxa studied, this invariably will update the existing data in the genus and ultimately in the family.

Conflict of Interest

Authors have declared no conflict of interest.

Authors' Contribution

Kadiri AB and Olowokudejo JD contributed to manuscript development and literary work as supervisors of Adeniran SA who did data acquisition, writing and laboratory work.

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Phytochemical composition of wild lemon balm (*Melissa officinalis* L.) from the flora of Bulgaria

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Bulgaristan florasından toplanan yabani melisa (*Melissa officinalis* L.)'nın fitokimyasal bileşimi

Abstract: This study aimed to identify the chemical composition (ash, cellulose, total chlorophylls, carotenoid, and essential oil (EO) content), antioxidant activity, and mineral composition of the lemon balm (*Melissa officinalis* L) obtained from Bulgaria. The chemical composition of the EO was analyzed using gas chromatography-mass spectrometry (GC-MS). The major constituents of the EO were caryophyllene oxide (33.99%), n-hexadecanoic acid (14.28%), α-citral (6.62%), (5E,9E)-farnesyl acetone (5.00%), β-caryophyllene (4.82%), (2E,6Z)-farnesal (4.56%), (5E,9Z)-farnesyl acetone (4.16%), β-citral (3.98%), and γ-eudesmol (3.13%). The antioxidant potential was assessed using DPPH (1,1-diphenyl-2-picrilhydrazyl) free radical, ferric reducing antioxidant power assay (FRAP), TEAC assay, determination of hydroxyl radical scavenging capacity and determination of superoxide scavenging capacity methods. The total phenol content was analyzed using the Folin-Ciocalteu method. The total flavonoid amount of the extracts was determined by optimizing the aluminum chloride colorimetric method. The total phenolic content, the total flavonoid composition, and the antioxidant potential of the lemon balm leaves included 184.33 mg GAE g^{-1}, 12.65 mg QE g^{-1}, and 62.83 µg ml^{-1}, respectively. Mineral and heavy metal contents were determined by the iCAP-Qc ICP-MS spectrometer. The highest amounts of macro and microminerals were determined for K, Ca, Mg, Sr, Rb, Ba, Mn, and Fe, respectively.

Key words: *Melissa officinalis* L., chemical composition, antioxidant activity, GS-MS, ICP-MS

Özet: Bu çalışma ile Bulgaristan florasından toplanan melisanın (*Melissa officinalis* L) kimyasal bileşenleri (kül, selüloz, toplam klorofil, karotenoid ve uçucu yağ (EO) içeriği), antioksidan aktivitesi ve mineral madde bileşenlerinin belirlenmesi amaçlanmıştır. Uçucu yağ analizinde gaz kromatografisi-kütle spektrometrisi (GC-MS) cihazı kullanılmıştır. Uçucu yağın ana bileşenleri, karyofilen oksit (%33.99), n-heksadekanoik asit (%14.28), α-sitral (%6.62), (5E,9E)-farnesil aseton (%5.00), β-karyofilen (%4.82), (2E,6Z)-farnesal (%4.56), (5E,9Z)-farnesil aseton (%4,16), β-sitral (%3,98) ve γ-eudesmol (%3.13) olarak tespit edilmiştir. Antioksidan kapasitesini belirlemek için DPPH (1,1-difenil-2-pikrilhidrazil) serbest radikali, ferrik indirgeyici antioksidan güç testi (FRAP), TEAC testi, hidroksil radikali süpürme kapasitesi ve süperoksit süpürme kapasitesi yöntemleri kullanılmıştır. Toplam fenol içeriği, Folin-Ciocalteu yöntemi kullanılarak analiz edilmiştir. Ekstraktların toplam flavonoid miktarı, alüminyum klorür kolorimetrik yöntemi optimize edilerek belirlenmiştir. Melisa yapraklarının toplam fenolik içeriği, toplam flavonoid içeriği ve antioksidan kapasitesi sırasıyla 184.33 mg GAE g^{-1}, 12.65 mg QE g^{-1} ve 62.83 µg ml^{-1} olarak tespit edilmiştir. Mineral madde ve ağır metal içerikleri, iCAP-Qc ICP-MS spektrometresi ile belirlenmiştir. En yüksek makro ve mikromineral miktarı sırasıyla K, Ca, Mg, Sr, Rb, Ba, Mn ve Fe olarak belirlenmiştir.

Anahtar Kelimeler: *Melissa officinalis* L., kimyasal kompozisyon, antioksidant aktivite, GS-MS, ICP-MS

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1. Introduction

Lemon balm *(Melissa officinalis* L.) is a perennial herbaceous plant of the Lamiaceae family, reaching a height of 1.25 m and is distributed in Central and Southern Europe, Russia, Ukraine, Caucasus, Asia, and the United States. Wild grows up to 1200 m above sea level. It is cultivated in Central and Southern Europe, Asia, and the United States mainly as a medicinal plant and is used as an essential oil due to its wich chemical composition and health benefits. It is wild in many regions of Bulgaria, but it is also cultivated (Georgiev and Stoyanova, 2006).

More than 130 ingredients have been identified in its EO composition, the amount of which varies depending on the habitat, stage of development, and plant variety. The main components determining the odor are geranial (33.60-48.82% and neral (22.18-33.39%), citronellal (0.2-

11.30%), caryophyllene oxide (1.3-8.35%), *etc*. (Holla et al., 1997; Pino et al., 1999; Sodre et al., 2012; Abdellatif et al., 2014). The composition of the EO revealed the wide range of lemon balm applications in food, medicine, pharmacy, herbal medicine, and other industries. The plant is generally used for treatment of inflammatory, antispasmodic, antitumor formations, and behavioral disorders (Georgiev and Stoyanova, 2006; Zarei et al., 2015). Studies have shown improvement in states of nervous tension, stress, and anxiety, and improved memory performance in people who have been treated with lemon balm extracts.

The following main ingredients are reported in the plant EO cultivated in Bulgaria: citral (16.9-40.4%), citronellal (4.5-25.1%), linalool (0.2-2.9%), geranyl acetate (1.9%), *β*-caryophyllene (11, 1-17.5%), etc. (Georgiev and Stoyanova, 2006). The EO has antimicrobial and antiviral action (Abdellatif et al., 2014), which correlates with the biologically active components contained in the composition of the plant species. The flavonoids contained in the composition (quercetin, luteolin, rhamnocitrin, and others) increased the application of the species in medicine. High levels of polyphenols improved the memory (Dehbani et al., 2019) and mood (Kennedy et al., 2002) of the people, and were used in the therapy of some digestive and gastrointestinal disorders.

In the food industry, lemon balm is more commonly used as a natural antioxidant in the composition of juices, infusions, aromatic compositions, confectionery, and the composition of edible antimicrobial packaging materials (Meftahizade et al., 2010). Lemon balm is often used as a component in the production of ice cream and herbal teas. It could be combined very well with other herbs, such as mint in cold infusions (lemonades). It was included in the composition of fruit dishes and candies, fish products, and pesto. The aim of the present study was to identify the phytochemical profile of *Melissa officinalis* L., its mineral composition, and antioxidant capacity.

2. Materials and Method

2.1. Plant material

The plants were collected in October 2016 from eastern Balkan mountains (South Bulgaria, at 553 m elev., 42.85 ° N 26.15 ° E), village of Bozhevtsi. The samples were collected by hand and dried in ventilated rooms in the absence of direct sunlight. The plant species was identified by the Department of Botany and Methods of Biology Teaching, Faculty of Biology, Paisii Hilendarski University of Plovdiv in Plovdiv, Bulgaria, according to the morphological features of the plant. The room air temperature was regulated (18±2 °C). After drying, the samples were placed in plastic bags for storage.

2.2. Preparation of Extracts

The plant leaf sample (4 g) was mixed by methanol (40 mL) (1/10 w/v). The prepared samples were incubated for 24 hours at 40 °C in an oven (Electo-mag M 5040 P). Then, it was filtered into balloon flasks using Whatman No 1 filter paper. The methanol in the samples was removed with the help of a rotary evaporator (Heating Bath B-491, BUCHI). The balloon bottles, which were blown up, were kept in the oven for 24 hours and completely dried. The extracts obtained (2 mL of methanol was added to the flasks and the extract was obtained by vortexing) were taken into falcon tubes and closed with parafilm and stored at +4 °C to be used in the analysis.

2.3. Chemical composition

The moisture of the leaves was determined by drying up to the constant weight at 105 °C (Anonymus, 1990) and the results from the chemical analyses were given on a dry weight (dw) basis.

The ash content was determined according to Horwitz and Latimer (2005), by mineralization of the samples at 550 °C for 5 h.

2.4. Isolation of essential oil and Gas chromatographic analyses

The leaves (50 g) were cut to a size of 0.5 cm. The EO was isolated by hydrodistillation (ratio leaves:water=1:10) for 3 h in a Clevenger-type laboratory glass apparatus of the British Pharmacopoeia, modified by Balinova and Diakov (1974). The oil obtained was dried over anhydrous sodium sulfate and stored in tightly closed dark vials at 4 °C until analysis.

A GC analysis was performed using an Agilent 7890A gas chromatograph, HP-5 column MS (30 m x 250 μm x 0.25 μm), temperature: 35 °C/3 min, 5 °C/min to 250 °C for 3 min, 49 min in total, helium as a carrier gas, 1 ml min^{-1} constant speed, 30:1 split ratio. A gas chromatography–mass spectrometric (GC/MS) analysis was carried out on an Agilent 5975C mass spectrometer, helium as a carrier gas, column and the temperature was the same as in the GC analysis. The identification of the chemical compounds was made compared to their relative retention time and library data (Adams, 2007; NIST 08 database). Components were listed according to their retention (Kovat's) indices, calculated using a standard calibration mixture of C_8-C_{40} n-alkanes in n-hexane. Compound concentration was computed as a percentage of the total ion current (TIC).

2.5. Protein content

The total protein content was analyzed according to Latimer (2016) method with a UDK 152 Kjeldahl System (Velp Scientiffica, Italy). The samples 1.0 g each, were mineralized in 15 ml concentrated H_2SO_4 and catalysts: anhydrous K_2SO_4 and $CuSO_4$. The process was run at 420 °C for 60 min. With this method, 40% NaOH was used to produce an alkaline distillation medium and 4% H_3BO_3 in order to collect the distilled ammonia. The titrations were carried out with a standard HCl (0.2 N) solution.

2.6. Cellulose content

The content of cellulose (crude fiber) in leaves was determined by a modification of the method by Brendel et al. (2000). Hydrolysis of cellulose and hemicellulose was carried out by boiling 1 g of leaves with 16.5 ml of 80% CH_3COOH and 1.5 mL concentrated HNO_3 for 1.5 h. After filtration of the suspension, the solid residue was dried at 105 °C for 24 h and weighed.

2.7. Total chlorophylls and carotenoid content

In order to evaluate chlorophyll a, chlorophyll b and the total carotenoid content, 0.5 g of fresh leaf sample was homogenized with 10 mL extract (80% alkaline acetone)

and stored in the dark at 25 °C for 24 h. After that, the homogenate was centrifuged at 1500 g for 10 min. Absorbance was measured at 470 nm, 645 nm and 663 nm; then, the results were calculated by the corresponding formulas (Corte-Real et al., 2017):

Chlorophyll a ($\mu g\ g^{-1}$) = ($9.784*A_{663}$-$0.990*A_{645}$)*1000 (1)

Chlorophyll b ($\mu g\ g^{-1}$) =($21.426*A_{645}$-$4.650*A_{663}$)*1000 (2)

Total carotenoids content ($\mu g\ g^{-1}$) = ($4.695*A_{470}$-0.268*(chl a + chl b))*1000 (3)

2.8. Total Phenolic Contents

Folin-Ciocalteu Reagent (FCR) method was used to determine the total phenolic content of the extracts (Singleton et al., 1999). For the study, 100 mL of sodium carbonate solution was prepared. 20g of Na_2CO_3 was first weighed and 80 mL of hot distilled water was added to it in order to prepare the saturated sodium carbonate solution. The lid of this solution was covered by boiling and dissolved thoroughly. After dissolution, the temperature of the solution was cooled down to room temperature (25 °C±2). Approximately 7 g of Na_2CO_3 was added on top and the solution was saturated. The resulting solution was left in the dark for 24 h. Samples were prepared for later analysis. First, 2.4 mL of pure water was placed in glass tubes and 40 µL of extract was added. 40µL methanol was added to the prepared control groups instead of extracts. Then, 200 µL of Folin and 600 µL of saturated Na_2CO_3 were added to the samples. In the next step, 760 µL of distilled water was added and vortexed for complete mixing of the added chemicals. The prepared samples were incubated at room temperature (25 ±2 °C) for 2 h and absorbance measurement was performed at 765 nm. Gallic acid (GA) was used for standard phenolic substance control. The values obtained are expressed as GA conjugate. Spectrophotometric measurements were made in order to determine the total phenolic content Perkin Elmer Lambda 25 UV/VIS.

2.9. Determinations of total flavonoid assay

The total flavonoid compound amounts of the extracts were determined by optimizing the $AlCl_3$ colorimetric method of Biju et al. (2014). One mg mL^{-1} extract was prepared. Plant extract 50 µL was mixed with 950 µL of methanol. Then, 4 mL of distilled water was added and mixed. After that, 0.3 mL $NaNO_2$ (5%) was added and incubated for 5 min and 0.3 mL of $AlCl_3$ (10%) was added and incubated for 6 min. After incubation, 2 mL of 1 mol L^{-1} NaOH was added. To the resulting solution, 2.4 mL of distilled water was added and completed to 10 mL. The solution was incubated for 15 min and then absorbance was measured at 510 nm. As result of quercetin equivalents (QE) g^{-1} of extract was calculated.

2.10. Determination of heavy metal

Plant samples were dried and 0.5 g was weighed. Then, each sample put into a porcelain crucible. All samples were burned until gray ash (550 °C). After burning the 0.5 g weighed samples, the ashes were dissolved in 4 ml 0.1 N HCl and filtered (Whatman No. 1), and completed with distilled water (10 mL) (Kaçar and İnal, 2010). Mineral and heavy metal contents were determined in Yozgat Bozok University, Science and Technology Application and Research Center using iCAP-Qc ICP-MS spectrometer (Thermo Scientific).

2.11. Antioxidant activity

2.11.1. DPPH Free Radical-Scavenging Activity

The free radical activities of the extracts were determined using DPPH (1,1-diphenyl-2- picrilhydrazyl) free radical, a known and commonly used radical (Gezer et al., 2006). Firstly, the amount of extract that defines a certain amount of DPPH radical has been determined, and a comparison has been made between these samples. Sixteen mg DPPH radical solution was prepared in 100 mL methanol. The DPPH solution was prepared as 0.1 µM. By setting 517 nm in the spectrophotometer, DPPH reading was done and dilution was made with methanol until the absorbance value was 1.000. 1 mg mL^{-1} extract solution was prepared as main stock and 6 different concentrations were obtained by dilution. Three mL samples were taken from each concentration (50, 75, 100, 150, 200, 300) and 1 mL 0.1 µM DPPH was added on top. The reaction mixture was incubated for 30 min in the dark. BHT (butyl hydroxytoluene) and BHA (butyl hydroxyanisol) were used as reference. Radical scavenging activity DPPH was determined as the inhibition percentage and the following formula is used:

Radical scavenging activity DPPH % = [A blank − A sample)/A blank] × 100

Spectrophotometric measurements for DPPH radical scavenging activity determination were performed with the aid of PerkinElmer Lambda 25 UV/VIS spectrophotometer device.

2.11.2. Ferric reducing antioxidant power assay (FRAP)

Ferric reducing antioxidant power assay (FRAP) was determined according to the method of Benzie and Strain (1999). Leaf sample (4 g) was extreated with distilled water (40 mL) (temperature from 80 to 105 °C) during 20 min (Fraction I). The crop residues were extreated with distilled water (60 mL) (temperature from 100 to 130 °C) during 30 min (Fraction II). Both fractions were filtered when cooled to 25 °C. This analysis evaluates the change in absorbance at 620 nm for the production of FeII-tripyridyltriazine from oxidised FeIII. The reagent was prepared via mixing 300 mmol/L acetate buffer with 10 mmol L^{-1} 2,4,6-tripyridyl-s-triazine with 40 mmol/L HCl and 20 mmol L^{-1} ferric chloride at low pH. Trolox was used as standard. Samples were quantified by a spectrophotometer (PerkinElmer Lambda 25 UV / VIS).

