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Owner
Prof. Dr. Abdullah KAYA

Corresponding Address
Gazi University, Science Faculty, Department of Biology, 06500, Ankara – Türkiye
Phone: (+90 312) 2021235
E-mail: anatolianjbot@gmail.com
Web: http://dergipark.gov.tr/ajb

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Differential regulation of antioxidant enzymes by resveratrol in healthy and cancerous hepatocytes

Aykut BOSTANCI[1], Esma Nur ŞENCAN[2], Ayşegül KÜTÜK[3], Gökhan SADİ[4]*
[1-4]*Karamanoğlu Mehmetbey University, Kamil Özdağ Science Faculty, Department of Biology, Karaman, Türkiye*
**sadi@kmu.edu.tr*, [1]*aykutbostanci22@gmail.com*, [2]*esmanursencan@gmail.com*, [3]*aysegulktk@hotmail.com.tr*

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Sağlıklı ve kanserli hepatositlerde antioksidan enzimlerin resveratrol tarafından farklı düzenlenmesi

Abstract: Resveratrol, mainly found in grapes, blueberries, raspberries, mulberries, and peanuts, is a naturally synthesized polyphenol that protects against several pathological issues. Increased oxidative stress in cancer cells and suppressed cellular antioxidant systems have been investigated and therefore herbal antioxidants could be used as an auxiliary substance in the treatment of several types of cancer. In this study, we inspected the effects of resveratrol, a powerful antioxidant, in the gene expression and activities of antioxidant enzymes in healthy and cancerous liver cells. After the liver cancer cells (HepG2) and healthy hepatocytes (THLE2) were treated with resveratrol at different doses (25-, 50-, 75- µM) for 48-hours, the gene expression levels of antioxidant enzymes were measured by qRT-PCR and their activities were measured spectrophotometrically. The results showed the suppression of all antioxidant enzymes in the THLE2 cells at all doses of resveratrol but conversely up-regulation of those in HepG2 cells. The reduction in gene expression in healthy cells was in line with the suppression of enzymatic activities of catalase and glutathione S-transferase, however, the activity changes in cancer cells were not significant. This differential regulation of antioxidant enzyme systems in healthy and cancerous hepatocytes demonstrates the stimulation of antioxidant enzymes in cancer cells as a protective function against oxidative damage, while decreased expression in healthy cells signifies the reduction of requirement for antioxidants, conversely. Therefore, resveratrol might be effective in cancer treatment through affecting the molecular mechanisms that can reduce cellular stress by activating antioxidant enzymes in cancer cells.

Key words: Resveratrol, antioxidant enzymes, gene expression, oxidative stress

Özet: Üzüm, yaban mersini, ahududu, dut ve yer fıstığı gibi bitkilerde bulunan resveratrol, çeşitli patolojik sorunlara karşı koruma sağlayan doğal olarak sentezlenmiş bir polifenoldür. Kanser hücrelerinde oksidatif stresin artışı ve hücresel antioksidan sistemlerin baskılanması belirlenmiş olup, bitkisel antioksidanların çeşitli kanser türlerinin tedavisinde yardımcı madde olarak kullanılabileceği öngörülmektedir. Bu çalışmada, güçlü bir antioksidan olan resveratrolün sağlıklı ve kanserli karaciğer hücrelerinde antioksidan enzimlerin gen ekspresyonu ve aktiviteleri üzerindeki etkileri araştırılmıştır. Karaciğer kanseri hücreleri (HepG2) ve sağlıklı hepatositler (THLE2) resveratrol ile farklı dozlarda (25-, 50-, 75- µM) 48 saat süreyle muamele edildikten sonra antioksidan enzimlerin gen ekspresyon seviyeleri qRT-PCR ile, aktiviteleri ise spektrofotometrik olarak ölçülmüştür. Sonuçlar, THLE2 hücrelerindeki tüm antioksidan enzimlerin resveratrolün tüm dozlarında baskılandığını, ancak bunun tersine HepG2 hücrelerinde antioksidan enzimlerin yukarı regülasyonunu göstermiştir. Sağlıklı hücrelerde görülen gen ekspresyonundaki azalma, katalaz ve glutatyon S-transferazın enzimatik aktivitelerinin baskılanmasıyla paralel değiştiği, ancak kanser hücresindeki aktivite değişikliklerinin anlamlı olmadığı belirlenmiştir. Sağlıklı ve kanserli hepatositlerde antioksidan enzim sistemlerinin farklı olarak düzenlemesi, kanser hücrelerinde antioksidan enzimlerin oksidatif hasara karşı koruyucu bir işlev olarak uyarılmasını gösterirken, sağlıklı hücrelerde azalmış ekspresyon, tersine antioksidan ihtiyacının azalmasına işaret etmektedir. Bu nedenle resveratrol, kanser hücrelerinde antioksidan enzimleri aktive ederek hücresel stresi azaltabilecek moleküler mekanizmaları etkileyerek kanser tedavisinde etkili olabilir.

Anahtar Kelimeler: Resveratrol, antioksidan enzimler, gen ekspresyonu, oksidatif stres

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1. Introduction

Free radicals, due to the unpaired electrons in their structure, are unstable and more energetic than non-radical molecules and so they deteriorate the structures of biomolecules such as lipids, carbohydrates, proteins, and nucleic acids (Studer and Curran, 2016). An imbalance between the accumulation of reactive species and their rate of removal is defined as oxidative stress which is considered as the main contributor to cellular pathologies (Sadi and Sadi, 2010). In the cells, several enzymatic and non-enzymatic antioxidant systems reduce or eliminate the stress conditions caused by elevated oxidative stress. The body's principal enzymatic defense mechanisms are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione-S-transferase (GST) (Sadi and Sadi, 2010). Besides, there are also molecular antioxidants such as glutathione, vitamin C, carotenoids, and vitamin E that form non-enzymatic defense systems. Free radicals and oxidative stress play effective roles in many disease conditions such as aging, atherosclerosis, ischemia-reperfusion, inflammation, rheumatoid arthritis, neurodegenerative diseases, cancer, and liver diseases (Khan et al., 2010; Abdul-Rahman et al., 2012; Klaunig, 2018). The development of hepatocellular carcinoma which

is the most common form of liver cancer cases has also been associated with the free radicals and oxidative stress (Fitzmaurice et al., 2017; Tarocchi and Galli, 2017).

Resveratrol (3, 5, 4′-trihydroxystilbene) is a natural antioxidant found in grapes, blueberries, raspberries, mulberries, and peanuts (Kataria and Khatkar, 2019). It reduces cellular stress, microbial infections, or the chemical effects of pesticide treatments in plants (Akbel et al., 2018). Besides, the potential effects of the resveratrol obtained from plant sources on human health are also being studied by many researchers. For example, antidiabetic (Zhang et al., 2018; Dai et al., 2019), anti-aging, anti-inflammatory, antioxidant, anti-cardiovascular disease, antimicrobial (Vestergaard and Ingmer, 2019), anti-estrogenic (Stivala et al., 2001; Berman et al., 2017), anti-carcinogenic (Athar et al., 2007; Bertelli, 2007), and vascular occlusion preventing roles (Catalgol et al., 2012) are well described. Many target molecules have been identified that mediate the protective effects of resveratrol such as endothelial nitric oxide synthetase, sirtuin 1, nuclear factor E2-related factor-2, and nuclear factor-kappa (Liu et al., 2015). In addition, resveratrol was also found to regulate antioxidant enzyme systems in several tissues (Karabekir and Özgörgülü, 2020). Recent studies have demonstrated either antioxidant or pro-oxidant effect of resveratrol depending on the specific cells, different tissues, or disease types. The exact mechanisms that make resveratrol a protective agent for normal cells as well as a radical generator with cytotoxicity against cancer cells are currently being debated (Khan et al., 2013). Besides, the effects of resveratrol on antioxidant enzymes' expressions and activities in various cancers are contradictive.

Therefore, in this study we evaluated the possible regulatory roles of resveratrol over main antioxidant enzymes in both healthy and cancerous hepatic cells. Differential expression patterns of antioxidant genes together with enzyme activities were assessed under different resveratrol concentrations the results of which might provide a better understanding of pharmacological uses of resveratrol in cancer prevention or treatment.

2. Material and Method

2.1. Cell culture and resveratrol treatment

Human healthy liver (THLE2) and human liver hepatocarcinoma (HepG2) cell lines were cultured using a Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS (fetal bovine serum), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 mg/ml) at 37ºC in the presence of 5% CO_2 and providing 95% humidity until they reached 90% confluency. Getting 90% confluency, cells were removed with trypsin/EDTA with phenol red solution and subcultured to the new growth media. After examining the cytotoxic properties of resveratrol over these cells, resveratrol in different concentrations (25 µM, 50 µM, and 75 µM) was applied to the cells for 48 hours. These doses of resveratrol are selected according to IC_{50} values for cellular cytotoxicity results (data not shown).

2.2. Total RNA isolation and cDNA library synthesis

The RNesy total RNA isolation kit (Qiagen, Hilden, Germany) was used to isolate total RNA from THLE2 and HepG2 cells according to the manufacturer's procedure. After isolation, spectrophotometry (Multiskan Go, Thermo Scientific, Waltham, MA, USA) at 260/280nm was used to assess the quantity and quality of total RNAs. Complementary DNA (cDNA) libraries were prepared using RNA samples using the First-Strand cDNA synthesis kit (Thermo Scientific, Waltham, MA, USA). Accordingly, 1 µg total RNA, 1 µl oligo$(dT)_{18}$ primer (100 µM), 4 µl 5X-M-MuLV reaction buffer, 1 µl RiboLock™ (20 U/µl) (Thermo Scientific, USA), 2 µl dNTP (10 mM) was mixed with 1 µl of M-MuLV Reverse Transcriptase (200 U/µl) and the total volume was made up to 20 µl. cDNA synthesis was carried out at 42ºC for 1 hour in a thermal cycler (Bioneer, South Korea).

2.3. Determination of antioxidant enzymes' genes expression with quantitative real-time polymerase chain reaction (qRT-qPCR)

A real-time quantitative polymerase chain reaction (qRT-PCR, LightCycler480 II, Roche, Basel, Switzerland) was used to assess the expression levels of *sod1*, *sod2*, *cat*, *gpx*, and *gstm*. Accordingly, 5 µl of SYBR Green Master Mix (Roche, Germany) was mixed with 2 µl forward and 2 µl reverse primers (2 µM each), and then 1 µl of cDNA was added. After initial denaturation at 95 °C for 10 minutes, denaturation at 94 °C for 15 seconds, annealing at 58 °C for 15 seconds, and extension at 72°C for 30 seconds were repeated 40 times, the resulting fluorescence after the extension step of each cycle was determined with the LightCycler 480-II (Roche, Germany) instrument. Primer pairs (Table 1) that were designed with the NCBI Primer Blast tool were also validated before the experiments. Measurements were performed in triplicate for each sample, and threshold cycle count (C_T) values were calculated for each amplification curve. The internal standard *gapdh* gene was used for normalization. The relative expressions of genes to *gapdh* were determined by the activity-corrected gene quantification tool of LightCycler™ 480 SW 1.5.1 software (Roche, Germany). Melt analysis was performed on the products to control the purity of the qPCR products and genomic DNA contamination was also tested using blank reaction tubes that did not contain any cDNA or DNA during the reaction.

Table 1. Primer sequences of *cat*, *sod1*, *sod2*, *gstµ*, *gpx*, and internal standard *gapdh* used for the mRNA expression determination.

Gene	Forward primer sequence (5'→3')	Reverse primer sequence (5'→3')
cat	GAACAGATAGCCTTCGACCC	AGTAATTTGGAGCACCACCC
gpx	CAGTCGGTGTATGCCTTCTC	TTCTTGGCGTTCTCCTGATG
sod1	AGATGACTTGGGCAAAGGTG	TTGGGCGATCCCAATTACAC
sod2	GCACATTAACGCGCAGATCA	AGCCTCCAGCAACTCTCCTT
gstmu	AGAAGCAGAAGCCAGAGTTC	GGGGTGAGGTTGAGGAGATG
gapdh	TGATGACATCAAGAAGGTGGTGAAG	TCCTTGGAGGCCATGTGGGCCAT

2.4. Protein isolation

Resveratrol treated THLE2 and HepG2 cells were scraped from the cell surface with a cell scraper (Sarstedt, USA) in 100 µl homogenization medium containing 50 mM Tris, 150 mM sodium chloride, 1 mM EDTA, 1% (w/w) NP-40, 0.25% (w/v) sodium deoxycholate, 1 mM sodium fluoride, 1 mM sodium orthovanadate and 1 mM PMSF (Phenylmethylsulfonyl fluoride), pH:7.4. After cell harvesting, they were incubated on ice for 45 minutes and then homogenized with an ultra sonicator (Sonopuls, Bandelin, Germany). Cell homogenates were centrifuged at 1.200*g* for 10 minutes at +4ºC and supernatants were removed. Total protein concentrations of the supernatants were determined by the Lowry method (Lowry et al., 1951).

2.5. Determination of Catalase and Glutathione S-transferase atıvities

Catalase enzyme activity was measured according to the protocol described by Aebi et al. (1974) with slight modifications. In this method, spectrophotometric measurement of the reduction in H_2O_2 to water through catalase activity was followed. Accordingly, 240 µl phosphate buffer (50 mM, pH: 7.0) and 10 µl cell homogenate were mixed thoroughly in a UV-permeable microtiter plate and the enzymatic reaction was initiated with the addition of 50 µl H_2O_2 (200 mM), and the absorbance changes were monitored for 2 min at 240 nm. Specific activity was calculated as the amount of decomposed hydrogen peroxide by 1 mg total protein in 1 minute and the extinction coefficient of hydrogen peroxide was 0.00364 ($L.mmol^{-1}.mm^{-1}$).

Total GST enzyme activity was determined according to the procedure developed previously (Habig et al., 1974). Accordingly, 15 µl cell homogenate was mixed with 20 µl GSH (50 mM), 15 µl CDNB (50 mM, dissolved in 2/3 ethanol), and 250 µl phosphate buffer (50 mM; pH 7.0) in a UV-permeable microtiter plate. The changes in absorbance were followed for 2 minutes at 340 nm. Total GST activity in cells was calculated as the amount of chromogenic product formed in one minute by the homogenate containing one mg of protein. The extinction coefficient was 9.6 $M^{-1}.cm^{-1}$ in specific activity calculations.

2.6. Statistical analysis

The GraphPad Prism 5.02 software was used to analyze the data. All data were given as mean ± standard error of the mean (SEM) values of three biological replicates with three technical replicate measurements.The corresponding *gapdh* levels were used to normalize the gene expression data. One-way ANOVA followed by a post-hoc test (Tukey's Honestly Significant Difference) was utilized for statistical evaluations. The statistical significance of comparisons with *P* values less than 0.05 was accepted.

3. Results

3.1. The changes in *cat, sod1* and *sod2* gene expressions with resveratrol in healthy and cancerous hepatocytes

Superoxide dismutase (SOD) isozymes; SOD1 and SOD2, which neutralize superoxide radicals in the cytoplasm and mitochondria, respectively, are the most important antioxidant enzymes in cells. The resultant hydrogen peroxide is then converted into non-toxic compounds with catalase (CAT) to eliminate the radical-induced stress conditions in hepatocytes. In this study, gene expressions of main antioxidant enzymes; *sod1, sod2,* and *cat* were determined by qRT-PCR for deep scanning of the exact modulation mechanisms over those enzymes with resveratrol in both cancerous and healthy hepatocytes. Results demonstrated that the gene expression levels of *cat*, *sod1,* and *sod2* were significantly repressed with resveratrol (Figure 1A, 1C, and 1E) in THLE2 cells. This reduction compromises the 80% of control expressions in all three doses and was found to be statistically significant ($P<0.05$). On the contrary to healthy cells, in HepG2 cells, gene expression of *sod1, sod2,* and *cat* were significantly induced in all doses of resveratrol in a dose-dependent manner (Figure 1B, 1D, and 1F). Gene expression of *cat* increased about two-fold in 25 µM, and about four-fold in 50- and 75 µM resveratrol treated HepG2 cells.

3.2. The changes in *gpx* and *gstm* gene expressions with resveratrol in healthy and cancerous hepatocytes

Glutathione-dependent antioxidant enzymes, the most important of which is glutathione peroxidase (GPx), catalyze the breakdown of hydrogen peroxide to water in the presence of glutathione, are another protective mechanism against oxidative stress. Some glutathione S-transferases (GSTs), which catalyze the conjugation of glutathione to a wide spectrum of electrophiles, share this function. The main isoform of this enzyme family having peroxidase-like antioxidant activity is the mu isoform (GST-mu). Herein, the expression levels of both *gpx* and *gstm* were also determined by qRT-PCR in both resveratrol treated THLE2 and HepG2 cells and the results are summarized in Figure 2.

Similar to *cat,* and *sod* expressions, *gpx,* and *gstm* levels were also suppressed in healthy THLE2 cells with all doses of resveratrol. Especially 50- and 75 µM resveratrol reduced the *gstm* expressions further ($p<0.05$) (Figure 2C). The behavior of these enzymes in cancerous hepatic cells (HepG2) is again contradictory in such a way that both *gpx* and *gstm* were induced tremendously with resveratrol. An approximately two-fold increase in *gpx* expression and a four-fold increase in *gstm* levels were observed at high doses of resveratrol in HepG2 cells and these changes were statistically significant ($p<0.05$).

3.3. The changes in CAT and total GST activities in THLE2 and HepG2 cells

Antioxidant enzyme systems catalyze processes that counteract free radicals and radical-induced byproducts, contributing the protection of cellular damage caused by free radicals. In this study, enzymatic activities of CAT and GST enzymes were determined, and the results are summarized in Figure 3. In line with the gene expression data, CAT and total GST activities were repressed with resveratrol in THLE2 cells (Figure 3A, 3C) showing that the suppressive effect starts at the gene expression level and continues up to the activity level. On the other hand, while resveratrol augmented gene expression levels of antioxidant enzymes in HepG2 cells, there was not any significant modulation in their activities (Figure 3B, 3D). This result indicated only gene expression regulatory function of resveratrol in cancerous hepatocytes and other

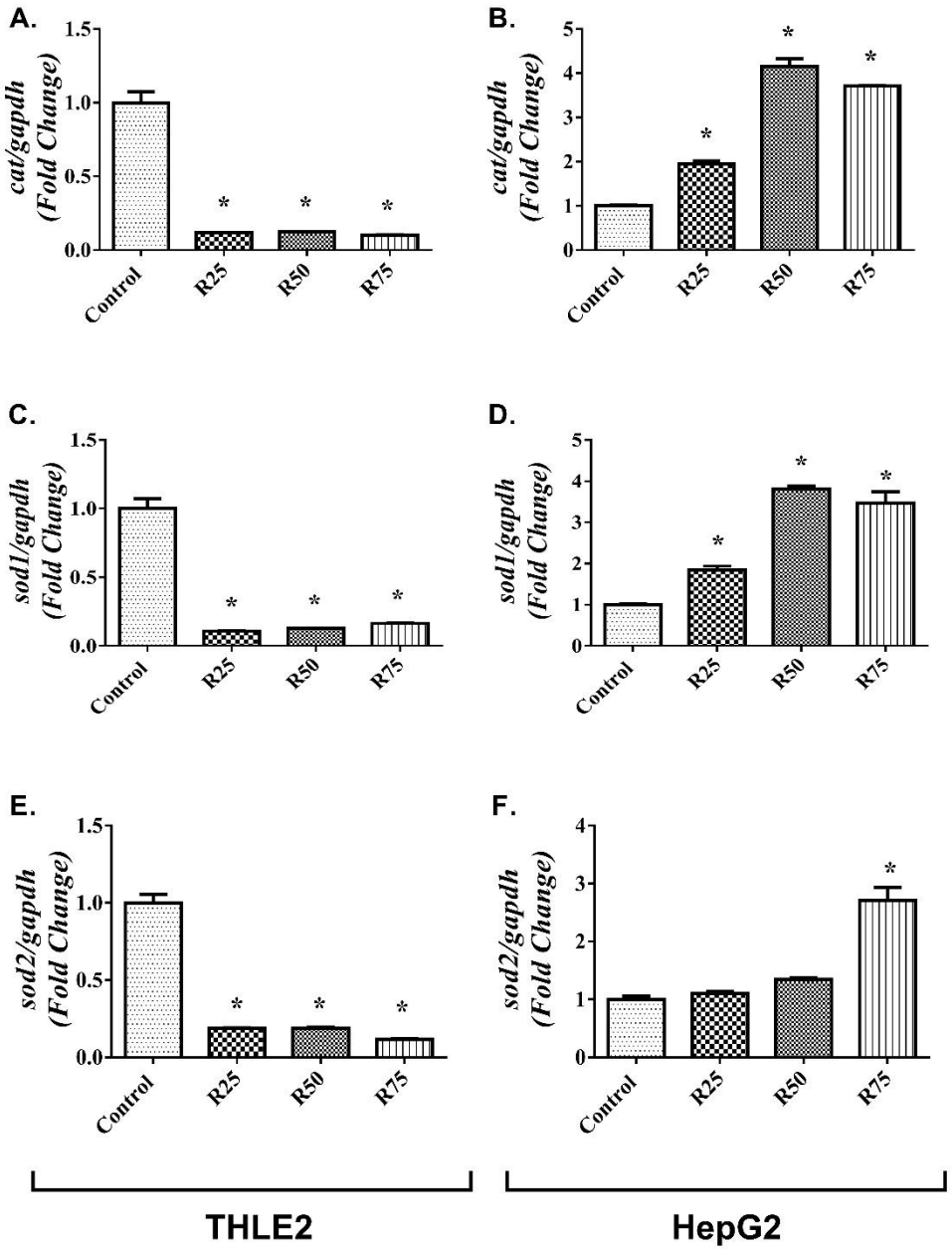

Figure 1. Genes expression levels of *cat* (A,B*), sod1* (C,D) and *sod2* (E,F) in resveratrol treated THLE2 and HepG2 cells. R25: cells treated with 25 µM resveratrol; R50: cells treated with 50 µM resveratrol; R75: cells treated with 75 µM resveratrol. The results are normalized to internal control *gapdh* gene and expressed as a fold change values over control cells. *Represents significance at $P < 0.05$ as compared with control group. All data were given as mean ± standard error of the mean (SEM) values of three biological replicates with three technical replicate measurements.

post-translational mechanisms that might stabilize the activities of CAT and GST in HepG2 cells.

4. Discussions

Cancer is a disease that spreads throughout the body from the organs and tissues in which cells divide uncontrollably. The causes of cancer have many genetic and environmental factors one of which is free radicals leading to damage to the nucleic acids, proteins, and lipids (Liou and Storz, 2010). Recent studies postulated that oxidative stress is a major contributor to carcinogenesis since it disrupts several intracellular signal transduction pathways (Alves et al., 2021) and it promotes carcinogenesis by providing sufficient components for cancer cell survival. Thus, cancer cells, in turn, might benefit from the advantages of elevated levels of free radicals to keep their survival, by adapting the content and regulation of their antioxidant machinery (Moloney and Cotter, 2018). Any pharmacological agent that prevents the build-up of radicals might sequentially increase the antioxidant capacity of cancer cells which could prevent the progression and induce apoptotic processes (Moloney and Cotter, 2018).

A plant-derived polyphenol, trans-resveratrol, exhibits strong antioxidant properties and has been previously shown in the prevention and treatment of cancer complications (Ko et al., 2017). It acts on cancer cells by

activating or deactivating molecular pathways and modulating the antioxidant enzymes' expression in diseases conditions such as cancer and diabetes (Khan et al., 2013; Sadi et al., 2013). Thus, inducing the expression of enzymatic antioxidants or enhancing their activities might be beneficial cancer prevention and treatment strategy. Despite the promising value of resveratrol to suppress tumor growth in various cancer research, the precise mechanism of anti-proliferative effects of resveratrol needs to be inspected in detail. Therefore, this study is conducted to reveal the modulatory effects of resveratrol over main antioxidant enzymes in both healthy and cancerous liver cells to enlighten the exact molecular mechanisms for antiproliferative action over cancer cells.

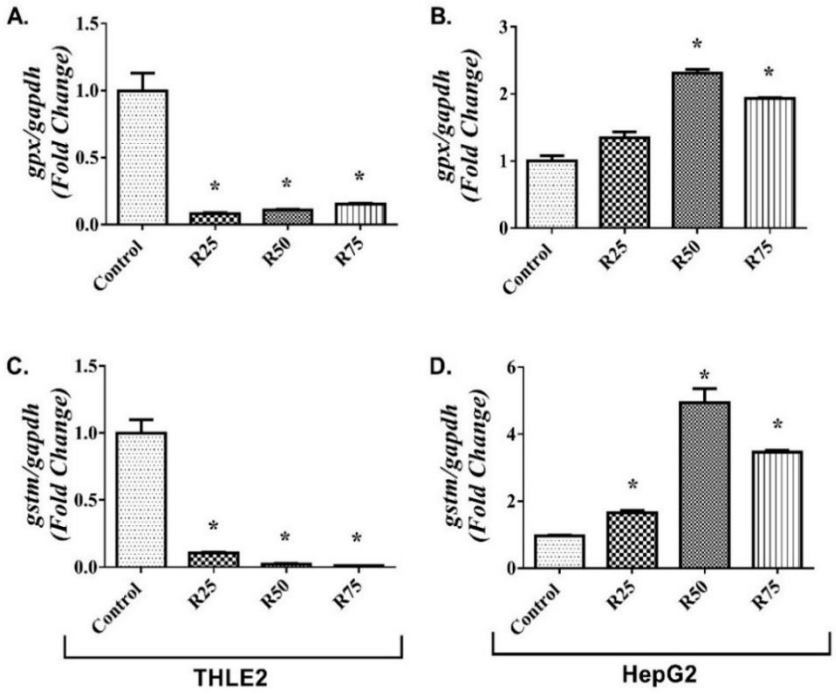

Figure 2. Genes expression levels of *gpx* (A,B*)* and *gstm* (C,D) in resveratrol treated THLE2 and HepG2 cells. R25: cells treated with 25 µM resveratrol; R50: cells treated with 50 µM resveratrol; R75: cells treated with 75 µM resveratrol. The results are normalized to the internal control *gapdh* gene and expressed as a fold change values over control cells. *Represents significance at $P < 0.05$ as compared with the control group. All data were given as mean ± standard error of the mean (SEM) values.

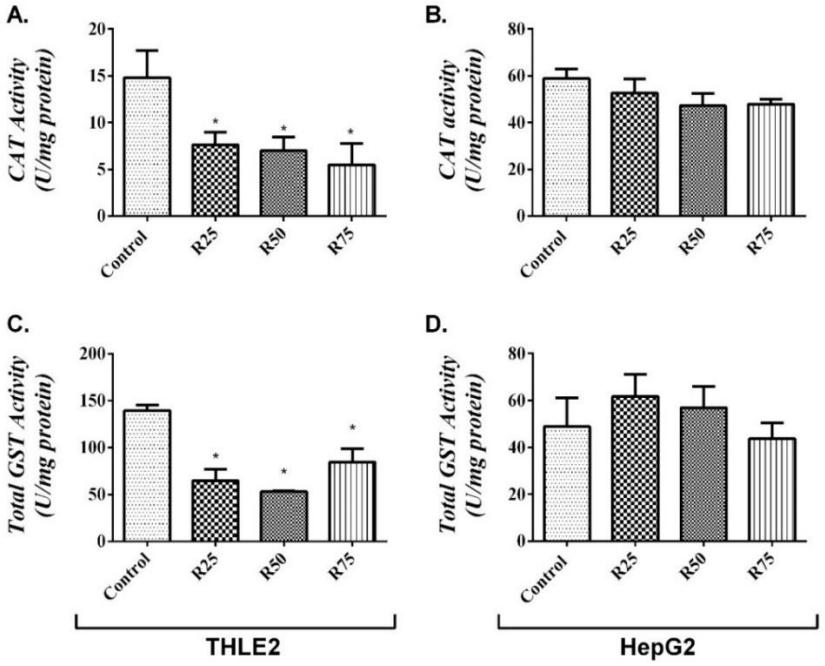

Figure 3. Specific activities of CAT (A,B) and GST (C,D) enzymes in resveratrol treated THLE2 and HepG2 cells. R25: cells treated with 25 µM resveratrol; R50: cells treated with 50 µM resveratrol; R75: cells treated with 75 µM resveratrol. *Represents significance at $P < 0.05$ as compared with the control group. All data were given as mean ± standard error of the mean (SEM) values.