2.11.3. TEAC assay

Trolox Equivalent Antioxidant Capacity (TEAC) assay is consisted in the reducing of the absorbance of the $ABTS^{+\bullet}$ (Re et al., 1999) at 734 nm. $ABTS^{\bullet+}$ was prepared by reacting ABTS solution with potassium persulfate (2.45 mM). The $ABTS^{\bullet+}$ solution at 734 nm was diluted with phosphate buffer. After addition to the diluted $ABTS^{\bullet+}$ Trolox standard, the mixture was incubated for 15 minutes. Next, inhibition in absorbance at 734 nm was evaluated. samples were examined with a PerkinElmer Lambda 25 UV/VIS spectrophotometer.

2.11.4. Determination of hydroxyl radical scavenging capacity

This method was analyzed according to the method of Halliwell and Gutteridge (2007). It was assessed by detecting the ability of leaf sample extracts to reduce the generation of 2-hydroxyterephthalate which is a strongly fluorescent in a reaction between terephthalic acid and hydroxyl radical. The mixture (2.5 mL) comprised TPA (500 µM), EDTA (10 µM), $FeSO_4$ (10 µM), ascorbate (100 µM) and H_2O_2 (100 µM) in a Na-phosphate buffer (50 mM, pH 7.2). The procedure was calibrated with ethanol and hydroxyl radical scavenging capacities were given as mM ethanol equivalent ml^{-1} leaf extract.

2.11.5. Determination of superoxide scavenging capacity

This method was specified as the superoxide radical inhibition caused to be decrescent of nitro blue tetrazolium to formazan (McCord and Fridovich, 1999). Formazan formation was assessed at 560 nm. The mixture (1.0 ml) consisted xanthine oxidase (0.015 U) in Na-phosphate buffer (50 mM, pH 7.2) comprising EDTA (0.3 mM), xanthine (0.2 mM) and nitro blue tetrazolium (1 mg mL^{-1}). Results were expressed as SOD unit equivalent mL^{-1} of the leaf extract.

2.12. Statistics

All measurements were carried out in triplicates. The results were expressed as mean ± SD and analyzed using MS-Excel software.

3. Results

The chemical composition of lemon balm leaves is shown in Table 1. In our study, moisture, essential oil yield, protein, cellulose, and ash content were determined in lemon balm leaves as follows 8.99±0.70%, 0.03±0.0%, 13.50±0.12%, 26.56±0.25%, and 9.94±0.08%, respectively.

The chlorophyll a, chlorophyll b, and total carotenoids content of lemon balm were analyzed. The results showed that lemon balm extracts were the richest for chlorophyll b (36.82±0.98 µg g^{-1} dw), chlorophyll a (32±0.03 µg g^{-1} dw), and carotenoid (1.5±0.47 µg g^{-1} dw).

The total phenol contents (mg g^{-1}) in methanol extracts, were determined from regression equation of calibration curve (y=0.001x – 0.006, R^2=0.9988) and expressed in gallic acid equivalents (GAE). Total phenol content was estimated as 184.33±0.50 mg GAE g^{-1} (Table 1). The average total flavonoid content was determined (12.65 mg±0.66 QE g^{-1}) (Table 1).

As a result of the analysis of the lemon balm leaves, 50% inhibition values was calculated. BHT (Butyl hydroxytoluene) and BHA (Butyl hydroxyanisol) were used as standard antioxidants.

The ability to scavenge the free radical DPPH (2,2 diphenyl-1-picrylhydrazyl) was estimated as 62.83±0.80 µg mL^{-1} (Table 2). IC_{50} value, µg/mL BHA (19.662±0.34) and BHT (13.818±0.50). According to the FRAP and TEAC assays, lemon balm showed 250.39±38.80 µmol L^{-1} and 60.25±1.52 µmol L^{-1}, respectively. Results of hydroxyl radical and superoxide scavenging capacities of lemon balm leaf extracts were detected 18.5±4.7 mM ethanol mL^{-1} and 20.0 ± 5.8 unit SOD mL^{-1}, respectively Table 2).

Table 1. The chemical composition of lemon balm leaves

Parameters	Leaves
Moisture, %	8.99 ± 0.70
Yield of essential oil, % (v/w)	0.03 ± 0.0
Protein, %	13.50 ± 0.12
Cellulose, %	26.56 ± 0.25
Ash, %	9.94 ± 0.08
Chlorophyll a, µg g^{-1} dw	32.0 ± 0.03
Chlorophyll b, µg g^{-1} dw	36.82 ± 0.98
Total carotenoids, µg g^{-1} dw	1.50 ± 0.47
Total phenol contents, mg GAE g^{-1}	184.33 ± 0.50
Total flavonoid assay, mg QE g^{-1}	12.65 ± 0.66

The chemical composition of the lemon balm essential oil is shown in Table 3. There were 27 essential oil constituents representing 98.67% of the total oil content that were identified in lemon balm essential oil. Thirteen of the EO constituents were with concentrations above 1%. The main EO constituents (over 3%) were: caryophyllene oxide (33.99%), n-hexadecanoic acid (14.28), α-citral (6.62%), (5E,9E)-farnesyl acetone (5.00%), β-caryophyllene (4.82%), (2E,6Z)-farnesal (4.56%), (5E,9Z)-farnesyl acetone (4.16%), β-citral (3.98%), and γ-eudesmol (3.13%).

Table 2. Antioxidant activity of lemon balm.

Methods	Leaves
FRAP assay, µmol L^{-1}	250.39 ± 38.80
TEAC assay, µmol L^{-1}	60.25 ± 1.52
DPPH assay IC_{50} value, µg mL^{-1}	62.83 ± 0.80
Hydroxyl radical scavenging capacity, mM ethanol mL^{-1}	18.5 ± 4.70
Superoxide scavenging capacity, unit SOD mL^{-1}	20.00 ± 5.80

Oxygenated sesquiterpenes (54.96%) were the dominant group in the EO, followed by oxygenated monoterpenes (18.83%), oxygenated aliphatics (17.02%), sesquiterpene hydrocarbons (6.44%), and monoterpene hydrocarbons (2.75%).

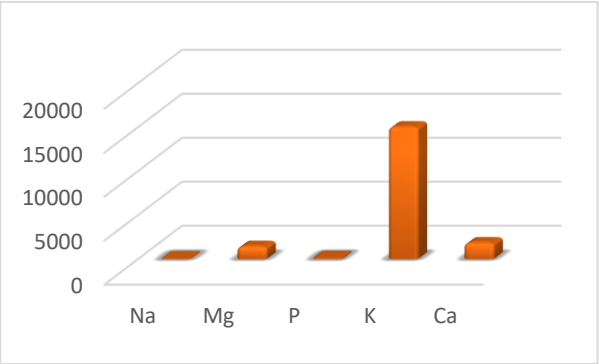

Figure 1. Macro minerals in lemon balm (ppm)

The content of macro and microelements in the raw material is presented in Figure 1 and Figure 2. The data showed that K and Mg contents were the highest among macronutrients. The amount of heavy metals was low, except of Sr, which was probably due to the nature of the soil in the region (Figure 3).

Table 3. Chemical composition of lemon balm essential oil

№	RT	RI [a]	Compound	Content (% of TIC [b])
1	10.05	930	α-Pinene	2.70 ± 0.02
2	17.04	1151	Citronellal	2.75 ±0.02
3	17.30	1158	(2E)-Nonen-1-al	0.63 ± 0.0
4	17.95	1177	(E)-Isocitral	0.86 ± 0.0
5	19.50	1238	β-Citral	3.98 ± 0.03
6	19.80	1262	Citronellic acid, methyl ester	0.86 ± 0.0
7	20.41	1270	α-Citral	6.62 ± 0.05
8	22.71	1322	Methyl geranate	0.90 ± 0.0
9	23.51	1380	Geranyl acetate	2.08 ± 0.02
10	24.58	1430	β-Caryophyllene	4.82 ± 0.04
11	25.49	1454	β-Caryophyllene	0.49 ± 0.0
12	26.00	1480	methyl-γ-Ionone	0.53 ± 0.0
13	26.17	1482	Germacrene D	0.58 ± 0.0
14	27.02	1523	δ-Cadinene	0.47 ± 0.0
15	28.37	1577	Spathulenol	0.80 ± 0.0
16	28.58	1581	Caryophyllene oxide	33.99 ± 0.31
17	29.04	1596	Fokienol	0.36 ± 0.0
18	29.29	1630	γ-Eudesmol	3.13 ± 0.03
19	30.25	1683	(2Z,6Z)-Farnesal	2.05 ± 0.02
20	30.57	1712	(2E,6Z)-Farnesal	4.56 ± 0.04
21	34.17	1861	(Z,Z)-Farnesyl acetone	0.54 ± 0.0
22	34.49	1883	(5E,9Z)-Farnesyl acetone	4.16 ± 0.04
23	36.30	1922	(5E,9E)-Farnesyl acetone	5.00 ± 0.04
24	36.69	1957	n-Hexadecanoic acid	14.28 ± 0.13
25	39.73	2130	Linoleic acid	0.56 ± 0.0
26	39.95	2141	Oleic acid	0.89 ± 0.0
27	40.03	2152	Linolenic acid	0.08 ± 0.0
Total, %				98.67
Oxygenated aliphatics				17.02
Monoterpene hydrocarbons				2.75
Oxygenated monoterpenes				18.83
Sesquiterpene hydrocarbons				6.44
Oxygenated sesquiterpenes				54.96

a RI – retention (Kovat's) index; b TIC – total ion current; c All data are presented as mean value ± standard deviation (n=3)

4. Discussions

4.1. Chemical composition and antioxidant activity

Total phenol content of the studied samples was estimated as 184.33±0.50 mg GAE g^{-1} (Table 1). We may say that the results in this study were in agreement with those reported in the literature. For instance, Moradi et al. (2016) reported average value of 227.6 mg GAE g^{-1} dw of lemon balm methanol extract. In another study, total phenol content was determined as 54.9±2.14 mg GAE g^{-1} (Spiridon et al., 2011). Hassan et al. (2019) obtained average value of 71.02 mg GAE g^{-1} dw for total phenol methanol extracts. On the other side, these results agreed with those of Tusevski et al. (2014), who determined average value of 70.86 mg GAE g^{-1} for total phenol content of lemon balm. Spiridon et al. (2011) found that total phenol content of lemon balm was 25.8±6.26 (mg R g^{-1}).

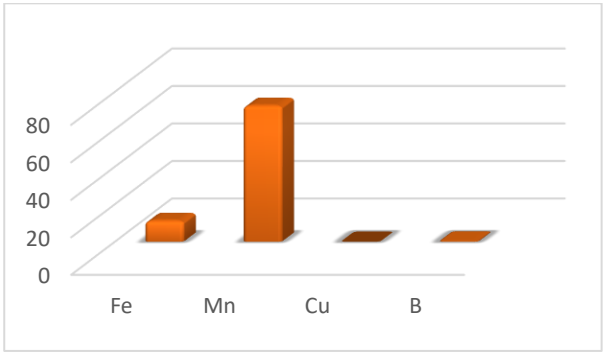

Figure 2. Micro minerals in lemon balm (ppm)

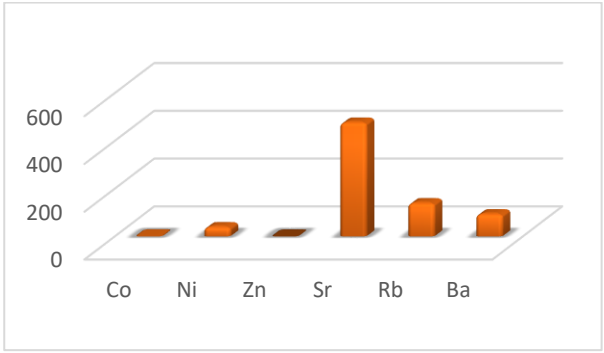

Figure 3. Heavy metals in lemon balm (ppm)

Previous studies of lemon balm alcoholic extracts have indicated that the total flavonoid content were 12.5±2.11 mg g^{-1} (Moradi et al., 2016). However, other studies gave average value of 45.71 mg g^{-1} dw (Tusevski et al., 2014) and 72.38 mg QE g^{-1} dw (Hassan et al., 2019) for TFC extracted from lemon balm leaves using methanol. These values were higher than that obtained in our study. In another study, lemon balm showed high level of – antioxidant activity (Dias et al., 2012). Many secondary metabolites, such as flavonoids, essential oils, and phenolic acids are produced in response to environmental stresses (Weitzel and Petersen, 2010). The results of the antioxidant activity of lemon balm leaves determined via five methods are presented in Table 2. According to the FRAP and TEAC assays, lemon balm showed 250.39±38.80 µmol L^{-1} and 60.25±1.52 µmol L^{-1}, respectively. Comparing of various analytical procedures is useful for better commentary and understanding of the data on the antioxidant ability of samples (Katalinic et al., 2004). Other studies the FRAP and TEAC method were also retrieved in the decoction of lemon balm leaves 1133.24±11.54 µM TE g^{-1} dw and 722.00±5.39 µM TE g^{-1}, respectively (Popova et al., 2016). According to Mihaylova et al. (2015), the highest value in the FRAP method was obtained in the 30 min decoction extract of fresh lemon balm leaves (116.58±1.55 µM TE g^{-1} FW). The lemon balm extract has the ability to scavenge both synthetic and natural free radicals (Dastmalchi et al., 2008). The ability to scavenge the free radical DPPH (2,2-diphenyl-1-picrylhydrazyl) was estimated as 62.83±0.80 µg mL^{-1} (Table 2). According to the other study, lemon balm (IC_{50} = 87.28 µg mL^{-1}) exhibited the most profound antioxidant activity DPPH method (Spiridon et al., 2011). Lemon balm scavenged DPPH radical in a concentration-dependent manner with IC_{50} values of 48.76 ±1.94 µg mL^{-1}

(Kamdem et al., 2013). In another study, Hassan et al. (2019), found IC_{50} value for lemon balm methanol extract was 125.72 µg mL^{-1}, this data are disagreed with our results. The variations between the results obained in this study and previously reported by Hassan et al. (2019) may be caused by the differences in the locality of the plant samples, soil compositions, environmental and climatic factors etc.

4.2. Essential oil content

Caryophyllene oxide was determined as major component (33.99%) in the composition of the studied essential oil. Basta et al. (2005) also found caryophyllene oxide as main component in lemon balm. Also, other major components were identified as n-hexadecanoic acid (14.28%), *α*-citral (6.62%), (5E,9E)-farnesyl acetone (5.00%), *β*-caryophyllene (4.82%), (2E,6Z)-farnesal (4.56%), (5E,9Z)-farnesyl acetone (4.16%), *β*-citral (3.98%), and *γ*-eudesmol (3.13%). According to Radulescu et al. (2021), the GC–MS analysis identified 36 components, the main constituents are beta-cubebene (27.66%), beta-caryophyllene (27.41%), alpha-cadinene (4.72%), caryophyllene oxide (4.09%), and alpha-cadinol (4.07%). Abdellatif et al. (2021) observed larger amounts of neral (31.72%), geranial (45.06%), and citronellal (6.42%) constituents in lemon balm EO. Chung et al. (2010) reported monoterpene hydrocarbons, sesquiterpene hydrocarbons and oxygenated monoterpenes in the lemon balm phytocomposition. Other studies have corroborated data collected in the current study (Sousa et al., 2004; Uyanik and Gurbuz, 2014; Bozovic et al., 2018). Several studies have reported that the essential oil contents and yield can vary with plant genotypes (Rajendra et al., 2016; Gholami-Zali and Ehsanzadeh, 2018). Differences in lemon balm EO content are strongly associated with biotic and abiotic conditions, different harvest years and genetic structure of genotypes (Kittler et al., 2017; Radulescu et al., 2021).

4.3. Determination of heavy metal, and nutrient contents

The results of lemon balm leaves mineral content are presented in Figure 1, 2 and 3. Lemon balm leaves contained macro and microminerals, and the most prodominant element was K, followed by Ca, Mg, Sr, Rb, Ba, Mn and Fe. In comparison with the other studies, the amount of K was higher, followed by Ca, Mg, Fe, Na, and Zn (Abdellatif et al., 2021). Mg is a vital mineral factor for plants, which can, directly and indirectly, affect diseases. Although Mg's more general physiological effects are not entirely known for active growth and resistance to infection, it is well-known that Mg is an significant contributor to plant health. On the other hand, structurally, Mg is a component of the middle lamella of the plants' and a constituent of the chlorophyll molecule (Huber and Jones, 2013).

Lemon balm is a potential medicinal and aromatic plant grown commonly in most wild areas. Its essential oil is currently used in medicine, pharmacology, the food and cosmetic industries. Our results showed that lemon balm leaves EO detected 27 compounds, representing 98.67% of the total oil composition. Our study revealed that due to the rich chemical composition and antioxidant potential of the lemon balm, it may be used as a potential source and raw material in various fields and industries, including pharmaceutical, food, and cosmetics.

Conflict of Interest

Authors have declared no conflict of interest.

Authors' Contribution

The authors contributed equally.

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A new record for the *Physalacriaceae* family in Turkey

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Türkiye'deki *Physalacriaceae* familyası için yeni bir kayıt

Abstract: *Cryptomarasmius minutus* (Peck) T.S. Jenkinson & Desjardin, is given as new record for the mycobiota of Turkey with a short description and figures related to its macro and micromorphology. This species is the second member of the genus *Cryptomarasmius* T.S. Jenkinson & Desjardin in Turkey and characterized by small, red-brown pileus, distant, often reduced lamellae, ellipsoid to narrowly ellipsoid basidiospores, and the presence of pileocystidia, dimorphic cheilocystidia, pleurocystidia, and caulocystidia.

Key words: *Agaricales, Basidiomycota,* biodiversity, new record, taxonomy

Özet: *Cryptomarasmius minutus* (Peck) T.S. Jenkinson & Desjardin, kısa bir betimleme ve makro ve mikromorfolojisine ilişkin şekillerle Türkiye mikobiyotası için yeni kayıt olarak verilmiştir. Bu tür *Cryptomarasmius* T.S. Jenkinson & Desjardin cinsinin Türkiye'deki ikinci üyesidir, ve küçük, kırmızı-kahverengi şapka, aralıklı, çoğunlukla indirgenmiş lameller, elipsoit veya dar elipsoit bazidiyosporlar, ve pilosistidlerin, çifte morfolojide cheilosistidlerin, ve caulosistidlerin varlığı ile karakterizedir.

Anahtar Kelimeler: *Agaricales, Basidiomycota,* biyoçeşitlilik, yeni kayıt, taksonomi

Citation: Kaplan D, Uzun Y, Kaya A (2021). A new record for the *Physalacriaceae* family in Turkey. Anatolian Journal of Botany 5(2): 120-123.

1. Introduction

Cryptomarasmius T.S. Jenkinson & Desjardin is an agaricoid fungal genus in the family *Physalacriaceae.* It was first proposed by Thomas S. Jenkinson & Dennis E. Desjardin (Jenkinson et al., 2014). All the members of the genus were previously placed in *Marasmius* Fr., section *Hygrometrici* characterized by a smaller pileus that is well-pigmented and broom cells mostly of the Rotalis-type or often in a combination with smooth cells, free to adnate attachment of lamellae, absence of collarium, a central and insititious stipe, usually presence of pleuro- and cheilocystidia, neither amyloid or dextrinoid nature of the pileus, lamellae or stipe trama, and medium-sized basidiospores (Singer, 1976; Dutta and Acharya, 2018).

Index Fungorum (2021) lists 15 *Cryptomarasmius* species among which only *C. corbariensis* (Roum.) T.S. Jenkinson & Desjardin is known to exist in Turkey. This species was first reported from by Nizip district of Gaziantep province by Uzun et al. (2017). Within the following year Bozok et al. (2018) also presented this species from Osmaniye province with molecular data.

Here we present *C. minutus* (Peck) T.S. Jenkinson & Desjardin as the second member of the genus *Cryptomarasmius* in Turkey. The current checklist (Sesli et al., 2020) and the latest contributions (Akçay, 2020; Çağli and Öztürk, 2020; Keleş, 2020; Sesli, 2020; Yeşil et al., 2020; Acar et al., 2021; Doğan, 2021; Kaygusuz et al., 2021; Sesli, 2021; Uzun, 2021) on Turkish mycobiota indicate that, *C. minutus* hadn't been reported from Turkey before, and this is the first distributional record of this species in Turkey. The study aims to make a contribution to the mycobiota of Turkey.

2. Materials and Method

The basidiocarps of *C. minutus* were collected during a field study in Silifke district of Mersin province. The material was photographed in the field using a Sony HX400V digital camera and extensive notes on the basidiomata were taken before drying. Then the collected samples were transferred to the fungarium within paper boxes and dried in an air conditioned room. Microscopic investigations were performed under a Nikon Eclipse Ci-S trinocular microscope by preparing free-hand sections of dry specimens. Identification was performed by comparing the obtained data with the available literature (Gilliam, 1976; Breitenbach and Kränzlin, 1991; Antonin and Noordeloos, 2010).

The specimens are kept at Karamanoğlu Mehmetbey University, Science Faculty, Department of Biology, Karaman.

3. Results

Fungi R.T. Moore

Basidiomycota R.T. Moore

Agaricales Underw.

Physalacriaceae Corner

Cryptomarasmius minutus (Peck) T.S. Jenkinson & Desjardin, Mycologia 106(1): 92 (2014)

Syn: **[***Chamaeceras capillipes* (Sacc.) Kuntze, *Chamaeceras minutus* (Peck) Kuntze, *Marasmius capillipes* Sacc., *Marasmius capillipes* var. *macrosporus* Kühner, *Marasmius minutus* Peck]

Macroscopic features: Pileus 0.6-2.5 mm in diam., almost hemispherical to deeply convex when young, becomes

conic convex, plano-convex to plane at maturity, often with a depressed central disc, radially striate or distantly striate, when fresh pale red brown with a darker centre, dark brown when dry, margin incurved when young, undulating at maturity. Flesh membranous. Lamellae almost invisible when young, distant, free to narrowly adnexed, without collar, whitish to light brownish. Taste and odor not distinguishable. Stipe 5-19.5 × 0.05-0.2 mm, central, filiform, equal, curved, twisted or curled, solid, brown to black-brown (Fig 1), somewhat lighter to whitish toward the pileus.

Microscopic features: Basidia 16-23 × 5-7.5 µm, cylindric-clavate, with 4-spored with a basal clamp (Fig 2a), basidioles 11-19 × 3.5-8.5, cylindrical-clavate to clavate. Basidiospores 6-8 × 2.2-4 µm, ellipsoid to narrowly ellipsoid, some amygdaliform, smooth, hyaline, inamyloid (Fig 2b). Pileipellis hymeniform, mainly composed of broadly clavate to pyriform, vesiculose or cylindrical broom cells of the Rotalis-type 10-21 × 6-15 µm, with numerous 0.8-2.3 long projections (Fig 2c). Pileocystidia 17-26 × 4-6.5 µm (Fig 2d), interspeaded among pileipellis. Cheilocystidia of two types: 1-broom cells of Rotalis-type, 10-16.5 × 6-11 µm, broadly clavate to vesiculose with 0.7-2 µm long projections; 2- fusiform to lageniform, smooth, 20-31 × 6.5-13 µm (Fig. 2e). Pleurocystidia 17-30 × 4-8 µm fusiform, ventricose or lageniform, smooth (Fig 2f).

Figure 1. Basidiocarps of *Cryptomarasmius minutus*

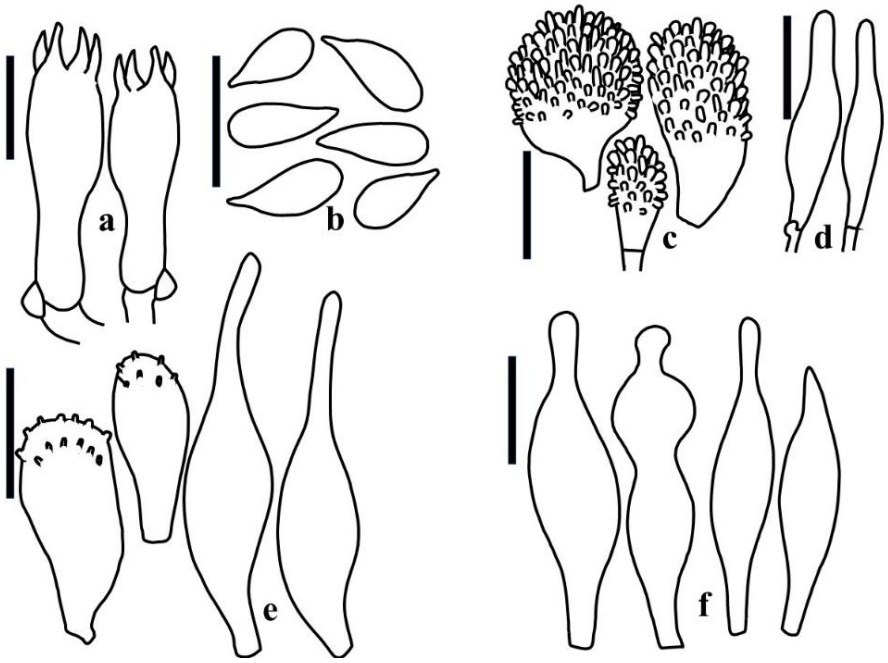

Figure 2. Basidia (a), basidiospores (b), pileipellis (c), pileocystidia (d), cheilocystidia (e) and pleurocystidia (f) of *Criptomarasmius minutus* (bars: 10 µm)

Cryptomarasmius minutus was reported to grow on fallen or decaying leaves of various deciduous trees such as *Populus* L., *Salix* L., *Fraxinus* L., *Ulmus* L., *Acer* L., *Syringa* L, especially of *Populus* and *Salix* spp. in damp, shady places (Gilliam, 1976; Breitenbach and Kränzlin, 1991; Antonin and Noordeloos, 2010; Jenkinson et al., 2014).

Specimens examined: Mersin, Silifke, Değirmendere village, Göksu river bank, on fallen *Populus* sp. leaves. 36°24′N-33°48′E, 40m, 09.11.2019, DerKap-305.

4. Discussions

Cryptomarasmius minutus was presented as a new member of the genus *Cryptomarasmius* in Turkey. In general, macro and micromorphology are in agreement with those given in literature (Gilliam, 1976; Breitenbach and Kränzlin, 1991; Antonin and Noordeloos, 2010; Jenkinson et al., 2014).

Though it has rarely been recorded, *C. minutus* is thought to be a widespread species in the temperate zone of Northern hemisphere and has been recorded from many European countries such as Austria, Czech Republic, France, Germany, Italy, Netherlands and Spain. Due to the tiny and easily drying-up fruit bodies which become almost indistinguishable from the substrate, it is an easily overlooked species (Antonin and Noordeloos, 2010).

Marasmius pseudominutus Singer and *M. pichinchensis Singer* are similar to *C. minutus.* But slightly larger spores (6.5-10 × 4.0-5.0 µm) of *M. pseudominutus* and the presence of rhizomorphs from which stipes arise, and the monotypic cheilocystidia of *M. pichinchensis* differ them from *C. minutus* (Antonin and Noordeloos, 2010).

Cryptomarasmius minutus is the second member of the genus *Cryptomarasmius* in Turkey. The previously reported Turkish species of the genus, *C. corbariensis*, have a larger fruit body (a pileus of 2-9 mm in diameter and a stipe of 10-30 × 0.2-0.3 mm), larger basidia (22-30 × 8-9.5 µm) and larger basidiospores (8-10 3.5-5.5 µm), though most other micro characters agree. The substrate of *C. corbariensis* was also different (Antonin, 2007; Uzun et al., 2017). *Cryptomarasmius corbariensis* was reported on rotting *Olea* L. sp. leaves, while *C. minutus* is largely confined to fallen *Populus* or *Salix* sp. leaves, and was collected on rotting *Populus* sp. leaves (Uzun et al., 2017; Henrici, 2018).

Conflict of Interest

Authors have declared no conflict of interest.

Authors' Contributions

The authors contributed equally.

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Cold sensitivity of *Casuarina cunninghamiana* (*Casuarinaceae*) saplings

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Casuarina cunninghamiana (*Casuarinaceae*) fidanlarının soğuğa duyarlılığı

Abstract: The Australian tree, *Casuarina cunninghamiana* Miq. (*Casuarinaceae*), is widely planted for agroecosystem and other benefits, and belongs to a family (sheoaks) with wide adaptation and tolerance to environmental extremes. However, extreme cold as a stressor is not common on the Australian continent, so compared to heat and drought, less work has been done on the cold tolerance of Australian flora. Therefore, saplings of *C. cunninghamiana* were exposed to 0 to 4 months of extreme winter temperatures below -5°C to about -15°C over 2 years in Niğde, Central Anatolia, Turkey to test their cold sensitvity. Damage was severe, however, most plants exposed to 1 or 2 months of the milder temperatures (down to -5°C) at the beginning of winter recovered. Four months exposure killed all the plants. However, even the milder, early-winter conditions are unlikely to be suitable for the long-term productivity or survival of *C. cunninghamiana* and perhaps all members of its family. The risk of extreme cold needs to be considered when planting sheoaks beyond their native range.

Key words: Abiotic stress, *Casuarina*, cold tolerance, frost damage, sheoaks

Özet: Avustralya ağacı, *Casuarina cunninghamiana* Miq. (*Casuarinaceae*) geniş bir adaptasyon ve çevresel uç noktalara toleransı olan bir familyaya (demirağacıgiller) ait olup, agroekosistem ve diğer faydalar için yaygın olarak yetiştirilmektedir. Bununla birlikte, Avustralya kıtasında aşırı soğuk bir stres etkeni olarak yaygın olmadığıdan, Avustralya florasının soğuğa toleransı üzerinde ısı ve kuraklığa kıyasla daha az çalışma yapılmıştır. Bu nedenle, *C. cunninghamiana* fidanları, soğuk toleranslarının belirlenmesi için Niğde'de (Orta Anadolu, Türkiye) 0 ila 4 ay süreliğine -5°C'nin altında ve yaklaşık -15°C'ye kadar aşırı düşük kış sıcaklıklarına 2 yıl boyunca maruz bırakılmıştır. Hasar şiddetli olmakla birlikte, kış başlangıcından itibaren 1 veya 2 ay daha ılıman sıcaklıklara (-5°C'ye kadar) maruz kalan bitkilerin çoğu toparlanmıştır. Dört aylık maruz kalma, tüm bitkilerin ölümüne neden olmuştur. Bununla birlikte, daha ılıman, erken kış koşullarının bile, *C. cunninghamiana*'nın ve belki de bu familyaya ait diğer tüm türlerin uzun vadeli üretkenliği veya hayatta kalması için uygun olması pek olası değildir. Kendi doğal aralığının ötesinde demirağacıgilleri dikerken aşırı soğuk riskinin dikkate alınması gerekmektedir.

Anahtar Kelimeler: Abiyotik stres, *Casuarina*, soğuğa tolerans, don hasarı, demirağacıgiller

Citation: Riley IT, Saygı AH (2021). A preliminary work on the cold sensitivity of *Casuarina cunninghamiana* (*Casuarinaceae*) saplings. Anatolian Journal of Botany 5(2): 124-126.