Herein, we used THLE2 cells as a control cell line to distinguish the effects of resveratrol on cancer cells (HepG2) from that on non-cancerous cells. The results demonstrated significant up-regulation of main antioxidant enzymes with resveratrol in cancerous HepG2 cells at gene expression levels in a dose-dependent manner due to a compansatory mechanism for increased oxidative stress levels. On the contrary to this result, gene expressions of antioxidant enzymes were down-regulated significantly in THLE2 cells with all used doses of resveratrol. We also noticed that the reduction in gene expression in control cells was in line with the enzymatic activities of CAT and GST enzymes, however, enzyme activity in HepG2 cells did not change significantly.

Simultaneous up-regulation of *cat*, *sod1*, *sod2*, and *gstm* in cancer cells, and their disproportional suppression in healthy cells would be valuable for cancer pharmacotherapy. Since free radicals keep the cancer cells malignant and provide resistance to normal cell cycle arrest mechanisms. Low radical content would lead to lower levels of DNA damage and genetic instability in cancer cells which might in turn decrease cell survival and proliferation. Teoh-Fitzgerald and coworkers have demonstrated that overexpression of SOD3 decreased breast cancer metastasis in vivo (Teoh-Fitzgerald et al., 2012). Besides, inhibition of cell growth with SOD2 overexpression in human pancreatic carcinoma (Ough et al., 2004), and suppression of the malignant phenotype in pancreatic cancer by overexpression of GPx glutathione peroxidase (Graves et al., 2006) supports our hypothesis that reducing radicals in cancer cells by up-regulating antioxidant enzymes might inhibit pro-tumorigenic signaling pathways in the treatment of cancer. We can better understand the significance of enhanced free radical generation in cancer and identify particular target pathways to more effectively treat cancer using this information.

In conclusion, the prevention of oxidative stress, inflammation, and cancer-cell proliferation, as well as the activation of tightly regulated cell-death processes, are all roles of resveratrol in preventing carcinogenesis. Because of the intricacy and number of biological systems involved, further research is needed to completely understand how resveratrol can be used to prevent the development and progression of cancer.

Conflict of Interest

Authors have declared no conflict of interest.

Authors' Contributions

AB: performed qPCR experiments, ENŞ: conducted cell culture studies, AK: measured the enzyme activities, GS: designed and supervised the study, made data analysis and wrote the manuscript.

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Total flavonoid, phenolic and antioxidant activities of *Pelargonium quercetorum* Agnew: Comparison of *in vivo* and *in vitro* grown plant

İbrahim Selçuk KURU[1]*, Pınar ORCAN[2], Mehmet FIRAT[3]
[1]*Batman University, Sason Vocational School, Department of Crop and Animal Production, Batman, Türkiye*
[2]*Batman University, Vocational School of Technical Sciences, Department of Food Processing, Batman, Türkiye*
[3]*Van Yuzuncu Yil University, Education Faculty, Department Math. & Science Education, 65080 Van, Türkiye*
**ibrahimselcuk.kuru@batman.edu.tr, [2]pinar.karakus@batman.edu.tr, [3]mehmetfirat@yyu.edu.tr*

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Pelargonium quercetorum Agnew'in toplam flavonoit, fenolik ve antioksidan aktiviteleri: *In vivo* ve *in vitro* yetiştirilen bitkinin karşılaştırılması

Abstract: Nowadays, natural compounds with phytochemical properties are considered human-friendly drugs because they do not have side effects. Therefore, the importance given to antioxidant compounds, which are also found in plants, continues to increase day by day. *Pelargonium* L'Hér. ex Aiton taxa are also used as curative in diseases such as respiratory tract infections, dysentery, liver complaints and diarrhea treatment. Plant tissue and cell culture techniques are a good tool for the production of some active metabolites such as polyphenols. It is also known that with these methods, secondary metabolite production is promoted and there are also changes in antioxidant capacity. In this context, it was aimed to determine the total phenolic, flavonoid and antioxidant capacities of *Pelargonium quercetorum* Agnew plant by growing *in vivo* (rhizome and above-ground part) and *in vitro* conditions. When the results obtained were examined, the highest phenolic and flavonoid content was found in the shoot extract in vitro; the lowest phenolic and flavonoid content was determined in the rhizome extract. In our study, 3 different methods (DPPH, ABTS, CUPRAC) were used to determine the total antioxidant activity. When the antioxidant activity results were evaluated in general, it was observed that the order of activity in all three methods was "in vitro shoot > *in vivo* above ground > *in vivo* rhizome". In the DPPH method, plant extracts showed better activity than BHT, which was used as a positive control, and better than BHA in the ABTS method. In addition, a positive correlation was observed between total phenolic-flavonoid content and antioxidant activity. The data obtained from this study, it is thought that the plant *P. quercetorum* has antioxidant activity, and our study will be a step in the search for natural origin antioxidants.

Key words: Antioxidant, flavonoid, *P. quercetorum*, phenolic

Özet: Günümüzde, fitokimyasal özelliğe sahip doğal bileşikler yan etkilerinin olmaması nedeniyle insan dostu ilaçlar olarak kabul edilirler. Bu nedenle bitkilerde de bulunan antioksidan bileşiklere verilen önem her geçen gün artarak devam etmektedir. *Pelargonium* L'Hér. ex Aiton taksonları da solunum yolu enfeksiyonları, dizanteri, karaciğer şikayetleri ve ishal tedavisi gibi hastalıklarda iyileştirici olarak kullanılmaktadır. Bitki doku ve hücre kültürü yöntemleri, polifenoller gibi bazı aktif metabolitlerin üretilmesi için iyi bir araçtır. Bu yöntemler ile sekonder metabolit üretiminin teşvik edildiği ve antioksidan kapasitede değişikliklere neden olduğu da bilinmektedir. Bu kapsamda araştırmamızda, *Pelargonium quercetorum* Agnew bitkisi *in vivo* (rizom ve toprak üstü kısım) ve *in vitro* şartlarda yetiştirilerek toplam fenolik, flavonoit ve antioksidan kapasitelerinin belirlenmesi amaçlanmıştır. Elde edilen sonuçlar incelendiğinde, en yüksek fenolik ve flavonoit içerik *in vitro* sürgün ekstresinde; en düşük fenolik ve flavonoit içerik ise rizom ekstresinde tespit edilmiştir. Araştırmamızda toplam antioksidan aktiviteyi belirlemek amacıyla 3 farklı yöntem (DPPH, ABTS, CUPRAC) kullanılmıştır. Antioksidan aktivite sonuçları genel olarak değerlendirildiğinde, her üç yöntemde de aktivite sıralamasının "*in vitro* sürgün > *in vivo* toprak üstü > *in vivo* rizom" şeklinde olduğu gözlenmiştir. DPPH yönteminde bitki ekstreleri pozitif kontrol olarak kullanılan BHT'ye göre, ABTS yönteminde ise BHA'ya göre daha iyi aktivite göstermiştir. Ayrıca toplam fenolik-flavonoit içerik ile antioksidan aktivite arasında pozitif korelasyon olduğu görülmüştür. Bu çalışmadan elde edilen veriler, *P. quercetorum* bitkisinin antioksidan aktiviteye sahip olduğunu, doğal kaynaklı antioksidan madde arayışında çalışmamızın bir basamak olacağı düşünülmektedir.

Anahtar Kelimeler: Antioksidan, fenolik, flavonoit, *P. quercetorum*

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1. Introduction

Benefiting from plants is as old as human history, and the use of plants for medicinal purposes dates back thousands of years. Thus, the interaction and relationship between plants and humans is the core of both human well-being and nature conservation (MEA, 2005).

People are always dependent on natural resources for basic needs such as clothing, food, cosmetics and medicine (Prance and Nesbitt, 2012). Although there are chemical active substances of herbal origin in the structure of many drugs used today, plants used directly as therapeutic drugs and auxiliary plants for treatment have also taken their place in modern medicine. In addition, the developed countries of the world have turned to herbal resources due to the undesirable side effects of drugs produced with pure, synthetic or semi-synthetic raw materials. For this reason, wild edible plants, which are traditionally used, existed in the past and are still a remedy for humans today, have gained acceptance and accuracy among both medicine and local people (Heinrich et al., 2017) and forming the basis of

practical use in medicine (Hamilton et al., 2003). In addition to being edible, wild plants contain important nutritional values such as vitamins, antioxidants, carbohydrates, fats, proteins and minerals (Geissler and Powers, 2017).

Plants contain a wide variety of free radical scavenging molecules such as flavonoids, anthocyanins, carotenoids, vitamins and endogenous metabolites. Nowadays, the intense interest in natural-origin phenolic antioxidants is not only due to their protective and therapeutic properties against various diseases caused by oxidative damage, but also to their prolonging the life of food products (Matkowski et al., 2008; Karatoprak et al., 2018).

Geraniaceae family is represented by 11 genus and 750 species in the world, and in Turkey with four genera as *Biebersteina* Stephan, *Geranium* Tourn. ex L., *Erodium* Aiton and *Pelargonium* L'Hér. ex Aiton and a total of 62 species (Trun et al., 2006; Brendlera and Van, 2008). Two species of *Pelargonium* (*Pelargonium endlicherianum* Fenzl and *Pelargonium quercetorum* Agnew), the most important genus of this family, are registered in the vegetation of Turkey. The taxon is generally distributed in Northern Iraq, it is distributed in Hakkâri province in Turkey. In addition to its medicinal use, this plant is also used for food purposes by the local people (Uce and Tunçtürk, 2014; Karatoprak et al., 2018). In our country, *P. quercetorum* is used to treat throat ailments and skin wounds, and also its seeds and leaves are used to burst boils. It has been stated that this plant, which is effective as a wormer and is very important for this feature, is also used for chronic headaches, neck pain and migraine (Uce and Tunçtürk, 2014).

Micropropagation techniques or *in vitro* cultures; unlike other classical production methods, are the process of culturing explants taken from various parts of the plant in sterile environments and aseptic conditions after sterilization and transforming these explants into plantlets in this environment (Başak and Candan, 2008). In previous studies, it has been reported that the production of secondary metabolites is promoted by plant tissue culture techniques and cause to differences in antioxidant systems (Tošić et al., 2019; Yaman et al., 2020).

There are various studies carried out by collecting plants from their natural environments but this situation leads to unconscious and excessive collection of plants from nature and thus carries risks to the extent that they endanger the generations of the plants in question. In *in vitro* culture techniques, both these risks can be eliminated and it is possible to reproduce and transfer endangered species to their natural environments. In this study, it was aimed to comparatively evaluate the total phenol, flavonoid and antioxidant capacities (ABTS, CUPRAC and DPPH) of *P. quercetorum* grown separately from their seeds with both *in vivo* (pot) and *in vitro* techniques.

2. Materials and Method

2.1. Plant Material

The flowering form of the plant material was collected in May 2021 in Şemdinli district of Hakkari province (1323 m, 37°20′51″ N, 44°26′03″ E). Mature seeds were collected in June 2021 and identified by Mehmet Fırat. Dried species samples were kept in Mehmet Fırat's personal herbarium and Van Yüzüncü Yıl University Faculty of Science Herbarium (VANF).

In tissue culture studies; mature seeds of *Pelargonium quercetorum* Agnew were washed with distilled water, kept in 70% alcohol for 30 seconds and pre-sterilized. Seeds were kept in 5% NaOCI solution for 10 minutes and rinsed in sterile distilled water to remove NaOCI. For the germination and growth of seeds, 30 g L^{-1} sucrose was added to ¼ MS medium and its pH was adjusted to 5.8 and then 5.6 g of agar was added to the medium and sterilized in an autoclave at 1 atm 121 °C for 25 minutes. For *in vitro* culture conditions; 1/1, 1/2 and 1/4 strength MS mediums were tested and the healthiest shoots were obtained in 1/4 MS medium. The prepared medium was divided into Magenta GA-7 culture dishes in a sterile cabine. 5 seeds were sown in each culture pot and allowed to germinate in a plant growth room with 25±2 °C temperature, 16/8 photoperiod and 3000 lux conditions. At the end of the three-week culture period, the shoots obtained from the germinated seeds were separated from the gelose and dried in the oven at 50 °C.

In pot (*in vivo*) studies, mature seeds of *P. quercetorum* were planted in pots containing soil:peat:perlite (1:1:1) and allowed to develop in a growing room with similar conditions of tissue culture. The above-ground and underground parts of the developing shoots were cleared from the soil and dried in an oven at 50 °C. Three gram of dried plant samples were taken and powdered in a mortar and then macerated with pure methanol. The extracts were filtered through Whatman No:1 filter papers and the solvents were evaporated in a rotary evaporator. Stock solutions were prepared from the obtained extracts at a concentration of 1000 ppm to be used in the determination of total phenolic, flavonoid and antioxidant activity.

2.2. Total Phenolic Content

The total phenolic content of the extracts was determined as equivalent to gallic acid using the Folin-Ciocalteu reagent (Slinkard and Singleton, 1977). 100 µL of the stock solutions of the extracts was taken and made up to 4.6 mL with distilled water. 100 µL of Folin-Ciocalteu Reagent (FCR) and 300 µL of 2% Na_2CO_3 solution were added to this mixture after 3 minutes and incubated at room temperature for two hours. The same procedure was applied for the gallic acid solutions prepared at different concentrations, and after incubation, spectrophotometric measurements were taken at a wavelength of 760 nm. The total phenolic contents of the extracts were calculated as the gallic acid equivalent (GAE) obtained from the standard gallic acid graph (GAEs, gallic acid equivalents ($y = 0.0319 x + 0.0026$ $R^2 = 0.9956$).

2.3. Total Flavonoid Content

Total flavonoid amounts in the extracts were determined as equivalent to quercetin using the aluminum nitrate method (Moreno et al., 2000). 100 µL of 1 M potassium acetate was added to the mixture and 100 µL of 10% aluminum nitrate was added after one minute. After an incubation period of 40 minutes, absorbances were read in UV spectrophotometer at 415 nm against the control. The total flavonoid amounts of the extracts were determined using the equation obtained from the standard quercetin graph (QEs, quercetin equivalents ($y = 0.0626 x + 0.0299$ $R^2 = 0.9969$).

2.4. Total Antioxidant Activity Measurements

In the DPPH method; free radical scavenging activities of the extracts were determined using 1,1-diphenyl-2-picrylhydrazil (DPPH) free radical (Blois, 1958). 1 mL of plant extracts prepared at different concentrations were taken and 4 mL of 0.1 mM DPPH solution was added. The prepared reaction mixtures were kept in the dark at room temperature for 30 minutes and then spectrophotometric measurements were taken at 517 nm in UV spectrophotometer.