1. Introduction

Australian plants have evolved with tolerance to a range of environmental extremes including extended drought, high temperatures, infertile or saline soils, and more (Turnbull, 1997). However, cold as a stressor is not particularly severe in Australia (Riley and Saygı, 2021), so Australian flora has not commonly been assessed for its cold sensitivity even though many species are now grown globally for economic and other benefits often under conditions that differ from their native range. The Australian sheoaks (*Casuarinaceae*) are an important group of actinorhizal plants that of potential use in agroecosystem in semiarid contexts, where conditions might be much colder than in Australia (Riley, 2019). However, there is limited data on their sensitivity to extreme cold. *Casuarina cunninghamiana* Miq. (river sheoak) was assessed for frost damage in California, USA and found to be potentially tolerant to -8°C but severely damaged at lower temperatures to -13°C (Merwin et al., 1995), but these data were only collected opportunistically after a few brief, extremely cold weather events, and no information was collected on recovery. More recently, it was reported that diaspore release from *C. cunninghamiana* is sensitive to freezing (Riley and Saygı, 2021) indicating freezing can mechanically damage the woody tissues of sheoak infructescences.

The objective of this study was to assess the cold sensitivity of *C. cunninghamiana* by exposing saplings to natural subzero winter temperatures for 0 to 4 months in Niğde, Central Anatolia, Turkey.

2. Materials and Methods

This study examined the sensitivity of container-grown *C. cunninghamiana* saplings when exposed to 0 to 4 months of natural subzero winter temperatures in Niğde, Turkey (37°56′37″N, 34°37′51″E; daily minimums for the 2 years of the are presented Figure 1, with both years having a similar pattern of temperatures. Seed of *C. cunninghamiana* (the common species in Turkey; Riley and Korkmaz, 2019) was sourced from Antalya, Turkey in October 2018 (36°53′06″N, 30°40′54″E) and plants grown in a greenhouse in a mixture of local soil and peat (1:3) repotting into square plastic pots (115 × 183 mm) at least six months before the first experiment in 2019. Fifty of these plants (mean height ~0.7 m) were arbitrarily allocated five groups of 10 (Fig. 2a) that were exposed to natural winter conditions for 0 to 4 months commencing 5 November 2019. The pots were fully imbedded into the field soil to prevent them freezing and to keep the plants upright (positions formally randomised in four blocks), and watered at least weekly. The experiment was repeated commencing 6 November 2020 with six plants per group

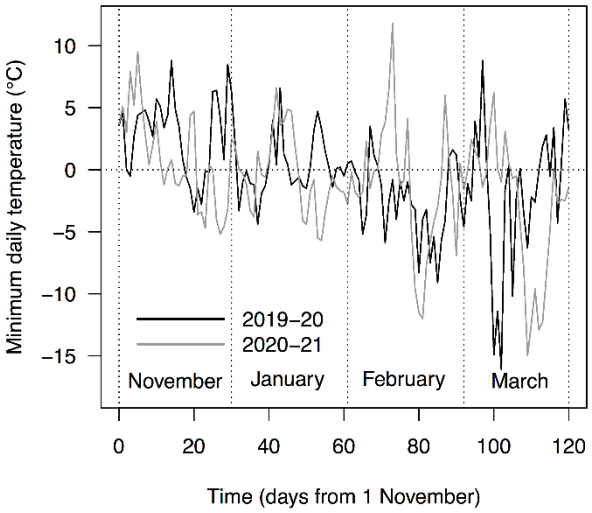

Figure 1. Minimum daily temperature in Niğde, Turkey (government weather station, 37°57′30″N, 34°40′46″E) for November to February 2019-20 and 2020-21

(mean height ~1.9 m; Fig. 2a) having been repotted into 300-m circular pots in mid-2020. When placed in the field (likewise randomised in four blocks), these pots were surrounded and covered with straw to prevent freezing and watered at least weekly. In both years, the plants were regularly examined of symptoms of frost damage and height (stem length) measured, and recovery monitored for 4 to 1 months after returning to the greenhouse for the 1- to 4-month exposure groups, respectively, with the final observations made in April. Data were analysed and visualised with R version 4.1.0 (R Core Team, 2021).

3. Results

In both years, nearly all foliage was frost damaged and had turned brown by early December having been exposed to minimums mostly above -5°C. With extended cold exposure, some damaged foliage and small branchlets were shed, and the main stems became bent with loss of mechanical strength exacerbated by snowfall events (Fig 3). The plant height decreased with longer exposure in both year (Fig. 2b,d), consistently for all exposure groups in 2019-20, but in 2020-21 with plants having stronger stems, the decrease in height was most evident in the longest exposure group. The control group (0 months exposure) continued to grow in the greenhouse (especially the younger plants) despite short day lengths and temperatures being typically below 20°C as the heating system did not fully compensate for the subzero ambient temperatures. Recovery (defined as an new shoot growth) in 2019-20 (recorded in April) was only observed in 8 and 6 of the 10 plants with 1 and 2 months of cold exposure, respectively (Figs 2c and 3a). In 2020-21, recovery was monitored from 1 month after returning plants to the greenhouse with half or more recovery occurring within a month and all recovery within 2 months for plants exposed for 1 and 2 months (Fig. 2c). Only one plant recovered after 3 months cold of exposure. These results were similar the first year (Fig 3b), but with a greater proportion recovering. Recovery (reshooting) mostly occurred along the entire stem but in a few plants, new shoots only developed at the base of the stem.

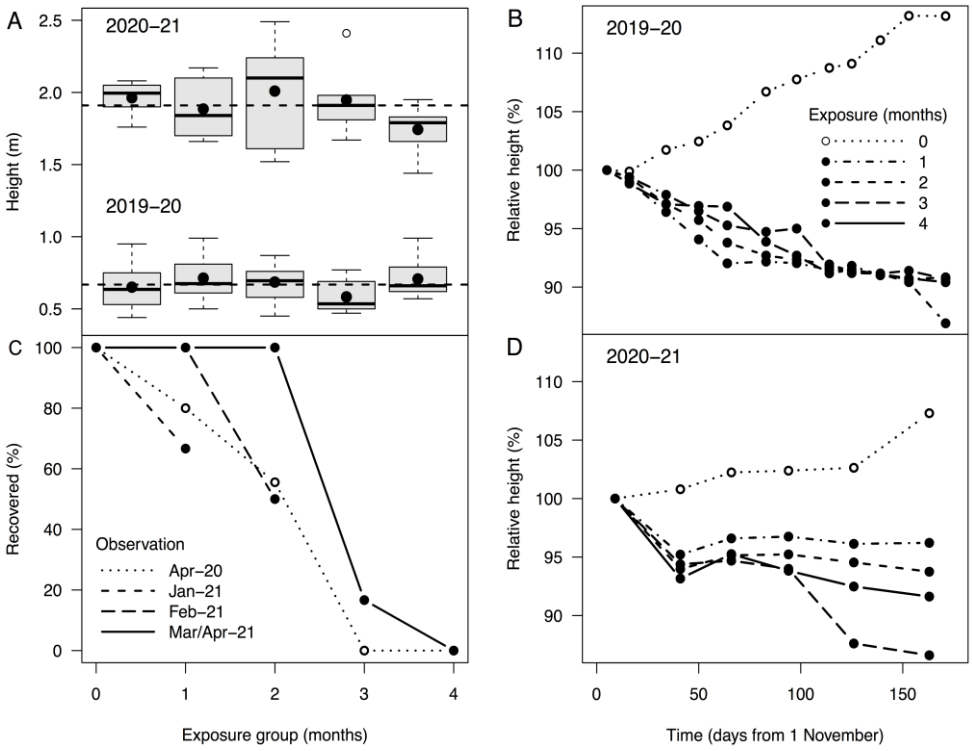

Figure 2. A. *Casuarina cunninghamiana* sapling height (main stem length) box plots for five low-temperature exposure groups for 2019-20 (n = 10) and 2020-21 (n = 6). Group means (black circle) were not significantly different (ANOVA, p = 0.05), however, subsequent heights are presented relative to the initial value to eliminate inter-plant variation. **B, D.** Mean relative height for saplings exposed to 0 to 4 months of natural subzero temperatures in the field in Niğde, Turkey, 2019-20 and 2020-21, respectively. **C.** Proportion of recovered plants in 2019-20 in April (1 month after the last group was returned to the greenhouse) and 2020-21 (1 month after each group was returned to the greenhouse). Recovery was defined as any fresh shoot growth indicating that the plant was alive.

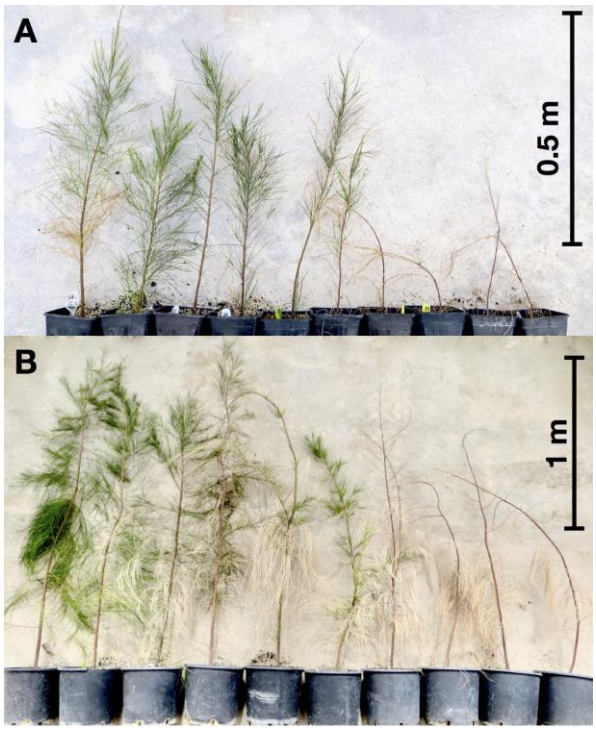

Figure 3. *Casuarina cunninghamiana* saplings of five low-temperature exposure groups from **A**, 2019-20, and **B**, 2020-21 (from right to left, 2 arbitrarily selected plants from each group of 0 to 4 months exposure) about months after return to the greenhouse. Although the photograph was taken about a month after (late April an and early May, respectively) the final observations presented in Figure 2c, no additional plants had recovered.

4. Discussion

These findings indicate that *C. cunninghamiana* is not tolerant to extended periods subzero temperatures, and clearly not extremely cold conditions, as experienced in the experimental site in January and February. It is not possible from these data to fully distinguish between degree and duration of cold exposure because the temperatures continued fall across 4 months (Fig. 1). However, the damage that occurred with only 1 month of cold exposure (November) was still quite problematic, with most foliage and some branchlets damaged, so even 1 month per year of such weather is likely to prove unsuitable for long-term productivity or survival of *C. cunninghamiana*. Therefore, it is concluded that *C. cunninghamiana* is unlikely to survive in areas where winter temperatures frequently drop to and below -5°C (consistent with the observations of Merwin et al., 1995). It is also highly probably that all members of the family are similarly cold sensitive having consistent morphology and anatomy. In addition, with global warming, extreme weather events will potentially become more frequent and, counterintuitively, this include extreme cold events as experienced in Texas, USA in February 2021 (Doss-Gollin et al., 2021). This means that even *C. cunninghamiana* planted in midlatitudes with mild winters could be subject to unanticipated damage from such events, and the risk of extreme cold needs to be considered when planting sheoaks beyond their native range.

Conflict of Interest

Authors have declared no conflict of interest.

Authors' Contributions

The authors contributed equally.

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Antioxidant potential of *Pseudevernia furfuracea* (L.) Zopf and its secondary metabolites on hepatocellular carcinoma cells: regulation of antioxidant enzymes

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Pseudevernia furfuracea (L.) Zopf ve sekonder metabolitlerinin hepatosellüler karsinom hücreleri üzerindeki antioksidan potansiyeli: antioksidan enzimlerin düzenlenmesi

Abstract: The use of medicinal plants and secondary metabolites increases in treating numerous diseases such as hepatocellular carcinoma (HCC), one of the leading causes of cancer-related death worldwide. Oxidative stress contributes to the development of liver cancer, and promoting antioxidant systems might provide better insights for the treatment. In the present study, the antioxidant potential of *Pseudevernia furfuracea* (L.) Zopf lichen species were investigated. Besides, effects of major secondary metabolites, olivetoric acid (OA) and physodic acid (PA), which were isolated from *P. furfuracea*, on hepatic gene expressions of antioxidant enzymes, were evaluated in both cancerous (HepG2) and healthy (THLE2) human liver cells. According to the results, the total phenolic content of *P. furfuracea* was 71.52 µg/mg and 8.16 µg/mg gallic acid equivalent for ethanolic and aqueous extracts, respectively. Likewise, β-carotene and lycopene contents were also higher in ethanolic extracts. In line with these antioxidant ingredients, DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity of ethanol extract (IC_{50}: 158.79 mg/L) was remarkably high as compared with its aqueous extracts (IC_{50}: 630.33 mg/L). OA and PA strongly augment all antioxidant enzymes' gene expressions in HepG2 cells, while only *gpx* expression was upregulated in healthy THLE2 cells. Conversely, these two metabolites suppress *cat, sod1*, and *sod2* expressions in THLE2 cells. These results together suggest that *P. furfuracea* not only has high antioxidant potential, but its secondary metabolites might also reduce oxidative stress in cancer cells by upregulating antioxidant enzymes, which would prevent oncogenesis and tumor progression in liver cancer.

Keywords: *Pseudevernia furfuracea*, olivetoric acid, physodic acid, antioxidant potential, antioxidant enzymes

Özet: Dünyada kansere bağlı ölümlerin önde gelen nedenlerinden biri olan hepatosellüler karsinom (HCC) gibi çok sayıda hastalığın tedavisinde tıbbi bitkilerin ve sekonder metabolitlerin kullanımı artmaktadır. Oksidatif stres, karaciğer kanseri gelişimine katkıda bulunur ve antioksidan sistemlerin teşvik edilmesi, tedavi için daha iyi bilgiler sağlayabilir. Bu çalışmada, *Pseudevernia furfuracea* (L.) Zopf likeninin antioksidan potansiyeli araştırılıştır. Ayrıca, *P.furfuracea*'dan izole edilen ana sekonder metabolitler olan olivetorik asit (OA) ve fisodik asitin (PA) hepatik antioksidan enzimlerin gen ekspresyonları üzerindeki etkileri hem kanserli (HepG2) hem de sağlıklı (THLE2) insan karaciğer hücrelerinde değerlendirilmiştir. Sonuçlara göre, *P. furfuracea*'nın toplam fenolik içeriği etanolik ve sulu ekstraktlar için sırasıyla 71.52 µg/mg ve 8.16 µg/mg gallik asit eşdeğeri bulunmuştur. Aynı şekilde β-karoten ve likopen içerikleri de etanolik ekstraktlarda daha yüksek olarak belirlenmiştir. Bu antioksidan bileşenlerle uyumlu olarak, etanol özütünün DPPH (2,2-difenil-1-pikrilhidrazil) radikal süpürme aktivitesi (IC_{50}: 158.79 mg/L), sulu ekstraktlar (IC_{50}: 630.33 mg/L) ile karşılaştırıldığında oldukça yüksektir. OA ve PA, HepG2 hücrelerinde tüm antioksidan enzimlerin gen ifade düzeylerini arttırırken, sağlıklı THLE2 hücrelerinde ise sadece *gpx* ekspresyonu yukarı yönde regüle olmuştur. Bundan farklı olarak, her iki metabolit de THLE2 hücrelerinde *cat, sod1* ve *sod2* ekspresyonlarını baskılamıştır. Bu sonuçlar *P. furfuracea*'nın sadece yüksek antioksidan potansiyeline sahip olmadığını, aynı zamanda sekonder metabolitlerinin de karaciğer kanserinde onkogenezi ve tümör ilerlemesini önleyecek antioksidan enzimleri yukarı regüle ederek kanser hücrelerinde oksidatif stresi azaltabileceğini göstermektedir.