ABTS cation radical scavenging activities of the extracts were determined using 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid). ABTS is reacted with strong oxidants such as $K_2S_2O_8$, MnO_2, H_2O_2 to form $ABTS^{+\bullet}$. 3.3 mg of $K_2S_2O_8$ was added to 7 mM 5 mL ABTS solution and left in the dark at room temperature for 16 hours. It was then diluted with ethanol to have 0.7 absorbance at 734 nm. 1 mL of extracts prepared at different concentrations were added to 4 mL of ABTS reagent and kept in the dark for 30 minutes and then spectrophotometric measurements were taken at 734 nm (Ree et al., 1999).

In the CUPRAC method, Cu(II)-Neocuproin (Nc) complex is reduced to colored Cu(I)-Nc chelate in the presence of antioxidant compounds in the samples and the absorbance of this chelate at 450 nm wavelength was measured (Apak et al., 2004). After adding 1 mL of 10 mM $CuCl_2$, 1 mL of 7.3 mM Neocuproine and 1 mL of 1 M ammonium acetate to the test tubes, the extracts prepared at different concentrations were added to a final volume of 4 mL and the absorbance was measured at 450 nm after 1 hour. The absorbance values of the samples were evaluated against to the control.

In all antioxidant activity methods, three parallel studies were performed from each sample and Butylated hydroxytoluene (BHT), Butylated hydroxyanisole (BHA) were used as positive controls.

3. Results

Developmental photographs of *Pelargonium quercetorum* plant grown *in vivo* and *in vitro* conditions are presented in Figure 1.

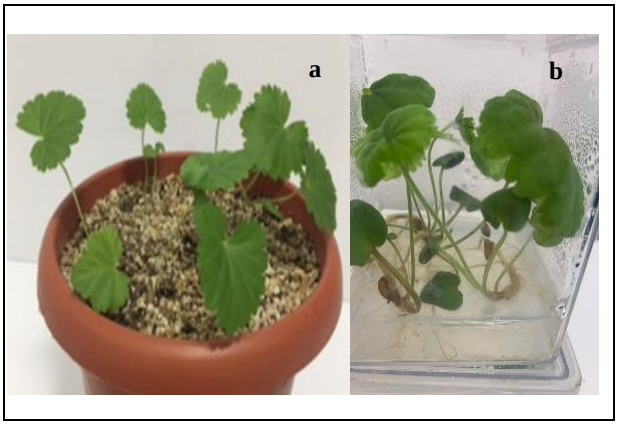

Figure 1. *In vivo* and *in vitro* development of *Pelargonium quercetorum* plants (a. *in vivo*; b. *in vitro*)

The total phenol content of the extracts was determined as gallic acid equivalent; the total amount of flavonoids was calculated as quercetin equivalent and the results are given in Table 1. The highest phenolic content was found in *in vitro* shoot extract (115.04±0.47 µg GAEs/mg extract) and the lowest content was determined in rhizome extract (101.98±0.089 µg GAEs/mg extract) in *Pelargonium quercetorum* Agnew. When the plant extracts were compared among themselves, it was seen that the order of total phenolic content was *in vitro* shoot > *in vivo* aerial parts > *in vivo* rhizome.

In terms of total flavonoid content, the highest flavonoid content was found in *in vitro* shoot extract (30.62.04±0.34 µg QEs/mg extract), the lowest content was found in rhizome extract (12.91±0.11 µg QEs/mg extract). The extracts showed similarity in terms of total phenolic and total flavonoid content, and as a result of both parameters, the highest order of values was determined as *in vitro* shoot > *in vivo* aerial parts > *in vivo* rhizome (Table 1).

Table 1. Total phenolic and flavonoid contents of *Pelargonium quercetorum* [a]

Samples	Phenolic content (µg GAEs/mg extract)	Flavonoid content (µg QEs/mg extract
Pq1	101.98±0.18^{c}	12.91±0.11^{c}
Pq2	110.34±0.54^{b}	27.48±0.32^{b}
Pg3	115.04±0.47a	30.62 ±0.34^{a}

Pq1: *In vivo* rhizome; Pq2: In vivo aerial parts; Pq3: In vitro shoot
a Values expressed are means ± SD of 3 parallel measurements , Values with different letters in the same column were significantly different ($p< 0.05$)

Table 2. Antioxidant activities of *Pelargonium quercetorum* [a]

	IC_{50} (µg/mL)		$A_{0.5}$ (µg/mL)
Samples	**DPPH**	**ABTS**	**CUPRAC**
Pq1	98.87±0.19^{b}	21.57±0.44^{a}	73.87±0.34^{a}
Pq2	91.32±0.48^{c}	16.52±0.31^{c}	73.17±0.42^{a}
Pq3	81.90±0.46^{d}	13.70±0.42^{d}	56.41±0.64^{b}
BHT	101.04±0.60^{a}	5.72±0.24^{e}	6.72±0.16^{d}
BHA	13.23±0.44^{c}	19.23±0.28^{b}	15.02±0.24^{c}

Pq1: *In vivo* rhizome; Pq2: *In vivo* aerial parts; Pq3:*In vitro* shoot
[a]Values represent averages ± standard deviations for triplicate experiments and values were calculated according to negative control, Values with different letters in the same column were significantly different ($p< 0.05$)

The DPPH free radical scavenging activity of the plant extracts was calculated according to the scavenging percentage of the radicals in the medium at different concentrations (25, 50, 100, 150, 200 µg/mL) (Figure 2). The activities of the extracts were evaluated among themselves and BHT and BHA was used as positive controls. In general, all plant extracts showed antioxidant activity and the best activity was observed at the highest concentration (200 µg/mL). At 200 µg/mL concentration; *in vitro* shoot 93.87±0.25%; *in vivo* aerial parts 90.89±0.68% ; *in vivo* rhizome 87.19±0.94%; BHT 83.55±1.80% and BHA showed 95.75±0.17% inhibition value. When the IC_{50} values of the plant extracts were compared, the order of activity was *in vitro* shoot > *in vivo* aerial parts > *in vivo* rhizome. In addition, plant extracts showed better activity than BHT, which was used as a

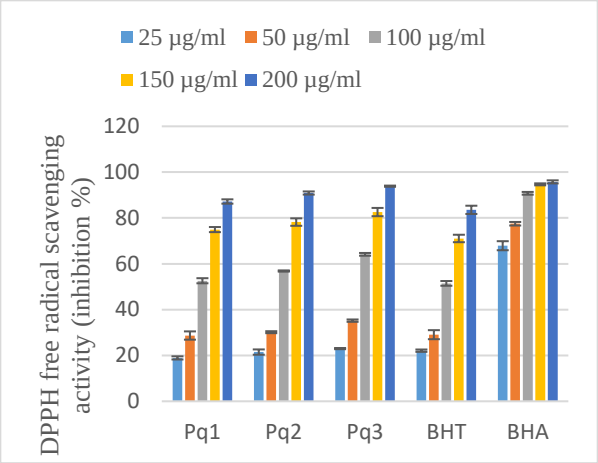

Figure 2. DPPH free radical scavenging activity of *Pelargonium quercetorum* and positive controls (Pq1: *In vivo* rhizome; Pq2: *In vivo* aerial parts; Pq3:*In vitro* shoot)

positive control, but BHA showed better activity than all plant extracts (Table 2).

ABTS radical scavenging effects of all extracts were evaluated at different concentrations (5, 10, 20,30, 40 µg/mL) and the results are given in Figure 3. The scavenging effects of all extracts and positive controls increased with increasing concentration. When the I% values were examined at the highest concentration (40 µg/mL); *in vitro* shoot 93.01±0.12%; *in vivo* aerial parts 91.56±0.18%; *in vivo* rhizome 84.30±0.38%, BHT 93.73±0.44% and BHA showed 91.37±0.14%. When the IC_{50} values were examined, *in vitro* shoot was 13.70±0.42; *in vivo* aerial parts 16.52±0.31; *in vivo* rhizome 21.57±0.44; BHT showed 5.72±0.24 and BHA showed 19.23±0.28 (Table 2). The order of activity was as *in vitro* shoot > *in vivo* aerial parts > in vivo rhizome (Table 2). The extracts obtained from *in vitro* shoot and *in vivo* aerial parts showed better activity than BHA but BHT showed better activity than all plant extracts.

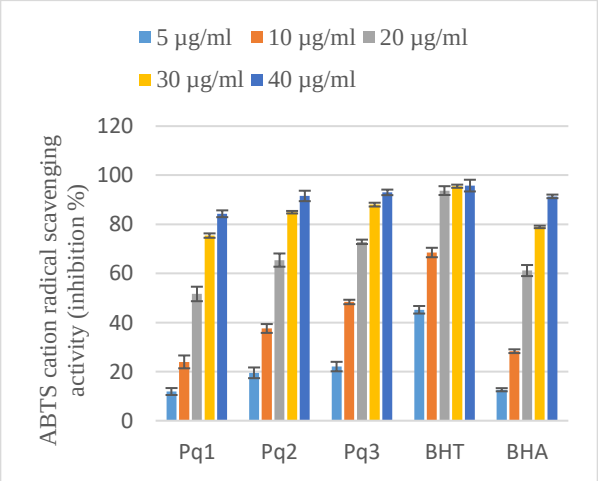

Figure 3. ABTS cation radical scavenging activity of *Pelargonium quercetorum* and positive controls (Pq1: *In vivo* rhizome; Pq2: *In vivo* aerial parts; Pq3:*In vitro* shoot)

The antioxidant activity of the extracts was studied with the CUPRAC method in the concentration range of 25 - 200 µg/mL and a comparison was made with BHT and BHA used as positive controls. In this method; the absorbance values obtained express the activity and high absorbance value means high activity. At 200 µg/mL concentration; absorbance values of *in vitro* shoot 1.64±0.15; *in vivo* aerial parts 1.26±0.06; *in vivo* rhizome 1.25±0.09; BHT 2.56±0.06 and BHA 1.92±0.11 were measured (Figure 4). When the $A_{0.5}$ values obtained from the CUPRAC method were compared, statistically the order of activity was in vitro shoot > in vivo aerial parts = in vivo rhizome.(Table 2) Among the plant extracts, the best activity was measured in *in vitro* shoot, but none of extracts were showed better activity than positive controls.

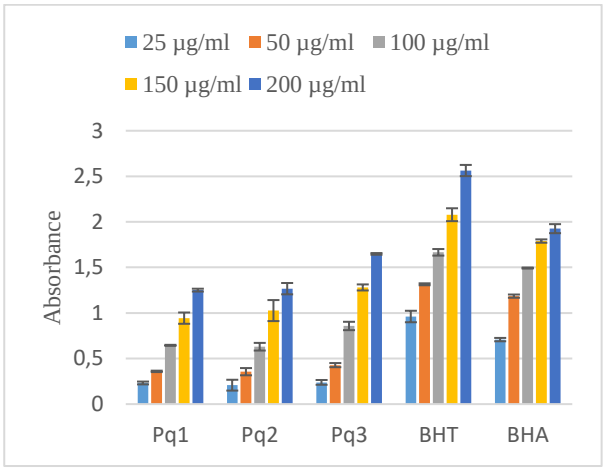

Figure 4. Cuprac reducing antioxidant capacity of *Pelargonium quercetorum* and positive controls (Pq1: *In vivo* rhizome; Pq2: *In vivo* aerial parts; Pq3:*In vitro* shoot)

4. Discussions

The plant-human relationship is as old as the existence of humanity and this interaction continues increasingly today. People first applied to plants for their food, shelter and protection needs and then for the treatment of diseases (Rahman et al., 2019). The use of many plants for medicinal purposes from ancient times to the present has been mostly by trial and error application method. However, with the developing technology, the problem of determining the cause-effect relationship by going beyond the traditional uses of plants has been the subject of researches. Plants have many uses area because of the secondary metabolites they contain, so the studies carried out in recent years have focused on the identification of the secondary metabolites of plants in terms of quantity and quality and the investigation of their biological activity potentials. Secondary metabolites are classified according to their biosynthetic origins, show different biological activities and used as drugs (antibiotics, antitumor agents, antiviral and antiparasitic agents, immunosuppressants, etc.), flavoring, odor or coloring agent, food additive or biopesticide in the industrial field (Murthy et al., 2014).

Recent studies have shown that the species belonging to the genus *Pelargonium* are very rich in phenolic compounds, and that this compounds with polyphenol structure have strong antioxidant properties (Koley et al., 2016; Stafussa et al., 2018). Among the species belonging to 5 different genera found in the flora of Turkey, the highest antioxidant activity was detected in the above-ground parts of *P. endlicherianum* belonging to the genus *Pelargonium* (Tepe et al., 2006). A drug with the international name Umckaloabo has been produced from the roots of *Pelargonium sidoides*, it has been stated that the drug alleviates the severity of symptoms and shortens the

duration of the disease by strengthening the immune system in upper respiratory tract diseases and colds (Bradt and Wagner, 2007). In a study on the comparison of total phenolic content in two different species of *Pelargonium*, the total phenol content of Umca® extract prepared from *P. sidoides* was considerably lower than *P. quercetorum* root extract, and it showed that *P. quercetorum* was rich in phenolic content.

The biosynthesis of secondary metabolites varies greatly depending on the cell type, developmental stage, and environmental factors, and these compounds are mobilized to different cells, tissues and organs of the plant (Patra et al., 2013). Therefore, the biosynthesis and accumulation of secondary metabolites may show organ or tissue specificity. In a study examining the total phenolic and flavonoid contents and antioxidant activities of the naturally collected samples of *P. quercetorum*, it was reported that the root extract contained higher phenols-flavonoids than the above-ground parts, and the antioxidant activity was similarly higher (Karatoprak et al., 2018). In our study, when the extracts prepared from *in vivo* aboveground, root parts and *in vitro* shoots of *P. quercetorum* were evaluated, the lowest extract in terms of activities was root extract. The inconsistency of these results with our study may be due to the plant materials studied under different conditions and at different developmental stages.

Secondary metabolism (alkaloids, phenolics, terpenoids, etc.) can also respond to oxidative stress and free radical production leading to accumulation of different compounds in plants grown *in vitro* or *in vivo*. This is due to both genetic factors and environmental factors affecting growth; Due to the formation of different oxidative stress conditions, different responses may be obtained in terms of secondary metabolite production. It has been determined in various studies that plants may have differences in secondary metabolites and biological activities when grown under different conditions (Buruni and Şahin, 2009; Kanungo and Sahoo, 2011).

In the literature, there are various studies comparing the natural environments of different plants with the samples grown in *in vitro* culture environment. However, there are no study has been found to compare the natural or artificial (*in vitro*) environments of *Pelargonium* species. Therefore, this study is the first research in this sense.

Kanungo and Sahoo (2011) stated that the antioxidant enzyme activities of two different ecotypes of *Withania somnifera* (L.) Dunal in samples grown separately *in vitro* and *in vivo* were higher in plants grown *in vitro*. The researchers concluded that the addition of essential nutrients for plants *in vitro* increases the antioxidant capacity. In our study, when the total phenol, flavonoid and antioxidant (DPPH, ABTS, CUPRAC) contents of *P. quercetorum* extracts were examined; *In vitro* shoot extract was found to be the richest extract in terms of content. Similarly, Barros et al. (2012) compared the phenolic contents of *Coriandrum sativum* L. samples grown *in vivo* and *in vitro*, and *in vitro* samples contained higher capacity polyphenols. Researchers emphasized that plant cell cultures are a good tool to study or produce some active metabolites such as polyphenols.

However, there are also several reports showing higher activity in *in vivo* extracts in plants grown *in vitro* and *in vivo*. Esmaeili et al. (2016) compared some biological activities (total phenolic and flavonoid content, antioxidant and antityrosinase) of methanol and hexane solvent of Asparagus grown in *in vivo* and *in vitro*. In the study, plants grown *in vivo* showed significantly higher total phenolic and flavonoid content, and the order of antioxidant activity was; *in vivo* plant > callus > *in vitro* plant.

5. Conclusion

It was determined that the total phenolic and flavonoid and antioxidant activities of the extracts prepared from the shoots of *P. quercetorum* obtained in vitro were higher than the *in vivo* extracts in this study. Total phenolic, flavonoid and antioxidant activities may be high in plants grown in the *in vitro* (artificial) environment due to the effect of various macro, micronutrients, sucrose and optimal conditions such as light, temperature, and humidity. As a matter of fact, there is a lot of evidence showing that secondary metabolite production is increased in plants grown using *in vitro* tissue culture methods. Therefore, we believe that *in vitro* cultures can be used to explore new pharmaceutical and medicinal potentials and the production of secondary metabolites tsuch as flavones, flavonols and anthocyanins, etc., both in Pelargonium species and in different plants.

Conflict of Interest

Authors have declared no conflict of interest.

Authors' Contributions

The authors contributed equally.

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Coprotus Korf & Kimbr.: A new coprophilous genus record for the mycobiota of Türkiye

Mustafa Emre AKÇAY[1*], Yunus DENĞİZ[2], Sedat KESİCİ[3]
[1]*Van Yüzüncü Yıl University, Science Faculty, Department of Biology, Van, Türkiye*
[2]*Haydarpaşa Technical High School, İstanbul, Türkiye*
[3]*Hakkari University, Yüksekova Vocational School, Department of Animal & Plant Production, Hakkari, Türkiye*
**memreakcay@gmail.com, [2]dengizyunus@gmail.com, [3]kesicisedat30@gmail.com*

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Coprotus Korf & Kimbr.: Türkiye mikobiyotası için yeni bir koprofil cins kaydı

Abstract: The genus *Coprotus* Korf & Kimbr, was given as new record for the mycobiota of Türkiye based on the collection and the identification of *Coprotus ochraceus* (P. Crouan & H. Crouan) J. Moravec from Şirvan district of Siirt province. A brief description of the species is given together its photographs related to its macro and micro morphologies.

Key words: New genus record, macrofungi, *Pezizales*, Turkiye

Özet: *Coprotus* Korf & Kimbr cinsi, *Coprotus ochraceus* (P. Crouan & H. Crouan) J. Moravec'un Siirt ili Şirvan ilçesinden toplanıp teşhis edilmesine bağlı olarak Türkiye mikobiyotası için yeni kayıt olarak verilmiştir. Türün kısa bir betimlemesi, makro ve mikromorfolojilerine ilişkin resimlerle birlikte verilmiştir.

Anahtar Kelimeler: Yeni cins kaydı, makromantarlar, *Pezizales*, Turkiye

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1. Introduction

Though the generic name *Coprotus* Korf & Kimbr. was first introduced by Korf in 1954 as a segregate of the genus *Ascophanus* Boud., it was validated by Kimbrough and Korf in 1967 (Kušan et al. 2018). Members of the genus have a coprophilous ecology and are characterised by an oblate to discoid or lenticular, whitish to yellowish or translucent apothecia; operculate, non-amyloid, eight to 256 spored asci; hyaline, smooth, de Bary bubbled and aguttulate ascospores; filiform, hyaline or pigmented paraphyses, generaly bent to uncinate or swollen at the apex; and an excipulum mainly composed of globose to angular cells (Kimbrough et al., 1972; Bell, 2005).

Kušan et al. (2018) gives the accepted species number in the genus *Coprotus* as 29. On the other hand IndexFungorum (2022) currently list 26 conformed *Coprotus* species. Considering the latest checklist (Sesli et al., 2020) and post-checklist publications on higher ascomycetous fungi of Türkiye (Akata and Erdoğdu, 2020; Akata et al., 2020; Çetinkaya et al., 2020; Kaplan et al., 2020; Uzun and Kaya 2020a,b; Uzun et al., 2020; Acar, 2021; Altuntaş et al., 2021; Berber et al., 2021; Çetinkaya and Uzun, 2021; Doğan, 2021; Kaplan et al., 2021; Kesici and Uzun, 2021; Uzun, 2021a,b; Uzun and Kaya, 2021a,b; Uzun and Kaya, 2022) any member of the genus have been reported from Türkiye.

The study aims to make a contribution to the mycobiota of Türkiye by adding a new ascomycete genus record.

2. Material and Method

Specimens were collected from Sit village of Şirvan (Siirt) district during a field study in 2014. Morphological and ecological characteristics of the samples were recorded during the field study and they were photographed in their natural habitats. Then, they were taken to the laboratory and microscopic investigations were carried out on them. Observations of apothecia were made using a Leica EZ4 stereo-microscope under magnifications up to 35×. Microscopic investigation was performed under a Leica DM500 light microscope mounted with a Leica ICC50 HD camera. Reagents such as 5% KOH and Iodine (IKI) were used as investigation media. Identification was performed with the aid of the relevant literature (Kimbrough et al., 1972; Thind et al., 1978; Bell, 2005; Thompson 2013; Melo et al., 2015; Kušan et al., 2018).

3. Results

Ascomycota Caval.-Sm.

Pezizomycetes O.E. Erikss. & Winka

Pezizales J. Schröt.

Coprotus ochraceus (P. Crouan & H. Crouan) J. Moravec

Macroscopic and microscopic features: Apothecia scattered to gregarious, without stalk, globular-discoid, pale yellowish to orange or ochraceous, 0.5-1.8 mm diam., smooth and without hairs (Fig. 1a,b). Excipulum of large celled textura globulosa-angularis with basal cells 20-40 µm diam., weakly to non-cyanophilous, marginal cells elongated. Paraphyses cylindric-clavate, sometimes curved at tips, septate, 2-3 µm thick at the base, enlarged towards the apices up to 5-7 µm, with yellowish cytoplasmic contents (Fig. 1f,g,h). Asci eight-spored, cylindrical, rounded above, tapers towards the base, 110-190 × 15-30

µm, no color change in iodine (Fig. 1e,f). Ascospores usually uniseriate, hyaline, smooth, ellipsoid, rounded at poles, 14-19 × 9-13 µm, each with a conspicuous de Bary bubble (Fig. 1c,d).

Coprotus ochraceus grows on dung of many animals especially of ruminants (cow, sheep, horse, deer and rabbit) from spring to autumn (Kimbrough et al., 1972; Thind et al., 1978; Thompson 2013; Melo et al., 2015).

Specimen examined: Siirt, Şirvan, Sit village, on accumulated herbivorous manure, 38°05′ N, 42°04′ E, 1328 m, 13.04.2014, Y. Denğiz 130.

4. Discussions

Coprotus ochraceus is reported for the first time from Türkiye. General characteristics of the investigated specimen are in agreement with those given in literature (Kimbrough et al., 1972; Thind et al., 1978; Thompson 2013; Melo et al., 2015).

The species seems to has a cosmopolitan distribution. It is currently known from four continent (Asia, America, Australia, Europe) and 20 countries (Argentina, Belgium, Bermuda, Brazil, Canada, Czech Republic, Denmark, France, Germany, U.S.A, India, Pakistan, Italy, Norway, Poland, Puerto Rico, Republic of Tajikistan, Sweeden, Venezuela, United Kingdom) (Melo et al., 2015).

Coprotus ochraceus shows general aspect similar to *Cheilymenia granulata*, but can be distinguished from *C. granulata* with the smaller and weakly or non-cyanophilous excipular cells. *Coprotus vicinus* and *C. luteus* are also similar to *C. ochraceus* in color and substrate choice, but *C. vicinus* has smaller asci and larger ascospores (up to 25 µm in lenght) and *C. luteus* has smaller ascospores (8-13 × 4.5-7 µm).

Conflict of Interest

Authors have declared no conflict of interest.

Authors' Contribution

The authors contributed equally.

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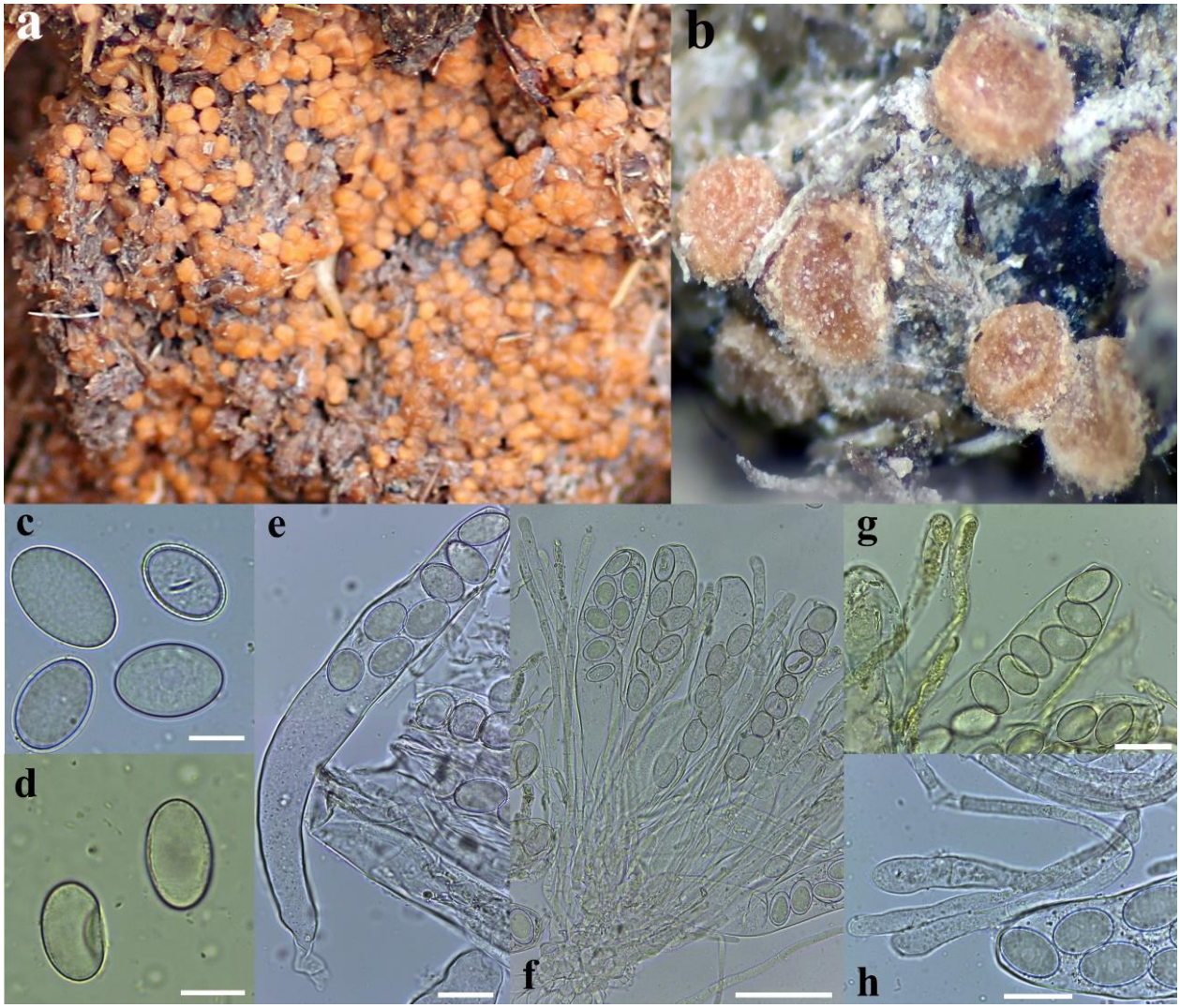

Figure 1. *Coprotus ochraceus:* a- apothecia on natural substrate, b- apothecia under stereo microscope (35× magnification), c- ascospores (in water, bar= 10 µm), d- ascospores (in IKI, bar= 10 µm), e- ascus (bar= 20 µm), f- asci and paraphyses (bar= 50 µm), g- apices of an ascus and paraphyses (in IKI, bar= 20 µm), h- apices of an ascus and paraphyses (in water, bar= 20 µm).

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Melanoleuca tristis (*Basidiomycota, Agaricales*), a new record from western Mediterranean region of Türkiye

Oğuzhan KAYGUSUZ[1]*, Meryem Şenay ŞENGÜL DEMİRAK[2]
[1]*Isparta University of Applied Sciences, Atabey Vocational School, Department of Plant and Animal Production, Isparta, Turkey*
[2]*Tokat Gaziosmanpasa University, Science and Arts Faculty, Department of Molecular Biology and Genetics, Tokat, Turkey*
**okaygusuz03@gmail.com, [2]senay.sengul@gop.edu.tr*

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Türkiye'nin Batı Akdeniz bölgesinden yeni bir kayıt, *Melanoleuca tristis* (*Bazidiyomikota, Agaricales*)

Abstract: A rare species, *Melanoleuca tristis* is reported for the first time from Türkiye on sandy soil under *Pinus nigra* subsp. *pallasiana* forest, a characteristic habitat of the Mediterranean region. Phylogenetic analysis based on a dataset of the nuclear ribosomal DNA (rDNA) internal transcribed spacer (ITS) sequences is performed to support the identification and to compare with similar taxa. The description, a color photograph of the basidiomata and microscopic drawings are given.