Anahtar Kelimeler: *Pseudevernia furfuracea*, olivetorik asit, fisodik asit, antioksidan potansiyeli, antioksidan enzimler

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1. Introduction

Hepatocellular carcinoma (HCC) is one of the most lethal cancer types worldwide, and accumulating evidence has pointed to the association between oxidative stress and the development of HCC; however, the mechanisms and overall impacts remain uncertain (Fu and Chung, 2018). Under normal physiological conditions, antioxidant enzymes play significant roles against oxidative stress. Among them, cytosolic (SOD1) and mitochondrial superoxide dismutase (SOD2) act on superoxide radicals to produce hydrogen peroxide that would eventually be detoxified by catalase (CAT) and glutathione peroxidase (GPx) enzymes. In addition to endogenous antioxidants in cells, exogenous antioxidants from natural species and secondary metabolites would protect cells against oxidative stress. Among the antioxidant compounds, especially phenolics have high capacities to act on radicals. Phenolic compounds have many different types, such as gallic acid, rutin, phloridzin, syringic acid, ferulic acid, and almost all of them show very high antioxidant properties (Mohammed et al., 2020; Emsen and Kolukisa, 2021). Many different plant species containing these compounds have traditionally been used by the public to treat diseases and are still highly preferred, especially in Asian countries (Bailly, 2021;

Bharti et al., 2021; Wang et al., 2021). Medicinal and aromatic plants can show different activities thanks to the various metabolites they contain (Ameh et al., 2010; Mohammed et al., 2021; Pachi et al., 2020).

Lichens have traditional uses dating back to ancient times. These organisms came into being by the symbiotic association of fungi and algae (Zambare and Christopher, 2012; Korkmaz et al., 2018). The presence of two different organisms in the structures of lichens contributes to the production of metabolites that are not found in other living organisms. Especially, secondary metabolites and extracts produced by lichens have the potential to show many biological activities (Ranković and Kosanić, 2019). Properties such as anticancer (Solárová et al., 2020a), genoprotective (Turkez et al., 2014), antiviral (Karagöz and Aslan, 2005), antifungal (Karabulut and Ozturk, 2015), antioxidant (Emsen, 2019), and antibacterial (Shrestha et al., 2014) reveal the different potentials of lichens. *Pseudevernia furfuracea* (L.) Zopf is a fruticose lichen that has been preferred in both traditional, alternative and complementary treatment processes since ancient times. Many different biological activities of this species are available in the literature (Ranković and Kosanić, 2019). Antioxidant, antimicrobial, anticancer (Kosanić et al., 2013), antifungal (Karabulut and Ozturk, 2015), pro-apoptotic (Šeklić et al., 2018), anti-inflammatory (Güvenç et al., 2012), and antibiofilm (Mitrović et al., 2014) activities are some of the major effects determined with *P. furfuracea*.

Olivetoric acid (OA) and physodic acid (PA) are the two primary secondary metabolites obtained from the lichen species, and they strongly improve the total antioxidant capacities of hepatic cells in vitro (Emsen et al., 2020; Emsen et al., 2021). Previously, we evaluated the apoptotic/necrotic impacts, cytotoxic, oxidative, antioxidative, genotoxic, and antigenotoxic effects of these secondary metabolites on the cancerous human liver (HepG2) and healthy human liver (THLE2) cell lines. However, the overall antioxidant potential of *P. furfuracea* and the effects of secondary metabolites over endogenous antioxidant enzymes remains to be elucidated. Therefore, this study is designed to evaluate the antioxidant potential of *P. furfuracea* by examining total phenolic contents, β-carotene, and lycopene contents, DPPH free radical scavenging activities together with metal chelating power. Additionally, the regulatory roles of secondary metabolites isolated from *P. furfuracea* over hepatic antioxidant enzymes; CAT, SOD1, SOD2, and GPx were evaluated in both cancerous (HepG2) and non-cancerous (THLE2) cells at the gene expression level.

2. Materials and Method

2.1. Lichen samples

Pseudevernia furfuracea samples were collected from the Oltu region of Erzurum province of Turkey. Samples were photographed in their natural environment and were carried to the laboratory and air-dried. To identify lichens, macroscopic and microscopic data were utilized using the published literature (Purvis et al., 1992; Wirth, 1995).

2.2. Preparation of the extracts and isolation of olivetoric and physodic acid

The lichen specimens were dried under room conditions and powdered with an ultra-centrifuge grinder (Retsch ZM 200, Germany). Then, ethanol and water extracts of *P. furfuracea* were obtained by 250 mL solvent systems using Soxhlet extraction apparatus. After filtering through Whatman No. 1 filter paper, the solvent was evaporated with a rotary evaporator and then lyophilized. OA and PA were isolated from *P. furfuracea* as we described in detail previously (Emsen et al., 2020; Emsen et al., 2021) using Soxhlet extraction and then silica column chromatography. The chemical structures of OA and PA were determined by proton and carbon-13 nuclear magnetic resonance spectrum.

2.3. Free radical scavenging activity of *P. furfuracea*

In measuring 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity of ethanol and water extracts obtained from *P. furfuracea*, applications were carried out with the final concentrations of the extracts in the plate wells of 200, 400, 600, 800, and 1000 mg/L. According to the method, 20 µL of the extracts were placed in each microplate well, and 180 µL of DPPH (0.06 mM in methanol) was added. The reduction of DPPH free radical was determined by measuring the absorbance values at 517 nm after 60 min incubation in the dark. The free radical scavenging activities of the extracts were calculated as a percentage using the following formula: Radical scavenging activity = [(Control absorbance – Extract absorbance) / Control absorbance)] × 100.

2.4. Metal chelating activity of *P. furfuracea*

In the measurement of the metal chelating activity of ethanol and water extracts, applications were carried out with the final concentrations of the extracts in the plate wells of 200, 400, 600, 800, and 1000 mg/L. According to the method, 50 µL of the extracts were added to each microplate well. 10 µL of ferrozine (5 mM), 5 µL of $FeCl_2$ (2 mM), and 185 µL of methanol were added and kept at room temperature for 10 min. Spectrophotometric measurements were performed at 562 nm. The metal chelating activities of the extracts were calculated in percentage with the following formula: Metal chelating activity = [(Control absorbance – Extract absorbance) / Control absorbance)] × 100.

2.5. Determination of total phenolic content of *P. furfuracea*

In the process of determining the total phenolic contents of ethanol and water extracts from *P. furfuracea*, gallic acid (0.01, 0.05, 0.1, 0.2, 0.4, 0.5, 1.0 mM) was used as a standard. 20 µL of ethanol and water extracts and standards up to a concentration of 1000 mg/L were added in the microplate wells. 20 µL of Folin reagent (2N) was applied, and the samples mixed by pipetting were incubated in the dark for 3 min. Then, 20 µL of 35% (w/v) sodium carbonate and 140 µL of dH_2O were added and kept in the dark for 10 min. Spectrophotometric reading was performed at 725 nm. Calculation in gallic acid equivalents was performed using the standard calibration curve.

2.6. Determination of β-carotene and lycopene content

The amounts of β-carotene and lycopene in ethanol and water extracts obtained from *P. furfuracea* were determined spectrophotometrically. According to the method, 1 mL of the extracts was mixed with 1 mL of acetone:hexane (4:6) and filtered through a filter paper after vortexing. Then, the amounts of β-carotene and lycopene were calculated using

the absorbance values at 453, 505, and 663 nm wavelengths, according to the following formulas:

β-carotene content (mg/100 mg) = 0.216 × Absorbance (663 nm) – 0.304 × Absorbance (505 nm) + 0.452 × Absorbance (453 nm)

Lycopene content (mg/100 mg) = -0.0458 × Absorbance (663 nm) + 0.372 × Absorbance (505 nm) – 0.0806 × Absorbance (453 nm)

2.7. Culture of the THLE2 and HepG2 cells

This study evaluated the effects of *P. furfuracea* secondary metabolites; OA and PA on normal human hepatocytes (THLE2) and human hepatocellular carcinoma (HepG2) cells. THLE2 cells were cultured in Bronchial Epithelial Cell Growth Medium (BEGM) enriched with gentamycin/ amphotericin, epinephrine, phosphoethanolamine, 10% fetale bovine serum (FBS) and epidermal growth factor (Lonza, Clonetics Corporation, Walkersville, MD 21793 (BEGM Bullet Kit)). HepG2 cells were grown in high glucose-containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and standard antibiotics (1% penicillin/streptomycin) in an incubator (Sanyo MCO 17AIC, USA) at 37ºC under 95% humidity and 5% CO_2 concentration. The cells were subcultured to their new growth media after reaching around 90% confluency. We determined the cell growth inhibitory potential of both OA and PA on THLE2 and HepG2 cells, previously (Emsen et al., 2020; Emsen et al., 2021).

2.8. Total RNA isolation and evaluation of antioxidant genes' expression profiles

HepG2 and THLE2 cells ($1x10^{+6}$ cells/well) were treated with 200 mg/L of OA and PA for 72-h. The dose of OA and PA were determined according to the median inhibitory concentration (IC_{50}) values that we published previously (Emsen et al., 2020; Emsen et al., 2021). After application, the cells were detached, and total RNAs were isolated with a miRNeasy RNA isolation kit (Qiagen, USA). Qubit 4.0 fluorometer (Thermo, USA) was utilized to evaluate the quality and quantity of isolated RNAs. Then, two µg of total RNA were reverse transcribed (First-strand cDNA synthesis kit, Thermo Scientific, USA) as described in the manufacturer protocol. Expression levels of antioxidant genes were determined with qRT-PCR (Light Cycler480 II, Roche, Switzerland). For gene expression measurements, 1 µL of cDNA were mixed with 5 µL SYBR Green Reaction Mix (Roche, Basel, Switzerland) and 4 µL primer pairs (Table 1) at 0.5 µM concentrations each in the final volume. qPCR reaction was initiated with 95 ºC incubation for 10 min. Then 40-repeated cycles of 95 ºC for 10 s for denaturation, 58 ºC for 15 s for annealing, and 72 ºC for 15 for extension were conducted. Green fluorescence values were measured at the end of each extension step, and a melt analysis was performed to confirm the unity of qPCR products. The relative expression of antioxidant enzymes with respect to the housekeeping gene (*gapdh*) was calculated with the advance relative quantification tool of LightCycler II 480 SW 1.5.1 software (Roche, Basel, Switzerland).

Table 1. Primer sequences of genes used in qPCR studies.

Gene		Primer sequences (5'→3')
cat	*Forward*	GAACAGATAGCCTTCGACCC
	Reverse	AGTAATTTGGAGCACCACCC
gpx	*Forward*	CAGTCGGTGTATGCCTTCTC
	Reverse	TTCTTGGCGTTCTCCTGATG
sod1	*Forward*	AGATGACTTGGGCAAAGGTG
	Reverse	TTGGGCGATCCCAATTACAC
sod2	*Forward*	GCACATTAACGCGCAGATCA
	Reverse	AGCCTCCAGCAACTCTCCTT
gapdh	*Forward*	CTTCTTTTGCGTCGCCAGCC
	Reverse	TGGAATTTGCCATGGGTGGA

2.9. Statistical analyses

All data were analyzed with one-way ANOVA followed by the appropriate post-hoc test, the Duncan test. Probit regression analysis was used to calculate the median inhibitor concentration (IC_{50}) values. All analyses were done using SPSS 21.0 software (IBM Corporation, Armonk, NY, USA).

3. Results

3.1. DPPH scavenging activity of *P. furfuracea*

For DPPH scavenging activity, a concentration-dependent increase was detected for both ethanol and water extracts. 800 and 1000 mg/L concentrations of the ethanol extract showed activity over 95%, and the difference between the two values was statistically ($p > 0.05$) insignificant. Similarly, there was no statistical ($p > 0.05$) difference between the concentrations mentioned above of the water extract. However, the highest DPPH activity of the water extract was 62.70% (Figure 1). The IC_{50} value of the ethanol extract (158.79 mg/L) was lower than the water extract (630.33 mg/L) (Table 1). Considering these results, it is clear that the DPPH scavenging activity of the ethanol extract was higher than that of the water extract.

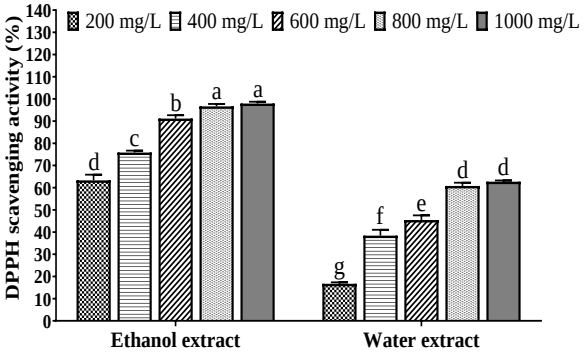

Figure 1. DPPH radical scavenging activities of ethanol and water extracts from *P. furfuracea* (Mean ± Standard Deviation, $n = 3$) (Values indicated by different letters differ from each other at the level of $p < 0.05$).

3.2. Metal chelating activity of *P. furfuracea*

In metal chelating activity, a concentration-dependent increase was detected in both ethanol and water extracts. The results of the 800 and 1000 mg/L concentrations showing the highest activity of both extracts were very close

to each other and posesses statistically (p > 0.05) insignificant difference (Figure 2). Based on the IC_{50} values, the water extract appears to be more effective than the ethanol extract with a lower value (860.54 and 906.34 mg/L, respectively) (Table 2).

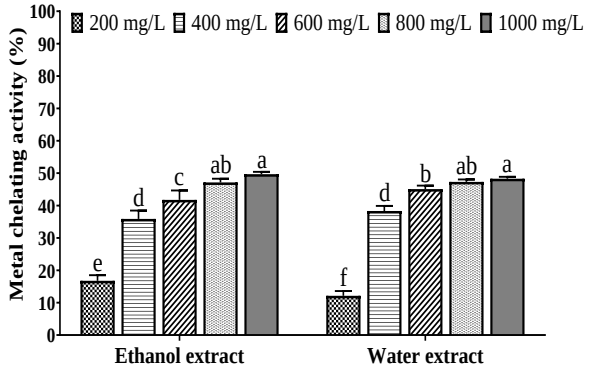

Figure 2. Metal chelating activities of ethanol and water extracts obtained from *P. furfuracea* (Mean ± Standard Deviation, n = 3). Values indicated by different letters differ from each other at the level of p < 0.05.

Table 2. Median inhibitory concentration (IC_{50}) values resulting from DPPH scavenging and metal chelating activities of extracts from *P. furfuracea*.

Activity	Treatment	IC_{50} (mg/L)	Slope ± Standard error of the mean (Limits)
DPPH scavenging	Ethanol extract	158.79	2.34 ± 0.17 (2.00-2.68)
	Water extract	630.33	1.86 ± 0.14 (1.57-2.14)
Metal chelating	Ethanol extract	906.34	1.32 ± 0.14 (1.04-1.60)
	Water extract	860.54	1.49 ± 0.14 (1.20-1.78)

3.3. Analysis of antioxidant compounds of *P. furfuracea*

Total phenol, β-carotene, and lycopene contents were investigated from the extracts obtained from *P. furfuracea*. Total phenolic contents were calculated based on the gallic acid equivalent that we used as a standard. The total phenolic amounts of ethanol and water extracts were 71.52 µg/mg gallic acid equivalent and 8.16 µg/mg gallic acid equivalent, respectively. According to these results, ethanol extract had a higher rate of phenolic compounds compared to the water extract. Likewise, when we look at the β-carotene and lycopene contents, it was seen that ethanol extracts had higher β-carotene and lycopene contents compared to water extracts (Table 3).

Table 3. Antioxidant compounds of extracts from *P. furfuracea*

Compound	Treatment	Content (µg/mg)
Total phenol (Gallic acid equivalent)	Ethanol extract	71.52 ± 2.68
	Water extract	8.16 ± 0.49
β-carotene	Ethanol extract	0.66 ± 0.03
	Water extract	0.27 ± 0.01
Lycopene	Ethanol extract	0.32 ± 0.01
	Water extract	0.18 ± 0.01

3.4. The effects of OA and PA on expression levels of antioxidant enzymes in THLE2 and HEPG2 cells

Relative changes in antioxidant enzyme gene expression levels of THLE2 and HepG2 cells were measured by qRT-PCR, and the results show the activation of main antioxidant enzymes; *cat*, *gpx*, *sod1*, and *sod2*, in cancerous HepG2 cells with OA and PA treatments (Figure 3). Similarly, these secondary metabolites also augmented *gpx* expression in THLE2 cells (Figure 3B). On the contrary to HepG2 cells, OA and PA have suppressive effects on *cat*, *sod1*, and *sod2* in non-cancerous THLE2 cells (Figure 3A, 3C, and 3D). The results also demonstrated that PA's stimulatory and/or suppressive potential is more than that of OA at similar concentration and treatment time.