Key words: Basidiomycota, *Melanoleuca*, new record, phylogenetic analysis, taxonomy, Türkiye

Özet: Nadir bir tür olan *Melanoleuca tristis*, ilk defa Türkiye'de, Akdeniz bölgesinin karakteristik bir habitatı olan *Pinus nigra* subsp. *pallasiana* ormanındaki kumlu toprak üzerinden rapor edilmiştir. Nükleer ribozomal DNA (rDNA) internal transcribed spacer (ITS) sekanslarının bir veri setine dayanan filogenetik analiz, türün belirlenmesini desteklemek ve benzer taksonlarla karşılaştırmak için gerçekleştirilmiştir. Bazidiyomatanın betimlemesi, renkli fotoğrafı ve mikroskobik çizimler verilmiştir.

Anahtar Kelimeler: Bazidiyomikota, *Melanoleuca*, yeni kayıt, filogenetik analiz, taksonomi, Türkiye

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1. Introduction

Melanoleuca Pat. (Yılanmantarı) is an agaric genus in *Tricholomataceae* R. Heim ex Pouzar (Singer, 1986; Sesli et al., 2020). According to the Index Fungorum, it is represented by more than 400 species, while only 60 species are accepted worldwide (He et al., 2019).

Melanoleuca species exhibit very similar macroscopic features that make morphological identification difficult or misleading. Furthermore, its species may have variable characters influenced by environmental conditions, which makes species delimitation more complicated (Bon, 1991; Boekhout, 1999). With the advent of molecular studies, it is now accepted that morphology-based classification of its species is insufficient. In addition to some features, such as basidioma colour, odour, stipe ornamentation, presence/absence of cheilocystidia, shape of cystidia, spore size and ornamentation, and pileipellis structure, phylogenetic methods should also be considered for a reliable taxonomic identification and infrageneric classification in this genus (Vizzini et al., 2011).

Melanoleuca species are often characterised by a collybioid to tricholomatoid basidioma, convex to slightly depressed pileus, emarginated to adnate to shortly decurrent lamellae, warty and strongly amyloid spores, white to pale-yellowish spore print, cutis to trichoderm pileipellis, mostly hymenial cystidia and lack of clamp connections (Singer 1986; Boekhout 1988, 1999; Bon 1991; Vesterholt 2008; Vizzini et al., 2011).

In the last few decades, molecular studies have revealed important information about the infrageneric classification and species identification in the genus *Melanoleuca* and have indicated that *Melanoleuca* species are closely related to those of Pluteaceae Kotl. & Pouzar and Amanitaceae E.-J. Gilbert (Moncalvo et al. 2002; Matheny et al. 2006; Garnica et al. 2007; Justo et al. 2011; Vizzini et al. 2011; Sánchez-García et al. 2014; Antonín et al., 2014, 2017, 2021; Binder et al. 2014; Yu et al. 2014; Kalmer et al., 2018; Xu et al., 2019; Pei et al., 2021). Vizzini et al. (2011) revealed that *Melanoleuca* is monophyletic, and divided into two subgenera, subg. *Urticocystis* and subg. *Melanoleuca*, in which the former includes taxa mainly with urticocystidia but also with macrocystidia and brightly coloured pilei, while the latter possesses basidiomata with non-septate macrocystidia, or rarely without cystidia.

In Turkey, only 28 *Melanoleuca* species have been reported so far, most of which lack molecular support (Sesli et al., 2020; Solak and Türkoğlu, 2022). In this study, molecular and morphological investigations of collections from the Meditteranean region of Turkey are provided. Our results indicate that the specimen is *Melanoleuca tristis* M.M. Moser, a new record from a non-European continent and for Turkish mycobiota.

2. Material and Method

2.1. Morphological studies

Specimens were collected from Isparta Province during the autumn season in 2012. Pictures were taken in the field and

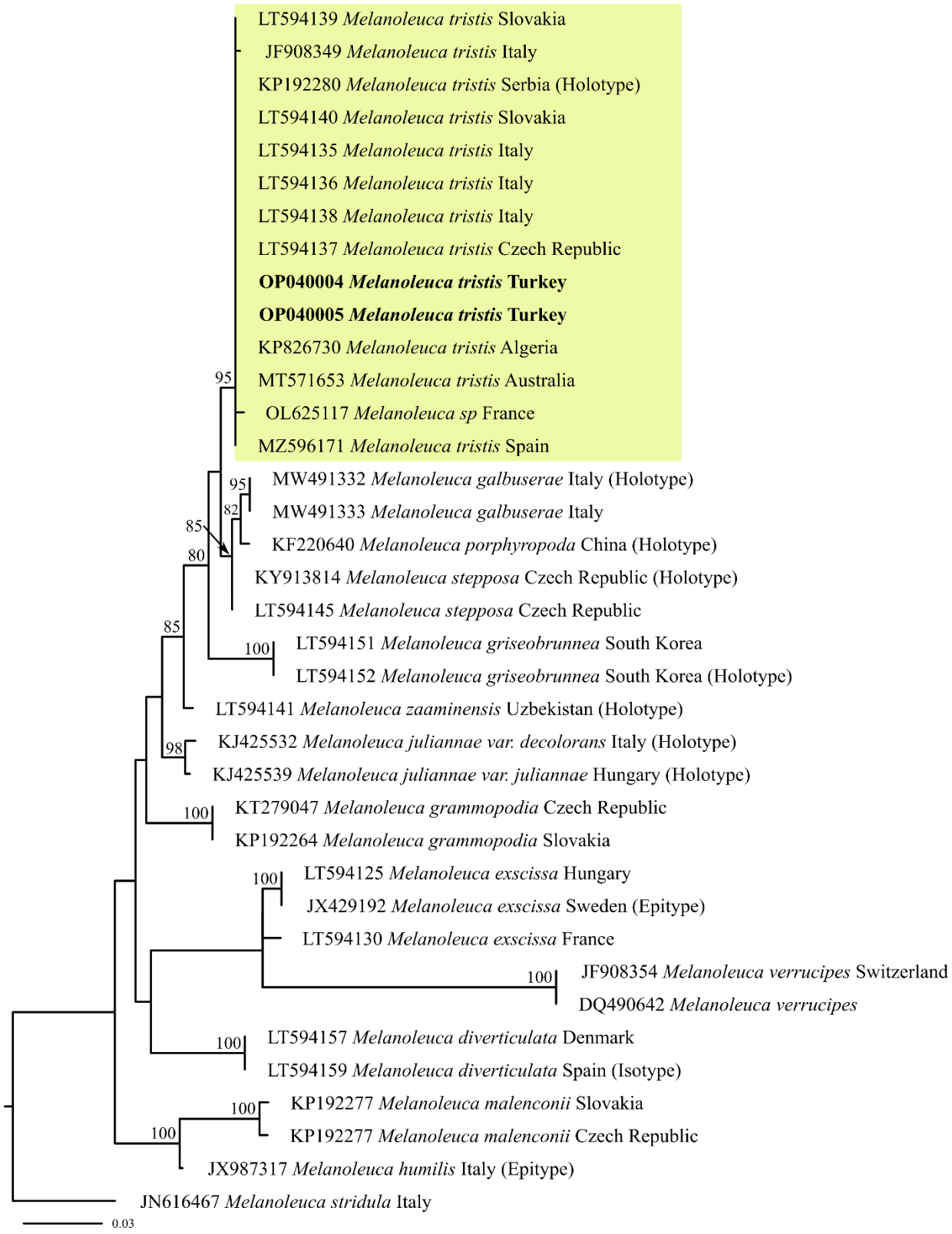

Figure 1. Maximum likelihood tree illustrating the phylogeny of *Melanoleuca tristis* and related taxa based on nrITS dataset (ML ≥ 80% is indicated). The tree is rooted with *Melanoleuca stridula* (Fr.) Singer (JN616467). Newly generated sequences are shown in bold.

macroscopic characters were recorded using fresh materials. Micro-morphological observations were made using dried samples and a Leica DM750 light microscope (Leica Microsystems, Wetzlar, Germany) at magnifications of up to 400× and 1000×. Specimens were deposited at the fungarium of Isparta University of Applied Sciences, Turkey.

2.2. Phylogenetic studies

2.2.1. DNA isolation, Polymerase chain reaction and Sequencing

Using the ZR Fungal/Bacterial DNA MiniPrep kit (Zymo Research, Irvine, California) in accordance with the manufacturer's instructions, the total genomic DNA was

extracted from specimens. To amplify the nrITS region, the primer pairs ITS1F/ITS4 (Gardes & Bruns 1993; White et al. 1990) were chosen. Polymerase chain reaction was performed as described by Kaygusuz et al. (2021). Using the same primer sets, sequencing was carried out (Source Bioscience, Berlin, Germany). Editing of sequence chromatograms was done with BioEdit 7.0.5. (Hall, 1999).

2.2.2. Phylogenetic analysis

The sequences for phylogenetic analysis were chosen based on Antonín et al. (2017). The Maximum Likelihood (ML) approach was used to build the phylogenetic tree. A dataset was used for the ML analysis in RAxML v8.2.10 (Stamatakis, 2014), with 1.000 bootstrap replicates and the GTRGAMMA substitution model.

3. Results

3.1. Molecular analysis

The phylogenetic tree inferred from nrITS gene sequences was obtained from 37 fungal samples representing 779 characters. The phylogenetic analysis based on the nrITS sequences resulted in the well-supported separation of the species belonging to subg. *Urticocystis* and subg. *Melanoleuca* and supported the monophyly of the *Melanoleuca* species. Additionally, Turkish collections presented as *Melanoleuca tristis* were grouped with the holotype from Serbia (KP192280) with high statistical support (MLB = 95%, Fig. 1).

3.2. Taxonomy

Melanoleuca tristis M.M. Moser, Boletus 15: 66 (1991)

(Fig. 2)

Mycobank 129636

Macroscopic and microscopic features:

Pileus 35–50 mm diam, at first convex, then applanate with undulating margin, depressed or umbilicate at the center, non-striate at the edge, not hygrophanous, non-pruinose, smooth, glabrous, brown to dark brown (Fig. 2a). Lamellae crowded, emarginate, sinuate, grayish to gray, with concolorous edge. Stipe 20–30 × 3–6 mm, cylindrical or slightly widened under pileus, longitudinally coarsely fibrillose, dark grey-brown to black-brown. Flesh dark grey-brown. Smell and taste indistinct.

Basidiospores (7.3–)7.8–9.7(–10.0) × (5.3–)5.8–6.2(–6.8) µm, ellipsoid to broadly ellipsoid, verruculose, irregular warts, thin walled, amyloid (Fig. 2b). Basidia 30–45 × 8–12 µm, clavate, hyaline, 4–spored (Fig. 2c). Cheilocystidia frequent, 30–50 × 6–10 µm, clustered, fusiform to subulate to subcylindrical, hyaline, thin-walled (Fig. 2d). Pleurocystidia absent or very scattered. Pileipellis ixocutis, composed of cylindrical, 4–10 µm wide hyphae, hyaline, thin-walled.

Habit and habitat: Solitary or in small groups, on sandy soil, under *Pinus nigra* Arnold. subsp. *pallasiana* (Lamb.) Holmboe.

Materials examined: Turkey, Isparta Province, Sav district, on soil under *Pinus nigra* subsp. *pallasiana*, alt. 1000 m, 01 November 2012, O. Kaygusuz, OKA-TR1220; GenBank: nrITS OP040004; idib, under *P. nigra* subsp. *pallasiana*, alt. 1050 m, 02 November 2012, O. Kaygusuz, OKA-TR1221; GenBank: nrITS OP040005.

4. Discussions

Melanoleuca tristis is a rare species which is distributed on the European continent. Its distinctive features include large basidiomata, a dark brown pileus, a stipe that varies in color from dark gray-brown to black-brown, grayish to gray lamellae, somewhat narrow basidiospores, a well-developed caulohymenium and large cheilocystidia (Moser, 1991; Antonín et al., 2017, 2018). The Turkish specimens also fit well with the characters of the originally described *Melanoleuca tristis* (Moser, 1991; Antonín et al., 2017, 2018).

Based on the molecular data, species of the genus *Melanoleuca* formed a monophyletic group, and our results are congruent with those of Vizzini et al. (2011). However, both molecular and morphological characters should be considered for a reliable identification. According to the ITS region phylogeny, *Melanoleuca tristis* is phylogenetically closely related to *Melanoleuca galbuserae* Antonín, Ševčíková, Para & Tomšovský, *M. porphyropoda* X.D. Yu and *M. stepposa* Vacek. However, morphologically, *Melanoleuca galbuserae* differs from *M. tristis* by smaller basidiomata (up to 40 mm broad), a light beige to dirty ochre-brown pileus, dirty whitish lamellae, ochre to pale brown or gray-brown stipe, and a different habitat (Antonín et al., 2021). *Melanoleuca porphyropoda*, originally described from China, differs from *M. tristis* by having an orange-cinnamon pileus, a purplish stipe, larger basidiospores (8.0–12.0 × 4.5–8.0 µm) and lacking cystidia (Yu et al., 2014). *Melanoleuca stepposa* has a dirty grey-yellow or yellow–brown pileus and white or pale cream lamellae (Antonín et al., 2017). *Melanoleuca tristis* also differs from *M. galbuserae* and *M. griseobrunnea* by a well-developed caulohymenium (Antonín et al., 2017).

Macroscopically *Melanoleuca tristis* is similar to *M. malenconii* Bon, *M. humilis* (Pers.) Pat., *M. grammopodia* (Bull.) Murrill and *M. exscissa* (Fr.) Singer. However, *Melanoleuca malenconii* differs from *M. tristis* in having a gray, ochraceous to dirty yellow pileus, a pale to grayish yellow lamellae and a more distinctly floccose stipe (Antonín et al., 2018). *Melanoleuca humilis* has a paler gray-brown pileus and stipe, which is paler in color (Antonín et al., 2018). *Melanoleuca grammopodia* differs in having grayish, whitish or brownish pileus, larger basidiomata (up to 125 mm broad) and gray-brown stipe (Antonín et al., 2018). *Melanoleuca exscissa* has a gray-brown or beige-gray pileus, whitish, grayish or pale ochraceous stipe, cream-coloured lamellae, and longer basidiospore size (7.5–11.0 × 5.0–7.0 µm) (Antonín et al., 2018).

Conflict of Interest

Authors have declared no conflict of interest.

Authors' Contributions

The authors contributed equally.