4. Discussion

The most widespread type of liver cancer is hepatocellular carcinoma (HCC), the mortality rate of which has increased over the past decade. Among the risk factors, oxidative stress and the reduction in antioxidant capacities might be contributors (Yahya et al., 2013). Even though there are different antioxidant systems to combat the generation of free radicals, the disruption of the balance between pro- and anti-oxidants might lead to oncogenesis and progression of this disease (Cheng et al., 2017).

Under normal circumstances, antioxidant enzymes function to reduce pathologies associated with oxidative stress (Sadi and Sadi, 2010), and exogenous antioxidant supplementations might reverse the detrimental effects of the dysregulated antioxidant network (Sadi and Sadi, 2011). Down-regulation of oxido-reductive enzymes functioning in the most important free radical scavenger systems such as CAT, SOD, and GPx are the characteristic pathological hallmark of HCC. Thus, the balance between oxidative stress and the endogenous antioxidant network is shown to impact the malignant progression of cancers (Marra et al., 2011).

Given the association with the oxidative stress and progression of HCC, the use of herbal products having a high antioxidant capacity for therapeutic purposes has become pronounced. Various plant species have been searched to find effective treatments for cancer with fewer side effects.

Numerous phenolic compounds are produced with lichen species such as xanthones, depsides, and depsidones, and a variety of beneficial effects have been determined. Among them, antioxidant, antiviral, antimicrobial, antifungal, and anticancer activities draw the attention (Ranković and Kosanić, 2019; Solárová et al., 2020b; Roychoudhury et al., 2021). The production of a great variety of secondary metabolites, many of which only appear in lichens, makes them gain pharmaceutical importance. Herein we determined the antioxidant power of *P. furfuracea*, and a remarkable amount of phenolics, β-carotene, and lycopene contents, especially in ethanolic extracts, have been found. Because of these compounds, *P. furfuracea* also provides good radical scavenging and metal-chelating properties. Previous studies demonstrated that methanol, aqueous, and acetone extracts from *P. furfuracea* exerted high reducing power, DPPH, and superoxide anion radical scavenging activities (Kosanić and Ranković, 2011; Bilgin Sökmen et al., 2012). In a study performed on the methanol extract of *P. furfuracea*, even though a high level of total phenol was

detected, the antioxidant capacity was found to be low (Odabasoglu et al., 2005). Similar to our results, another study also demonstrated a good correlation between radical scavenging activity and the amount of antioxidant compounds (Aoussar et al., 2017).

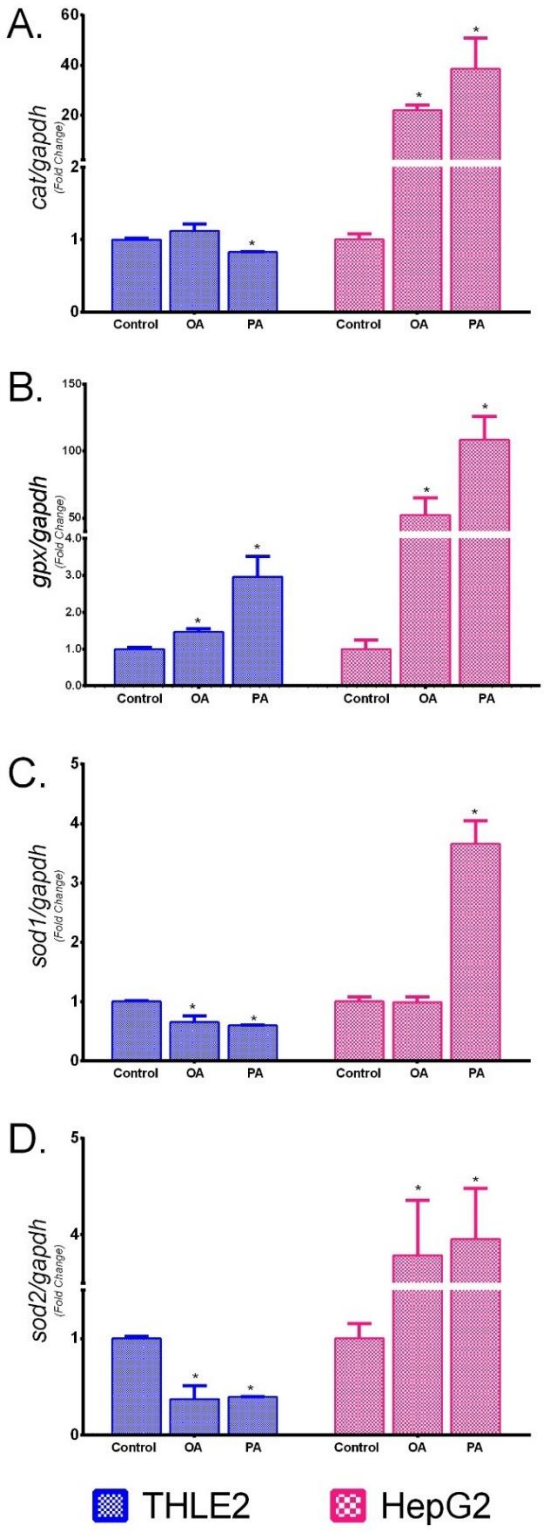

Figure 3. Changes in expression levels of *cat* (A), *gpx* (B), *sod1* (C), and *sod2* (D) mRNA levels in THLE2 and HepG2 cells with OA and PA treatments. Data were normalized using the data of *gapdh*. Each bar represents the means from three biological replicates.* $p < 0.05$, significantly different from the control; OA: Olivetoric acid; PA: Physodic acid.

In addition to whole extracts of *P. furfuracea*, its isolated secondary metabolites have different antioxidant capacities and biological activities. Physodic acid isolated from *P. furfuracea* had high DPPH and superoxide anion radical scavenging activities (Kosanić et al., 2013). OA and PA isolated from *P. furfuracea* increased the total antioxidant capacity on primary rat cerebral cortex cells (Emsen et al., 2016), human amnion fibroblasts (Emsen et al., 2017) and human lymphocytes (Emsen et al., 2018). Recently, we have demonstrated cytotoxic (apoptotic and necrotic), antioxidant, pro-oxidant, genotoxic, and apoptosis-related genes expression modulatory effects of PA and OA which were isolated from *P. furfuracea*. Results also revealed the lower side effects of these metabolites on healthy cells since HepG2 cells' apoptotic and necrotic genes were highly upregulated in HepG2 but not as much in THLE2 cells (Emsen et al., 2020; Emsen et al., 2021). Herein, we further evaluated the modulatory effects of these metabolites on the gene expression profiles of antioxidant enzymes in cancerous and healthy hepatocytes. Accordingly, the gene expressions of the main antioxidant enzymes, *cat*, *gpx*, *sod1*, and *sod2*, were upregulated in cancerous HepG2 cells with OA and PA treatments. However, OA and PA have suppressive effects on the *cat*, *sod1*, and *sod2* in non-cancerous THLE2 cells. The results also demonstrated that PA's stimulatory and/or suppressive potential is more than OA at similar concentration and treatment time. These results suggest that OA and PA promote the antioxidant defense system by increasing antioxidant enzyme expression, inhibiting oxidative stress and tumorigenesis in cancer cells. These metabolites could also hinder cancer progression because of the association between oxidative stress and carcinogenesis.

Activating major cellular antioxidant networks with *P. furfuracea* secondary metabolites could help us to understand the pharmacology of lichens secondary metabolites and their possibilities in the treatment of hepatocellular carcinoma. Our previous data showed the inhibition of cancer cell growth with OA and PA but not on non-cancerous cells, and the data in this study indicates the disproportional upregulation of *sod1*, *sod2*, *cat*, and *gpx* expression and in cancer cells. These two data together might be an evidence for the induction of apoptosis with a redox dependent mechanisms which might, in turn, induces cancer cell apoptosis. Considering all results together, the importance of *P. furfuracea* and its metabolites, OA and PA, were revealed as an alternative treatment against liver cancer. It not only has high antioxidant potential, but might also reduce oxidative stress in cancer cells by upregulating antioxidant enzymes, which would prevent oncogenesis and tumor progression in liver cancer.

Conflict of interest

Authors have declared no conflict of interest.

Authors' contributions

AA collected and identified the lichen samples. NŞ and BE performed the experiments, BE made the statistical analysis and prepared the manucript draft. GS designed the study, organized the research, made critical reviews on manuscript.

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In vitro axillary shoot regeneration from cotyledonary node of Bishop's weed (*Trachyspermum ammi* L.)

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Bishop otunun (*Trachyspermum ammi* L.) kotiledon boğum eksplantından in vitro aksil sürgün rejenerasyonu

Abstract: Bishop's seed (*Trachyspermum ammi* L.) is a vital neutraceutial plant used as spice and also used as medicinal plant for curing different ailments and diseases of humans and animals. The study presents the in vitro regeneration of Bishop's seed using cotyledonary node explant taken from in vitro germinated seeds. All mediums used in this study were comprised of MS (0.44%) medium having 3.0%sucrose and 0.65% agar with pH of 5.8. Explants were inoculated on MS medium enriched with 0.10-1.60 mg/L Thidiazuron (TDZ) alone or in combination with 0.10 mg/L IBA (Indole-3-butyric acid) for seven weeks under 16/8 light photoperiod provided with cool-white fluorescent lamps. Thereafter, explants were sucultured on MS medium without any plant growth regulators (PGRs) for five weeks more under similar culture conditions. Results revealed 100% shoot regeneration frequency with 42.85-100.00%. The shoot count and shoot length ranged 3.43-19.40 and 1.04-2.74 cm respectively. The highest shoot count (19.40) and shoot length (2.74 cm) were observed on MS medium enriched with 0.20 mg/L TDZ and 0.20 mg/L TDZ+0.10 mg/L IBA respectively. Relatively low rooting was recorded on IBA containing medium with low adaptation percentage of plantlets in pots containing peat moss.

Key words: Bishop's weed, cotyledonary node, in vitro, thidiazuron

Özet: Bishop otu (*Trachyspermum ammi* L.) çok önemli baharat olarak kullanılan nutrasötik bitki olup, aynı zamanda insan ve hayvanların farklı rahatsızlık ve hastalıklarını tedavi için kullanılan tıbbi bitkidir. Bu çalışmada Bishop otunun in vitro çimlenmiş fidelerden elde edilen kotiledon boğum eksplantlar kullanılarak in vitro çoğaltım yapılmıştır. Tüm çalışmalarda %0.44% MS, %3.0 sukroz ve %0.65 agar ile 5,8 pH içeren besi ortamı kullanılmıştır. Eksplantlar 0.10-1.60 mg/L Thidiazuron (TDZ) veya 0.10-1.60 mg/L TDZ+ 0,1 mg/L Indole-3-butyric acid (IBA) içeren besi oratmlarında kültüre alınarak yedi hafta boyunca soğuk beyaz floresan lambalar ile desteklenmiş 16/8 saat ışık fotoperyotında bekletilmiştir. Daha sonra eksplantlar her hangi hormone içeremeyen MS besi ortamına alt kültüre alınarak benzer koşullarda beş hafta boyunca bekletilmiştir. Sürgün rejenerasyon yüzdesi 100 olurken kallus rejenerasyon yüzdesi ise %42,85-100,00% arasında kaydedilmiştir. Sürgün sayısı ve sürgün uzunluğu ise sırasıyla 3,43-19,40 ve 1,04-2,74 cm olarak kaydedilmiştir. En fazla sürgün sayısı (19,40) ve sürgün uzunluğu (2,74 cm) sırasıyla 0,20 mg/L TDZ ve 0,20 mg/L TDZ+0,10 mg/L IBA içeren ortamında elde edilmiştir. Genel olarak IBA içeren ortamlarında düşük oranda köklendirme kaydedilirken, saksılarda aktarıldığında da düşük oranda adaptasyon sağlanmıştır.

Anahtar Kelimeler: Bishop otu, in vitro, kotiledon boğum, tidiazuron

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1. Introduction

Bishop's seed (*Trachyspermum ammi* L.) with common name of Ajowan, Ajwain, Carom or Ethopian cumin (Ashraf and Orooj, 2006) is one of the significant neutraceutial spice and medicinal shrub of Apiaceae or Umbelliferae family (Jeet et al., 2012). It is widely cultivated herb mainly on saline soils of arid and semi-arid regions of Central, South and West Asia (Moosavi et al., 2015). The plant is normally tall (60-90 cm) having branched structure. The flowers bloom from month of July upto September and bear small sized fruit having relatively bitter pungent taste (Sharifi- Rada et al., 2013; Koca and Aasim, 2015). The oil of seeds contain essential oils having thymol as major component (Ashraf and Orooj, 2006; Bairwa et al., 2012; Moosavi et al., 2015) and used for treating diseases and disorders like anti-inflammatory (Thangam and Dhananjayan, 2003), antifilarial (Mathew et al., 2008), antilithiasis (Ramaswamy et al., 2010), bronchitis (Singh et al., 2003), digestive stimulant (Platel and Srinivasan, 2001), and stomach disorders (Jain et al., 2011). Bishop's weed is used as aflatoxin and detoxificant in perfumery and preservative in foods (Velazhahan et al., 2010), nematicide, (Kwon et al., 2007) and fungicide (Ashraf and Orooj, 2006).

Medicinal plants are highly significant for humans due to containing wide array of bioactive compounds used in different areas. These medicinal plants are gaining popularity in recent years and have been used as raw or in processed form in both traditional and modern pharmaceutical industry (Niazian et al., 2017a). The application and safe usage of organic materials in pharmaceutical industries replacing synthetic materials enhance the significance of medicinal plants (Moghaddam et al., 2015). Some of these medicinal plants also used as spice, flavor and perfume industries, which enhance the economic importance of such medicinal plants. However,

the major issue of such medicinal plants is relatively low production with quality issues (Nomani et al., 2021). In order to cope the demand with high quality compounds, biotechnologiccal techniques have been employed (Tripathi and Tripathi, 2003; Nomani et al., 2021). Among these techniques, plant tissue culture is major and these techniques are the most effective for the manipulation of significant seccondary metabolites. In recent years, *in vitro* propagation of Bishop's weed have been developed for callus induction, somatic embryogenesis, direct or indirect shoot induction (Koca and Aasim 2015; Niazian et al 2017b; Teymourian et al., 2017; Nomani et al., 2021). However, there is always need of new *in vitro* regeneration protocols for medicinal plants. This study presents the response of cotyledonary nodes explant cultured on mediums enriched with TDZ-IBA for in vitro shoot proliferation of Bishop's weed.

2. Materials and Method

The seeds of Bishop's weed were purchased from Pakistan and seeds were placed in water in order to remove floated seeds followed by surface sterilization process reported by Koca and Aasim (2015). Surface sterilized seeds were inoculated on MS (Murashige and Skoog, 1962) medium without any PGRs for germination. Cotyledonary node explants used in this study were isolated from two weeks old *in vitro* germinated seedlings under sterile conditions. The isolated explants were transferred immediately to MS medium enriched with variable concentrations (0.10, 0.20, 0.40, 0.80 and 1.60 mg/L) of Thidiazuron (TDZ) used alone or with combination of 0.10 mg/L of Indole-3-butyric acid (IBA). The explants were initially cultured for seven weeks followed by subculture to MS medium without TDZ-IBA for five more weeks. The medium used in this study for *in vitro* regeneration and rooting medium was prepared by adding 0.44% MS medium (Duchefa), 3.0% sucrose (Duchefa), 0.65% agar (Duchefa) followed by adjusting the pH between 5.6–5.8. All cultures (regeneration and rooting) were incubated in the growth room equipped with cool-white fluorescent lamps for 16/8-h (light/dark) photoperiod and temperature of 24° ± 1º C.

For rooting, healthy and normal shoots (not showing hyperhydricity) were used for *in vitro* rooting. Shoots were isolated under sterile conditions followed by immidiate transfer to MS rooting medium provided with 0.2, 0.4, 0.60, 0.80 and 1.0 mg/L IBA for three weeks. In vitro rooted plantlets were transferred to pots containg peat moss. The pots were wrapped with polyethylene bags for moisture conservation and placed in the growth room at 23±1 °C. After one week, holes were made in the bags for 3-4 days followed by complete opening of pots and exposed to growth room conditions.

The experiment was carried out in three replication with seven explants per replication having 10 different PGRs type and concentration. The data regarding shoot induction frequency (%), callus induction frequency (%), shoot counts and shoot length (cm) were taken after twelve weeks of culture and analysis of variance (ANOVA) was done using SPSS 20.00 for Windows. Post hoc tests using Duncan's multiple range test (DMRT) was used for comparing means difference among PGRs treatments. Prior to statistical analysis, data was transformed into Arcsine square root transformation (Snedecor and Cochran, 1967).

3. Results and Discussion

Callus induction and shoot induction from cotyledonary node explant started simultaneously within two weeks of culture irrespective of PGRs type, concentration and combination. However, extensive callus induction was observed after four weeks of culture (Fig 1a) alongwith multiple shoot induction. After seven weeks of culture (Fig 1b), explants were sub-cultured to MS medium devoid of PGRs for next five weeks for shoot proliferation (Fig 1c). After twelve weeks of total culture, data regarding callus induction frequency (%), shoot induction frequency (%), shoot counts and shoot length (cm) were recorded. Shoot induction frequency (%) was recorded 100% and hence not subjected to statistical analysis. On the other hand, callus induction frequency (p<0.01), shoot counts (p<0.05) and shoot length (p<0.01) were statistically significant to different concentration of TDZ-IBA.

Table 1. Impact of TDZ-IBA concentration on callus induction (%) from cotyledonay node explant of *T. ammi*

TDZ (mg/L)	Callus Regeneration Frequency (%)	TDZ-IBA (mg/L)	Callus Regeneration Frequency (%)
0.10	85.71^{ab}	0.10+0.10	71.42^{bc}
0.20	71.42^{bc}	0.20+0.10	71.42 ^{bc}
0.40	100.00^{a}	0.40+0.10	42.85^{d}
0.80	42.85^{d}	0.80+0.10	85.71^{ab}
1.60	57.14^{cd}	1.60+0.10	52.38c^{d}

Means followed by different small letters within columns are significantly different using DMRT test at P<0.01

Table 2. Impact of TDZ-IBA concentration on shoot counts from cotyledonay node explant of *T. ammi*

TDZ (mg/L)	Shoot Counts	TDZ-IBA (mg/L)	Shoot Counts
0.10	4.30^{d}	0.10+0.10	4.00^{d}
0.20	19.40^{a}	0.20+0.10	6.45^{c}
0.40	7.29^{bc}	0.40+0.10	8.25^{b}
0.80	7.43^{bc}	0.80+0.10	8.00^{b}
1.60	3.43^{d}	1.60+0.10	4.00^{d}

Means followed by different small letters within columns are sigificantly different using DMRT test at P<0.05

Table 3. Impact of TDZ-IBA concentration on shoot length (cm) from cotyledonay node explant of *T. ammi*

TDZ (mg/L)	Shoot Length (cm)	TDZ-IBA (mg/L)	Shoot Length (cm)
0.10	2.08^{c}	0.10+0.10	1.57^{d}
0.20	1.49^{d}	0.20+0.10	2.74^{a}
0.40	1.52^{d}	0.40+0.10	1.51^{d}
0.80	1.50^{d}	0.80+0.10	1.58^{d}
1.60	2.39^{b}	1.60+0.10	1.04^{e}

Means followed by different small letters within columns are sigificantly different using DMRT test at P<0.01

The nature/type of explant is highly significant for *in vitro* shoot induction and proliferation and cotyledonary node explant is very potent explant reported for various important crops (Chaudhary et al., 2007; Kendir et al., 2008; Teymourian et al., 2017). The results revealed the supermacy of cotyledonary node explant and recorded 100.00%. The other studies on this plant also revealed relatively high shoot regeneration using different explants and PGRs. The previous study on *T. ammi* using seed explant cultured on TDZ-IBA containing medium also yielded high shoot regeneration frequency that was recorded in the range of 77.77-94.44 % (Koca and Aasim,

2015). Shoot regeneration frequency of 44-100% was reported in *T. copticum* using cotyledonary node explant (Teymourian et al., 2017). Whereas, 41.1-92.6% shoot regeneration frequency was reported from zygotic embryos explant of *T. ammi* (Nomani and Tohidfar, 2021). Callus inducion is an important parameter of *in vitro* regeneration studies and dependant mainly on type and combination of PGRs along with plant and explant. Results on callus induction frequency (%) revealed the clear impact of TDZ-IBA concentration. Results also reveal the variable impact of TDZ or TDZ+IBA concentration on callus induction and recorded 43.85-100% and 42.85-71.42% respectively (Table 1). The highest callus induction frequency (100 %) was observed on MS medium enriched with 0.40 mg/L TDZ. On the other hand, minimum callus induction frequency (42.85 %) was recorded on 0.80 mg/L TDZ and 0.40 mg/L TDZ+0.10 mg/L IBA. 13.88-80.00 %. Callus induction in response to different PGRs (combination of auxin and cytokinin) has been reported from hypocotyl explant of *C. coptimum* (Niazian et al., 2017b) cotyledonary node explant of *T. copticum* (Teymourian et al 2017), hypocotyl (Nomani et al., 2021) and zygotic embryos (Nomani and Tohidfar, 2021) of and *T. ammi*.

The provision of different concentrations of TDZ alone or TDZ-IBA exerted significant impact on shoot counts. Shoot counts on MS medium supplemented with TDZ alone yielded 3.43-19.40 shoots per explants (Table 2). The maximum shoot counts of 19.40 was documented on MS medium supplemented with 0.20 mg/L. However, a sharp decline on shoot counts was recorded thereafter with elevated TDZ concentration and minimum shoot counts was documented on MS medium supplemented with 1.60 mg/L TDZ. The negative impact of elevated TDZ concentration on shoot counts have been reported in Hungarian vetch (Sahin-Demirbag et al., 2008) and *T. ammi* (Koca and Aasim, 2015). On the other hand, combination of TDZ-IBA yielded 4.00-8.5 shoots per explant with maximum shoot counts (8.25) were recorded on Ms medium enriched with 0.40 mg/L TDZ+0.10 mg/L IBA. The study by Teymourian et al (2017) revealed shoot count ranged 1.1-3.5 (shoots per explant) from different culture medium with maximum from medium containing no PGR. Shoot length of *in vitro* regenerated shoots was also showed the clear impact of TDZ-IBA concentration. Shoot length on MS medium containing different concentration of TDZ ranged 1.49-2.39 cm with longer shoots were attributed to 1.60 mg/L TDZ followed by 0.10 mg/L TDZ (Table 3). The general perception about high TDZ concentration is the inhibitory impact on shoot length (Karataş and Aasim 2014). On the other hand, TDZ-IBA containing medium yielded shoot length of 1.04-2.64 cm with longer shoots were attained on MS medium inoculated with 0.40 mg/L TDZ+0.10 mg/L IBA. The study carried out by Teymourian et al. (2017) revealed shoot length range of 0.5-2.8 cm with longer shoots from medium containing no PGR.

In vitro regenerated shoots were shifted to MS medium provided with IBA induced relatively low rooting frequency due to induction of hyperhydric shoots in response to TDZ. The induction of hyperhydric shoots due to high concentration of TDZ or cytokinin is reported in most of the plants (Aasim et al., 2011). The study presented by Niazian et al. (2017b) revealed the massive rooting of shoots of *C. copticum* when cultured on ½ MS medium having 0.1 mg/L NAA. Whereas, Nomani et al. (2021) reported root induction of *T. ammi* plantlets inoculated on Ms medium enriched with 0.57 µm/L IAA. The rooted plantlets in this study failed to adapt in the pots containing peat moss. The previous study by Koca and Aasim (2015) on *T. ammi* revealed the successful rooting and adaptation by first done hardening of the shoots on MS medium enrihed with different sucrose cocentration followed by acclimatization of shoots in the growth chamber under controlled conditions. Similarly, high adaptation rate of approximately 60.00% was reported by Niazian et al. (2017b). Similarly, successful aclimatization have been reported for *T. ammi* plantlets by (Nomani and Tohidfar, 2021).

Development of *in vitro* regeneration protocol either through direct or indirect regeneration is highly significant for practicing the biotechnological techniques like genetic transformation, isolation of secondary metabolites. The results of this study reveal the high shoot regeneration and callus induction frequency with high shoot counts and this protocol can be employed for future biotechnological studies.

Conflict of Interest

Authors have declared no conflict of interest.

Authors' Contributions

The authors contributed equally.

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Accumulation of cadmium, copper and zinc in selected natural *Viola* taxa in Turkish Mediterranean serpentine soils

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Türkiye Akdeniz serpantin topraklarında seçilmiş doğal *Viola* taksonlarının kadmiyum, bakır ve çinko akümülasyonu

Abstract: Currently, 55 serpentine areas have been determined in Turkey. More than 60 Ni-accumulators and more than 43 serpentine-endemics are known from these areas. Within our field studies in the Mediterranean phytogeographic region, 8 *Viola* taxa distributed in serpentine areas and their respective soil samples were collected. Cd, Cu, and Zn concentrations of the soil and plant samples were investigated. After the plant and soil samples were digested in the microwave, metal measurements were made using a ICP-OES device. As a result of the Cd, Cu, and Zn measurements, none of the 8 *Viola* taxa collected from different localities were found to be hyperaccumulators. However, it was determined that *Viola kizildaghensis* has an accumulator feature in terms of Zn.

Key words: accumulator, serpentine soil, *Viola* taxa, Zinc

Özet: Ülkemizde 55 serpantin alan belirlenmiş ve bu alanlarda 60'dan fazla Ni akümülatörü ve 43'den fazla sayıda serpantin endemiği olduğu tespit edilmiştir. Akdeniz fitocoğrafik bölgesinde yapmış olduğumuz arazi çalışmalarında serpantin alanlarda yayılış gösteren 8 *Viola* taksonu ve bunların yetiştiği toprak örnekleri toplandı. Toplanan 8 *Viola* taksonunun yetiştiği topraklar ve bitkilerdeki Cd, Cu, ve Zn konsantrasyonları araştırılmıştır. Bitki ve toprak örnekleri mikrodalgada çözüldükten sonra metal ölçümleri ICP-OES cihazında yapılmıştır. Farklı lokalitelerden toplanan 8 *Viola* taksonunda yapılan Cd, Cu, ve Zn ölçümleri sonucunda hiçbir bitkide hiperakümülatör özelliği tespit edilememiştir. Ancak, *Viola kizildaghensis* bitkisinin Zn yönünden akümülator özelliğinin olduğu belirlenmiştir.

Anahtar Kelimeler: akümülatör, serpantin topraklar, *Viola* taksonları, çinko

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1. Introduction

It is becoming increasingly important to use hyperaccumulator or accumulator plants in stabilizing environments contaminated with heavy metals. Among heavy metals, Cd, Cu, and Zn are toxic elements that are easily taken up by plants and translocated to different parts of the plant.

Some of the earlier dry matter concentration thresholds for hyperaccumulation of trace elements in terrestrial plants have been revised recently. Recommendations by Krämer (2010), van der Ent et al. (2013), Remigio et al. (2020) and Peng et al. (2020), for plants that are growing in their natural habitat, are as follows: Cd > 100 mg kg^{-1}; Cu 300 mg kg^{-1}; Zn 3,000 mg kg^{-1}. The global hyperaccumulator database (www.hyperaccumulators.org) includes 721 hyperaccumulators, and their number is growing. Most hyperaccumulators species (532 species) are identified as Ni hyperaccumulators, while hyperaccumulators of Cd (7 species) and As (5 species), which currently pose the greatest threat to human health, are notably scarce (Reeves et al., 2018). At the same time, hyperaccumulator plants must manage to grow and to survive in an environment rich in metals.

Many studies have been carried out about serpentine areas in Turkey. Until now, 55 serpentine areas have been examined in Turkey and 62 Ni-accumulators and more than 43 serpentine-endemics have been reported from these areas (Reeves, 1998; Reeves et al., 1983, 2001, 2009; Reeves and Adıgüzel, 2004, 2008; Adıgüzel and Reeves, 2012; Altınözlü et al., 2012; Aksoy et al., 2015; Çelik et al., 2018). According to the Flora of Turkey, it is reported that there are 248 plants specific to serpentine areas, of which 119 are serpentinophytes and 129 are serpentinophages (Kurt et al., 2013; Özdeniz et al. 2017). According to Kurt et al. 2013 and Özdeniz et al., 2017, *Viola* species were not recorded in the serpentine flora of Turkey.

The hyperaccumulator/accumulator properties of some *Viola* species have been investigated by various researchers. Hyperaccumulator/accumulator properties have been determined for *Viola calaminaria* (Ging.) Nauenb., *V. guestphalica* Nauenb. (Zn and Pb), *V. baoshanensis* W.S.Shu, WLiu & CY.Lan (Cd), *V. principis* H.Boissieu (As, Cd and Pb), *V. kopaonikensis*, Pančić ex Tomović & Niketić, *V. elegantula* Schott and *V. beckiana* F.Fiala ex Beck (Ni, Cd, and Pb), as well as *V. arsenica* Beck, *V. allcharensis* G.Beck and *V. macedonica* Boiss. & Heldr. (As, Sb and Tl) (Brooks, 1998; Jedrzejczyk et al., 2002; Liu et al., 2004; Lei et al., 2008; Stevanović et al., 2010; Bačeva et al., 2014: Tomović et al., 2021).

The genus *Viola* L. is a member of the family *Violaceae* and is represented by approximately 600 taxa in the World (Marcussen et al., 2015; Düşen et al., 2018). In Turkey the genus *Viola* is represented by 36 taxa, of which 14 are endemic (Coode and Cullen, 1965; Davis et al., 1988; Yıldırımlı, 2000; Dinç, 2012; Adıgüzel and Reeves, 2012; Knoche and Marcussen, 2016; Düşen et al., 2018).

According to the current literature, there is no study that investigated the hyperaccumulator/accumulator properties

of *Viola* taxa from Turkey. In this study, 8 *Viola* taxa were collected from serpentine areas of the Mediterranean Region and Cd, Cu and Zn concentrations of the soils in which these plants grow were determined. The aim of this study was to investigate whether some of the *Viola* taxa studied have hyperaccumulator/accumulator properties for Cd, Cu and Zn.

2. Materials and Methods

2.1. Materials

Soil samples and specimens of the investigated 8 *Viola* taxa were collected from serpentine areas in the Mediterranean phytogeographical region in 2016 (Table 1). For each taxon, three soil samples (24 in total) were collected from the areas where the *Viola* taxa (Table 1) were growing. Plant and soil samples were collected in 3 replicates for heavy metal analysis. Further, the collected specimens were stored in the Herbarium of Akdeniz University.

2.2. Sampling

Soils were taken from the 0-5 cm zone, brought to the laboratory, sieved with a standard 4 mm sieve and were than air-dried. At least 15-50 adult plants were randomly selected and collected from each site. Two to ten plants were retained as herbarium specimens. The remaining other plants from each site were divided into above- and below-ground parts. These were transferred to the laboratory in plastic bags. Plant samples were washed with tap water, followed by deionized water. They were dried in an oven (80 °C) until they reached constant weight and brittleness. The samples were subsequently ground with a pestle and mortar. Homogenized plant materials and soil samples were then stored in clean paper bags before heavy-metal analysis.