Acknowledgements
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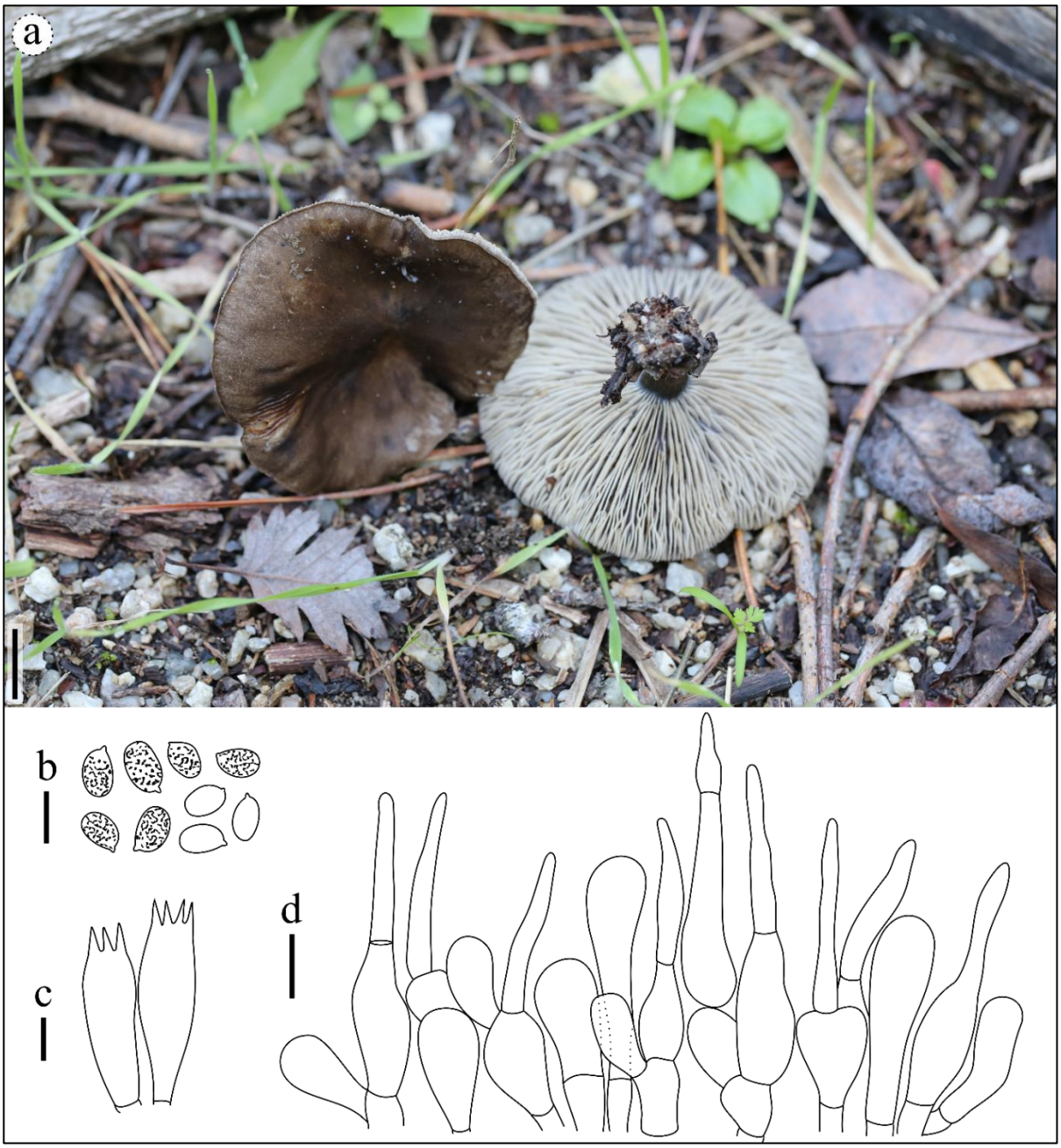

Figure 2. *Melanoleuca tristis*: a. Basidiomata, b. Basidiospores, c. Basidia, d. Cheilocystidia Scale bars: a = 10 mm, b-d = 10 μm.

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Interactive effect of thiourea application on morphological and physiological characteristics in *Cicer arietinum* L. grown at different temperatures

Sema LEBLEBİCİ[1,2], Fadime DONBALOGLU BOZCA[1*]
[1]*Bilecik Şeyh Edebali University, Science Faculty, Department of Molecular Biology and Genetics, Bilecik, Turkiye*
[2]*Bilecik Şeyh Edebali University, Biotechnology Application and Research Center, Bilecik, Turkiye*
**fadime.donbaloglu@bilecik.edu.tr, [2]leblebicisema@gmail.com*

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Farklı sıcaklıklarda yetiştirilen *Cicer arietinum* L.'de tiyüre uygulamasının morfolojik ve fizyolojik özellikler üzerine interaktif etkisi

Abstract: Global warming affects many metabolic events in plants and significantly reduces yield and product quality. One of the physiological events most affected by heat stress is nitrogen metabolism. In this study, 5 and 10 mM thiourea was applied to chickpea plants grown at 15, 25, and 35 °C and it was aimed to determine how the plant can cope with heat stress with nitrogen supplementation. It was determined that the root length decreased significantly at all three temperatures depending on the increasing thiourea concentration, while the shoot length increased at 15 and 35 °C compared to the control. There was a decrease in root fresh weight in all three experimental groups due to increasing thiourea concentrations. Only at 5 mM at 15 °C was a highly significant increase seen over the control. When the experimental groups at all temperatures were compared, the highest chlorophyll a, b, and total chlorophyll values were found at 35 °C. It was determined that SOD activity decreased at all three temperatures compared to the control, while CAT and APX activity increased. A significant increase in NR and GS activity was determined in both thiourea treatments at 25 and 35 °C compared to the control.

Key words: Antioxidant enzymes, chickpeas, morphological parameters, heat stress, nitrogen metabolism

Özet: Küresel ısınma bitkilerde birçok metabolik olayı etkilemekte, verimi ve ürün kalitesini önemli ölçüde düşürmektedir. Sıcaklık stresinden en çok etkilenen fizyolojik olaylardan biri de azot metabolizmasıdır. Bu çalışmada, 15, 25 ve 35 °C'de yetiştirilen nohut bitkisine 5 ve 10 mM tiyoüre uygulanmış ve azot takviyesi ile bitkinin sıcaklık stresi ile nasıl başa çıkabileceğinin belirlenmesi amaçlanmıştır. Artan tiyoüre konsantrasyonuna bağlı olarak her üç sıcaklıkta da kök uzunluğunun önemli ölçüde azaldığı, sürgün uzunluğunun ise kontrole göre 15 ve 35 °C'de arttığı belirlendi. Artan tiyoüre konsantrasyonlarına bağlı olarak her üç deney grubunda da kök taze ağırlığında bir azalma olmuştur. 15 °C'de sadece 5 mM'de kontrol üzerinde oldukça önemli bir artış gözlenmiştir. Tüm sıcaklıklardaki deney grupları karşılaştırıldığında en yüksek klorofil a, b ve toplam klorofil değerleri 35 °C'de tespit edilmiştir. Her üç sıcaklıkta da kontrole göre SOD aktivitesinin azaldığı, CAT ve APX aktivitesinin ise arttığı belirlenmiştir. Kontrole kıyasla 25 ve 35°C'de her iki tiyoüre uygulamasında da NR ve GS aktivitesinde önemli bir artış tespit edilmiştir.

Anahtar Kelimeler: Antioksidan enzimler, nohut, morfolojik parametreler, sıcaklık stresi, azot metabolizması

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1. Introduction

Today, global warming causes an increase in temperature and drought throughout the world, and changing climatic conditions pose a serious threat to plant yield and production (Ahmad et al., 2014). In cases where the temperature is below or above the optimum degree, plants create one or more of the escape, avoidance, tolerance, resistance, adaptation, and adaptation responses by creating physiological, biochemical, metabolic, and molecular changes (Bohnert et al., 2006). High temperature causes acceleration of molecular movements in the cell, loosening of intermolecular bonds, and more fluidity of the cell membrane (Kabay and Şensoy, 2017). Apart from these, when the temperature rises above 40 °C, the photosynthesis level of most plants is seriously affected. In addition, it causes protein denaturation, degradation, and enzyme inactivation and adversely affects the protein mechanism (Akladious, 2014). These negative effects cause starvation, growth inhibition, a decrease in ion flow, increase of toxic compounds and reactive oxygen species (ROS) in the plant (Wahid, 2007). It has been reported that the nitrogen source added externally in some plants facilitates the plant to cope with heat stress (Akladious, 2014).

Plants are affected by low temperatures as well as high temperatures. Low-temperature stress is generally observed at temperatures of 15 °C and below (Kumar et al., 2011). Plants under cold stress synthesize various molecules based on carbohydrates and amino acids, which have antifreeze properties in their sap. However, changes occur in the amount of protein and enzyme activity. There are protective enzymes such as SOD, CAT, and APX, which scavenge the reactive oxygen radicals accumulated in plants exposed to both low and high-temperature stress and minimize the resulting stress damage (Aslantaş et al., 2010). Low and high-temperature stress affects almost all physiological events and chemistry of the plant as well as nitrogen metabolism. It has been stated in studies that ambient temperature is effective on nitrogen uptake by plants and that temperature increase reduces nitrate (NO^{-3}) and ammonium ($NH4^{+}$) uptake (Clarkson and Warner, 1979).

Chickpea (*Cicer arietinum* L.), a plant rich in starch, vitamins, and minerals, is one of the most important legumes cultivated in more than 50 countries on all continents of the world. One of the most important features of leguminous plants, of which chickpea is a member, is that it is very rich in nitrogen maintenance. Plants take nitrogen from the soil in the form of inorganic nitrate and ammonium (Nasr Esfahani et al., 2014). Nitrate, which is taken up by the roots and transported to the target organs via the xylem, is reduced to nitrite by cytosolic nitrate reductase (NR), and nitrite, which passes into the chloroplast, is reduced to ammonium by nitrite reductase. Ammonium taken directly from the soil or converted from nitrate is assimilated into organic compounds via glutamine synthetase (GS)/glutamate synthase (Nasr Esfahani et al., 2014).

This study mainly aimed to investigate the eco-physiological changes caused by high and low temperatures in a *Fabaceae* Lindl. Member, *C. arietinum*, which is important in terms of nitrogen content. It also aimed to determine the effects of temperature change on nitrogen metabolism, to compare the morphological and physiological characteristics of the plant with nitrogen supplementation, and to reveal the extent to which the plant can cope with heat stress.

2. Material and Method

2.1. Abiotic stress applications and morphological measurements

In this study, the seeds of the Işık-05 cultivar of the Chickpea plant were used as experimental material. Seeds were kept in 10% NaClO for 5 minutes and then sterilized by passing through distilled water 3 times. 6 seeds were planted in each pot and the experiments were carried out in 3 repetitions. The seeds were irrigated with pure water only until the cotyledons emerged, and then each pot was watered with 100-150 ml of distilled water (control group) and 2 different thiourea solutions, 5 and 10 mM. The sown seeds were grown for 6-8 weeks at 15/10, 25/20, and 35/30˚C, respectively, in a 16-hour light and 8-hour dark photoperiod. Root-stem lengths (cm), and root-stem fresh and dry weights (g) of the grown plant samples were determined.

2.2. Chlorophyll analysis

To determine the chlorophyll content of the plant samples, the fresh leaf sample of each concentration was weighed 0.05 g and homogenized with 15 ml of 80% acetone, filtered through filter paper, and the final volume was completed to 15 ml with 80% acetone. The absorbance values of the homogenate were measured in 3 replicates at 645 nm and 663 nm wavelengths in the spectrophotometer. The chlorophyll a, b, and total chlorophyll contents of the leaf samples were determined as mg/ml (Arnon, 1949).

2.3. Protein extraction of leaf samples

For the extraction process, 0.5 g of the leaf samples were weighed, powdered with liquid nitrogen, and homogenized with 5 ml extraction buffer. The obtained homogenate was centrifuged at 20000 g for 20 min at +4 °C and the supernatant portion was used for enzyme activity determination. Differently from other enzymes, 5 mM ascorbic acid was also added to the extraction buffer to measure APX activity (Sairam et al., 2000).

2.4. Determination of total protein content

Since protein amounts were used while calculating enzyme activities, the protein contents of all leaf extracts were determined. The Bradford method and bovine serum albumin (BSA) were used as standards for determining the protein concentrations of leaf samples (Bradford, 1976).

2.5. Determination of antioxidant enzyme activities

In the study, SOD and CAT activity of plant leaf samples were determined according to Tepe and Aydemir (2011), and APX, NR, and GS activities were determined according to Cervilla et al. (2007) with minor modifications.

2.6. Statistical analysis

Each study with control and experimental groups was performed with at least 3 independent and 3 dependent replications. For statistical analysis, the p-value was calculated by using a two-way analysis of variance (Two-Way ANOVA) in the Graphpad program.

3. Results

3.1. Determination of morphological parameters

In this study, the control temperature of the study was established as 25°C. It is used in 15°C low-temperature applications and 35°C high-temperature applications. When the temperature experimental groups were compared with the plant samples at the control temperature, it was determined that the root length increased while the stem length decreased in the control group and 10 mM thiourea at low temperature ($p<0.05$; $p<0.001$; $p<0.01$, respectively).

In the application of 5 mM thiourea at high temperature, only the root length decreased compared to the experimental groups at control temperature ($p<0.05$); it was determined that the stem length showed a significant decrease in control, 5 and 10 mM thiourea application ($p<0.001$; $p<0.01$; $p<0.001$, respectively) (Table 1).

Compared to the experimental groups at control temperature, the root fresh weight significantly increased at 5 and 10 mM thiourea at low temperature stress ($p<0.001$; $p<0.01$, respectively), while at high temperature stress the control and both thiourea application it was determined that there was a significant decrease ($p<0.001$) (Table 2 and 3). There was no statistically significant change in root dry weights of all experimental groups.

3.2. Photosynthetic pigment amounts

Low and high temperatures experimental groups were compared with the control temperature, and a significant increase was observed in the amount of chlorophyll a, b, and total chlorophyll only in the control group ($p<0.001$) at 15 °C, and in the control, 5 and 10 mM thiourea application at 35 °C ($p<0.001$) (Fig. 1).

3.3. Total amount of protein

It was determined that while low-temperature stress increased the total protein amount ($p<0.001$), high-temperature stress decreased it ($p<0.05$). The thiourea application with low temperature caused a significant decrease in only 5 mM concentration ($p<0.05$). The interactive effect of thiourea application at high temperatures caused a significant decrease in both thiourea concentrations ($p<0.001$; $p<0.01$, respectively) (Fig. 2).