2.3. Chemical and statistical analyses

Soil samples (0.5 g dry weight) were digested with 10 ml of pure HNO_3 (65%), using a CEM-MARS 5 microwave digestion system (digestion conditions were the following: maximum power 1200 W; power 100%; ramp time 20 min, pressure 180 psi; temperature 180 °C; and hold time 10 min). After digestion, the volume of each sample was adjusted to 25 mL by using double-deionized water. Homogenized plant samples (0.5 g dry weight) were also prepared using the same procedure as for heavy-metal analysis. The soil and plant samples were analyzed for Cd, Cu, and Zn by ICP-OES; Varian-Liberty II. All chemicals were of analytical reagent grade. Standard peach leaves (NIST, SRM-1547) were used as a reference material. All analytical procedures were performed using this reference material. Soil and plant samples were digested in triplicate and analyzed. The means and standard deviation (SD) of the data were calculated using SPSS v15.0 (SPSS Inc., Chicago, IL, USA).

Table 1. Collecting sites and protection categories of *Viola* taxa.

No	Collected species	Turkish name	Collecting places	IUCN
1	*Viola alba* Besser subsp. *dehnhardtii* (Ten.) W. Becker	Meşe menekşesi	B5: Kayseri, Yahyalı Çamlıca village, Kayapınar location, 1410m, 11.08.2016, *Aksoy2645*	-
2	*V. dirimliensis* Blaxland	Dirmil menekşesi	C2: Burdur-Altınyayla, Dirmil Pass,1630 m, 21.04.2016. *Aksoy 2569*	CR
3	*V. heldreichiana* Boiss.	Gök menekşe	B5: Kayseri, Yahyalı Çamlıca village, Kayapınar location, 1410m, 04.04.2016, *Aksoy 2605*	
4	*V. kitaibeliana* Roem. & Schult.	Yabani menekşe	B5: Kayseri, Yahyalı Çamlıca to Ulupınar village, 2 km from Ulupınar village 1370 m, 04.04.2016, *Aksoy 2608*	-
5	*V. kizildaghensis* Dinç & Yıld.	Pembe menekşe	C3: Konya-Derebucak, Çamlık village, Kızıldağı, Akçeşme location 1450n, 26.00.2016. *Aksoy 2642*	CR
6	*V. modesta* Fenzl	Sahra menekşesi	C4: Konya-Bozkır, Üçpınar village, Tufan Deresi,1990, 28.04.2016. *Aksoy 2581*	-
7	*V. sandrasea* Melch. subsp. *sandrasea*	Sandras menekşesi	C2: Muğla-Köyceğiz, Sandras Dağı 1830 m, 21.04.2016. *Aksoy 2563*	EN
8	*V. suavis* M. Bieb.	Akgöz menekşe	C5: Hatay- Arsuz, above the village of Kale, Kızıldağ, 17.04.2016 *Aksoy 2586*	-

3. Results and Discussion

The minimum, maximum and mean concentrations of Cd, Cu, and Zn in the investigated soils and aboveground and underground parts of the *Viola* taxa are given in Table 2 and the mean concentrations of Cd, Cu, and Zn are further shown in Figure 1.

While the Cd concentrations ranged between 3.40-33.75 mg kg^{-1} in the studied soils, it was found that the values varied between 0.15-1.86 mg kg^{-1} in the underground parts and between 0.68-1.71 mg kg^{-1} in the aboveground parts of the *Viola* taxa. The highest Cd concentration (33.75 mg kg^{-1}) was detected in the soils where *V. heldreichiana* grew and the lowest Cd concentration (3.40 mg kg^{-1}) was found in the soils where *V. modesta* samples grew (Table 2). It has been reported that the Cd concentration of *V. baoshanensis*, which grows in the Baoshan lead / zinc mine field in Hunan, China, varies between 456-2310 mg kg^{-1} in the aboveground parts and between 233-1846 mg kg^{-1} in the underground parts (Wei et al., 2004).

Cu concentrations ranged between 7.51-33.30 mg kg^{-1} in the studied soils, between 1.21-6.31 mg kg^{-1} in the underground parts and between 2.57-4.69 mg kg^{-1} in the aboveground parts of the investigated *Viola* taxa. The highest Cu concentration (30.85 mg kg^{-1}) was detected in the soils where the sampled *V. heldreichiana* grew while the lowest Cu concentration (7.51 mg kg^{-1}) was found in the soils where *V. kizildaghensis* grew (Table 2).

While the Zn concentrations ranged between 5.65-95.25 mg kg^{-1} in the studied soils, Zn concentrations for the investigated *Viola* taxa varied between 4.65-69.25 mg kg^{-1} in the underground parts and between 12.15-64.65 mg kg^{-1} in the aboveground parts. The highest Zn concentration (33.30 mg kg^{-1}) was detected in the soils where the investigated *V. heldreichiana* samples grew and the lowest

Zn concentration (9.68 mg kg^{-1}) was found in the soils where *V. sandrasea* subsp. *sandrasea* samples grew (Table 2).

The investigated Cd and Cu concentrations were found to be higher in the soil compared to the underground and aboveground parts of the plants. However, Zn concentrations in *V. dirimliensis*, *V. kizildaghensis*, *V. suavis* and *V. sandrasea* subsp. *sandrasea* were found to be higher, both in the underground and aboveground parts of the plant, than the concentrations of the soil samples (Fig. 1).

Table 2. Minimum, maximum and mean concentration of Cd, Cu, and Zn in underground and aboveground parts of the investigated *Viola* species and soils collected from serpentine areas in the Mediterranean region of Turkey (mg kg^{-1} dry weight ± SD).

		Soil			Underground			Aboveground		
Plant species	Elements	**Cd**	**Cu**	**Zn**	**Cd**	**Cu**	**Zn**	**Cd**	**Cu**	**Zn**
V. alba* subsp. *dehnhardtii	min	10,68	16,82	30,94	1,44	5,74	42,99	1,00	2,95	23,72
	max	10,83	16,84	31,40	1,50	5,76	43,35	1,03	2,98	23,80
	mean	**10,75**	**16,83**	**31,17**	**1,47**	**5,75**	**43,17**	**1,02**	**2,96**	**23,76**
	SD	±0,11	±0,01	±0,32	±0,04	±0,01	±0,25	±0,02	±0,02	±0,06
V. dirimliensis	min	7,77	10,24	13,31	0,97	2,12	15,78	0,93	2,57	19,13
	max	7,80	10,35	13,49	1,06	2,16	15,80	0,96	2,60	19,49
	mean	**7,78**	**10,30**	**13,40**	**1,01**	**2,14**	**15,79**	**0,94**	**2,58**	**19,31**
	SD	±0,02	±0,08	±0,13	±0,06	±0,30	±0,01	±0,02	±0,02	±0,26
V. heldreichiana	min	33,65	32,70	94,45	0,15	1,20	4,65	1,61	3,50	38,20
	max	33,75	33,30	95,25	0,17	1,30	4,75	1,71	3,55	40,15
	mean	**33,70**	**32,91**	**94,88**	**0,16**	**1,25**	**4,71**	**1,66**	**3,51**	**38,90**
	SD	±0,13	±0,30	±0,40	±0,02	±0,05	±0,05	±0,32	±0,02	±0,98
V. kitaibeliana	min	30,51	23,70	86,05	0,57	2,30	17,15	1,44	3,05	34,10
	max	30,85	24,35	88,25	0,59	2,35	17,40	1,50	3,15	34,85
	mean	**30,68**	**24,00**	**86,96**	**0,58**	**2,31**	**17,25**	**1,47**	**3,10**	**34,56**
	SD	±0,36	±0,32	±1,10	±0,02	±0,26	±0,12	±0,12	±0,05	±0,37
V. kizildaghensis	min	11,46	7,51	29,67	1,84	5,05	68,25	1,08	2,77	64,36
	max	11,49	7,52	29,96	1,86	5,11	69,25	1,10	2,75	64,62
	mean	**11,47**	**7,52**	**29,82**	**1,85**	**5,08**	**68,75**	**1,09**	**2,76**	**64,49**
	SD	±0,27	±0,01	±0,21	±0,01	±0,05	±0,70	±0,16	±0,01	±0,18
V. modesta	min	3,40	10,72	35,50	1,11	5,17	17,50	1,11	4,04	21,20
	max	3,45	10,83	35,76	1,15	5,19	17,53	1,14	4,05	21,29
	mean	**3,42**	**10,78**	**35,63**	**1,13**	**5,18**	**17,53**	**1,12**	**4,04**	**21,25**
	SD	±0,04	±0,08	±0,18	±0,03	±0,01	±0,02	±0,02	±0,01	±0,06
V. sandrasea* subsp. *sandrasea	min	6,89	9,68	5,65	1,31	6,27	26,47	0,68	3,92	12,15
	max	6,93	9,76	5,87	1,33	6,31	26,91	0,70	3,96	12,48
	mean	**6,91**	**9,72**	**5,76**	**1,32**	**6,29**	**26,69**	**0,69**	**3,94**	**12,32**
	SD	±0,03	±0,06	±0,16	±0,01	±0,03	±0,32	±0,01	±0,03	±0,23
V. suavis	min	9,37	12,92	25,85	1,20	5,49	45,36	0,92	4,64	33,03
	max	9,55	13,17	26,09	1,22	5,51	45,85	0,94	4,69	33,43
	mean	**9,46**	**13,05**	**25,97**	**1,21**	**5,50**	**45,61**	**0,93**	**4,66**	**33,23**
	SD	±0,13	±0,18	±0,17	±0,01	±0,02	±0,34	±0,012	±0,04	±0,28

Among the eight *Viola* taxa studied, Zn concentrations of *V. dirimliensis* were highest in the aboveground (19.31 mg kg^{-1}) and slightly lower in the underground parts (15.79 mg kg^{-1}) and in the soil (13.40 mg kg^{-1}). The Zn concentrations in the aboveground (64.49 mg kg^{-1}) and underground (68.75 mg kg^{-1}) parts of *V. kizildaghensis* were found to be twice as high as in the soils (29.82 mg kg^{-1}). The fact that the Zn concentration in the aboveground parts of *V. kizildaghensis* is markedly higher than in the soil where the plants grew indicates that *V. kizildaghensis* has a Zn accumulator feature. Stevanovic et al. (2010) and Bačeva et al. (2014) examined whether some *Viola* species in the Alchar region of Macedonia are hyperaccumulators for several heavy metals including Zn. They found that the *Viola* species studied had an accumulator feature for Zn.

Different ecosystems in many parts of the world have been contaminated by various heavy metals such as Cd, Cr, Cu, Ni, Pb, Zn. In recent years, hyperaccumulator or accumulator plants have been used as a model in the cleaning of these contaminated environments, and thus to reduce this pollution (Aksoy and Öztürk, 1997; Baker et al., 2000; Prasad, 2005).

Many studies have been carried out about serpentine areas in Turkey with 62 Ni-hyperaccumulator plants being recorded in these areas. Most of these plants belong to the *Brassicaceae* and *Asteraceae* family. According to these studies, no plant species with Cd, Cu and Zn hyperaccumulator properties were found in Turkey. (Reeves, 1988; Reeves et al., 1983, 2001, 2009; Reeves and Adıgüzel, 2004, 2008; Adıgüzel and Reeves, 2012; Altınözlü et al., 2012; Aksoy et al., 2015; Çelik et al., 2018).

A large number of hyperaccumulators in *Viola* species have been reported in Asian and European countries, for example, *V. baoshanensis* for the hyperaccumulation of cadmium (Liu et al., 2004; Tonin et al., 2001) and *V. calaminaria* (Tonin et al., 2001) and *V. lutea* (Sychta et al., 2018) for the hyperaccumulation of zinc. In their study on *V. boashanensis* in China, Wu et al. (2010) found Cd concentrations of 1090 mg kg^{-1}, Pb concentrations of 1902

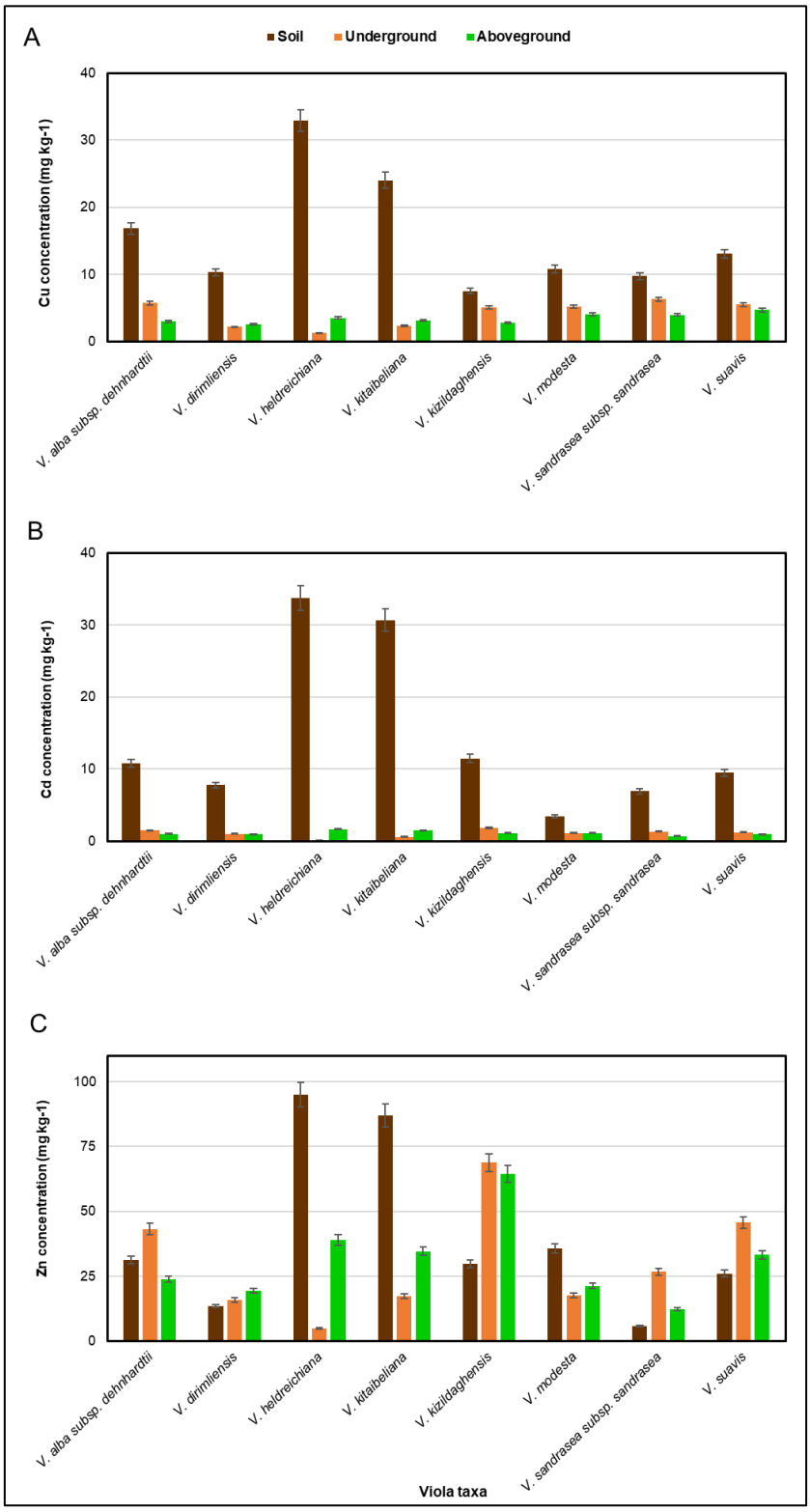

Figure 1. Mean concentrations of Cd (**A**), Cu (**B**) and Zn (**C**) in the soil and the aboveground and underground parts of the *Viola* taxa, together with standard errors (S.E.).

mg kg^{-1}, and Zn concentration of 3428 mg kg^{-1}. They reported that *V. boashanensis* was a hyperaccumulator plant for Pb and Zn as well as for Cd. As a result of this study, no hyperaccumulator properties could be detected for the investigated *Viola* taxa. However, it has been determined that *V. dirimliensis*, *V. kizildaghensis*, *V. suavis* and *V. sandrasea* subsp. *sandrasea* accumulate zinc to a certain extent.

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