Table 1. Morphological characteristics of plant samples

		Root Length (cm)	Stem Length (cm)	Root Fresh Weight (g)	Root Dry Weight (g)	Stem Fresh Weight (g)	Stem Dry Weight (g)
15°C	Control	27.89±0.16*	24.25±0.82*	1.54±0.01	0.11±0.003	1.63±0.08***	0.25±0.02*
	5 mM	24.40±0.81	25.02±0.69	2.47±0.06***	0.12±0,000	1.83±0.07***	0.28±0.04
	10 mM	21.18±0.64**	19.42±1.44***	1.00±0.10**	0.07±0.006	1.20±0.17*	0.22±0.02
25°C	Control	23.52±1.29	29.59±0.59	1.60±0,10	0.13±0.007	2.22±0.04	0.45±0.01
	5 mM	22.23±0.99	28.77±1.36	0.97±0.16	0.08±0.014	0.95±0.00	0.22±0.01
	10 mM	16.15±0.07	29.40±0.28	0.78±0.04	0.06±0.007	0.78±0.04	0.27±0.01
35°C	Control	22.50±2.83	14.90±0.14***	0.49±0,08***	0.04±0.002	0.71±0.11***	0.10±0.02**
	5 mM	16.45±3.18*	19.39±0.97**	0.32±0.02***	0.03±0.002	0.92±0.01	0.13±0.02
	10 mM	13.75±1.20	17.70±1.84***	0.41±0.01***	0.04±0.003	0.84±0.15	0.12±0.02

*, 15°C and 35°C experimental groups compared with 25°C control groups. * p<0.05; ** p<0.01; *** p<0.001

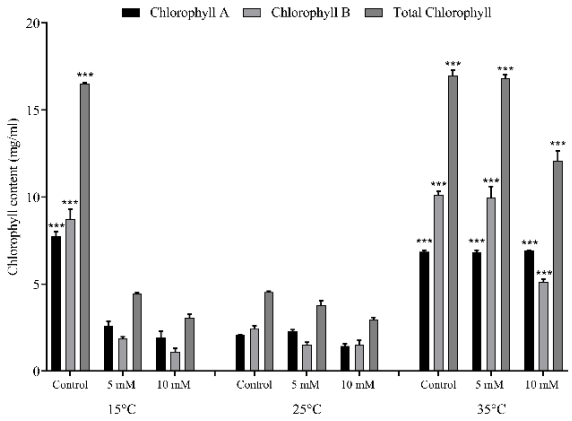

Figure 1. Photosynthetic pigment amounts of chickpea leaves treated with thiourea (mg/ml) (*, 15°C, and 35°C experimental groups compared with 25°C control groups. * p<0.05; ** p<0.01; *** p<0.001)

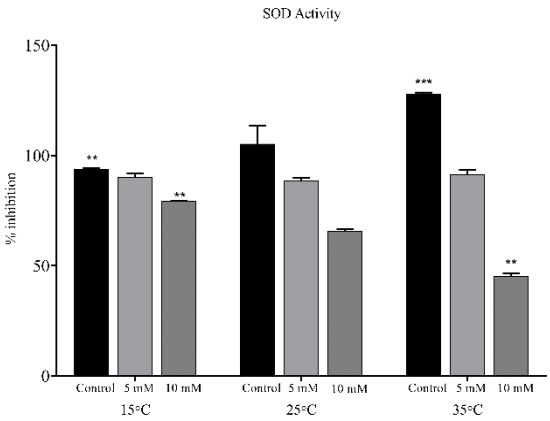

Figure 3. SOD activity of chickpea samples treated with thiourea (%inhibition) (*, 15°C, and 35°C experimental groups compared with 25°C control groups. * p<0.05; ** p<0.01; *** p<0.001)

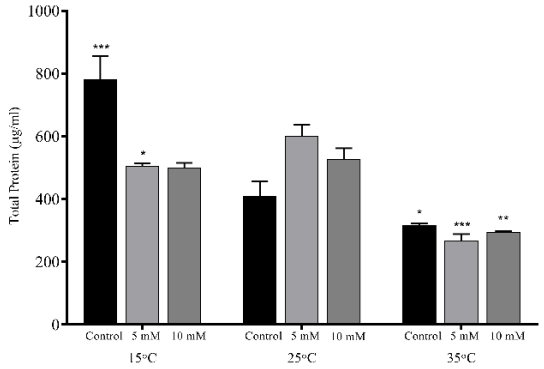

Figure 2. Total protein amounts of chickpea leaves treated with thiourea (mg/ml) (*, 15°C, and 35°C experimental groups compared with 25°C control groups. * p<0.05; ** p<0.01; *** p<0.001)

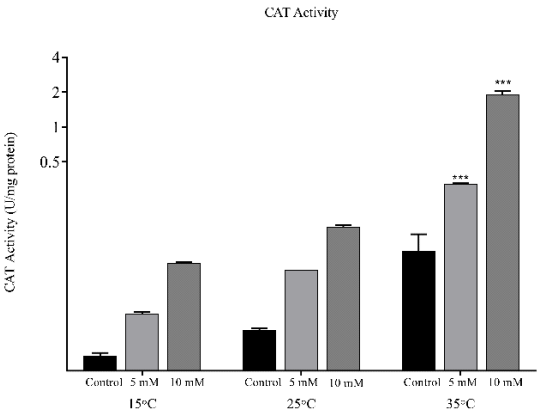

Figure 4. CAT activity of chickpea samples treated with thiourea (U/mg protein) (*, 15°C, and 35°C experimental groups compared with 25°C control groups. * p<0.05; ** p<0.01; *** p<0.001)

3.4. Antioxidant enzyme activities

While determining the antioxidant enzyme activities, the experimental groups of plant samples grown at three different temperatures and applied to two different concentrations of thiourea (5 and 10 mM) were compared. When experimental groups in the low and high temperatures were compared with the control temperature, a decrease at low temperature and an increase at high temperature were observed (p<0.01; p<0.001, respectively).

Low temperature and 10 mM thiourea application increased SOD activity (p<0.01), and high temperature and 10 mM thiourea application decreased SOD activity (p<0.01 (Fig. 3). The experimental groups of the control temperature were compared, and only the samples applied to 5 and 10 mM thiourea at high temperature showed a quite significant increase at CAT activity (p<0.001) (Fig. 4). While low-temperature application alone decreased APX activity, high-temperature application increased APX activity. (p<0.001; p<0.01, respectively). The interactive effect of thiourea with a low temperature significantly increased the APX activity at both thiourea concentrations (p<0.01;

p<0.001, respectively). Similarly, the combined application of high temperature and thiourea increased APX activity only at 5 mM concentration (p<0.001) (Fig. 5).

3.5. Nitrogen metabolism enzyme activities

Both low temperature and high-temperature stress alone caused a significant decrease in GS activity compared to the control temperature (p<0.01). Both low temperature and thiourea combined application and high temperature and thiourea combined application significantly increased GS activity at 5 mM concentration and decreased it at 10 mM concentration (p<0.001; p<0.01, respectively) (Fig. 6).

It was determined that there was a significant increase in NR activity at both low and high temperatures compared to the control temperature (p<0.01; p<0.001, respectively). The combined effect of low temperature and thiourea caused a significant decrease in NR activity in both thiourea concentrations (p<0.001), while the combined application of high temperature and thiourea caused a significant increase (p<0.001) (Fig. 7).

4. Discussions

4.1. Changes in morphological parameters

Global warming, which is the main subject of the study, causes sudden low temperatures as well as high temperatures. Low temperatures cause changes in the morphology and yield of plants, just like high temperatures. Some plants are extremely sensitive to cold, especially in germination and early seedling development. Cold stress significantly impairs germination and reduces seedling viability, negatively affecting root-stem length and biomass, thereby restricting growth (Croser et al., 2003; Aslantaş et al., 2010; Hussain et al., 2018; Donbaloglu Bozca and Leblebici, 2022). For this reason, the response of plants to stress first emerges in morphological characteristics. In this study, it was determined that low-temperature stress did not significantly affect root length and root fresh-dry weight of chickpea plants. Stem length and stem fresh weight decreased significantly under low-temperature stress. No significant change was detected in the dry weight of the stem (Table 1). In the literature, studies on low-temperature stress, in which different plant species were used as materials, revealed both similar and different results. In a study with chickpea, 12°C cold stress was applied to plant samples and it was found that low temperature reduced root and stem length and total dry weight (Kaur et al., 2008). Another study also reported that low-temperature stress reduced stem length and stem fresh and dry weight in chickpeas (Turan and Ekmekçi, 2011, 2014). The decrease in morphological features observed in the roots and stems of plants under cold stress can be attributed to the fact that cold stress prolongs the cell cycle and reduces division (Rymen et al., 2007; Wu et al., 2022). In addition, cold stress causes a significant reduction in root growth, branching, and root surface area. This, in turn, affects the uptake and transport of water, nutrients, and minerals. For this reason, a significant decrease is observed in the above-ground biomass of the plant (Hassan et al., 2021; Wu et al., 2022).

The increase in global temperature due to climate change is the biggest concern and is known to have harmful effects on many agricultural products (Yadav et al., 2018). Chickpea is a winter-growing legume, and when it is exposed to heat stress, significant yield losses are experienced. For chickpeas, a temperature of 35°C is a critical temperature for plant growth and yield (Gaur et al., 2014). Therefore, 35°C was chosen for high-temperature stress application in this study. As a result, it was observed that high-temperature stress significantly reduced stem length, root fresh weight, and stem fresh-dry weight.

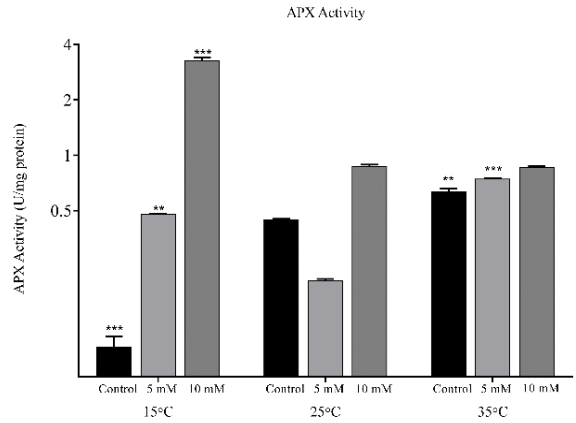

Figure 5. APX activity of chickpea samples treated with thiourea (U/mg protein) (*, 15°C, and 35°C experimental groups compared with 25°C control groups. * p<0.05; ** p<0.01; *** p<0.001)

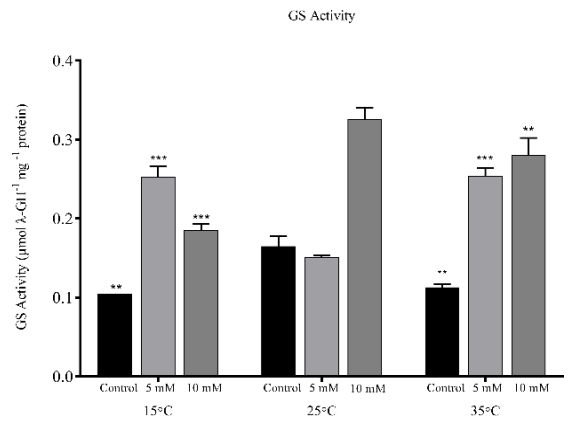

Figure 6. GS activity of chickpea samples treated with thiourea (μmol λ-GH^{-1} mg^{-1} protein) (*, 15°C, and 35°C experimental groups compared with 25°C control groups. * p<0.05; ** p<0.01; *** p<0.001)

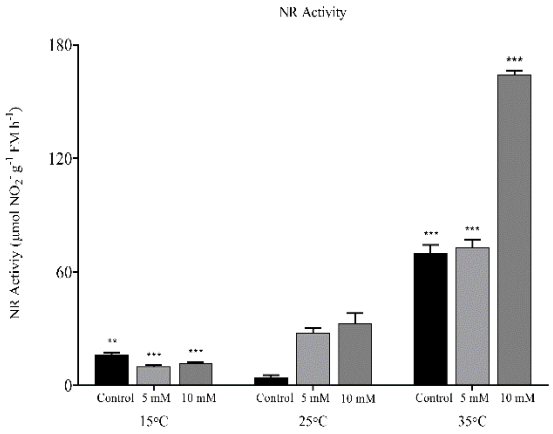

Figure 7. NR activity of chickpea samples treated with thiourea (μmol NO_2^- g^{-1} FM h^{-1}) (*, 15°C, and 35°C experimental groups compared with 25°C control groups. * p<0.05; ** p<0.01; *** p<0.001)

Contrary to this, root length and dry weight were not affected by high-temperature stress (Table 1). In another study with chickpeas, three different heat stresses were applied to the plants at the point of 35, 40, and 45°C. It was reported that heat stress reduced root-stem length (Kaushal et al., 2013). In a study with rice and corn, high-temperature stress was applied to the plants (35, 40, 45°C). It was stated that high-temperature stress reduced root-stem length in both plants (Kumar et al., 2012). Similar to cold stress, heat stress elongates the cell cycle and decreases cell division. This may be the reason for the decrease in morphological parameters in plants exposed to heat stress (Carvalho et al., 2018).

Stress tolerance can be given to the plant by administering some stress-reducing chemicals to the plant. Among the stress-reducing compounds, thiourea; is an important molecule with two functional groups: "thiol", which is an oxidative stress response, and "imino", which partially meets the nitrogen requirement under abiotic stress conditions. It provides tolerance to abiotic stresses because it dissolves in water and is easily absorbed in living tissues (Wahid et al., 2007; Akladious, 2014). Therefore, in our study, the role of thiourea in mitigating and eliminating the effects of heat stress on plants was investigated and the combined effects of heat stress and nitrogen supplementation in chickpea plants were demonstrated. In this study, thiourea application to plants subjected to low-temperature stress further reduced root and stem length. In other words, the interactive effect of the two applications stopped the division in the growing parts of the plant and delayed the plant development. Thiourea application at low temperature increased root and stem fresh weight at 5 mM concentration and decreased it at 10 mM concentration. There was no significant change in root and stem dry weights (Table 1). In addition to high-temperature stress, the interactive effect of thiourea on the root was greater than the effect of high temperature alone. Similarly, root wet weight was further reduced by the combined effect of both high temperature and thiourea application. On the contrary, the application of thiourea in plants subjected to high-temperature stress increased the stem length at both concentrations. There was no significant change in root dry weight, stem fresh and dry weight (Table 1). In the literature, there are studies that reveal the effects of thiourea application on plants and support the results of our study. In a study, thiourea was applied to chickpeas in two different ways, with irrigation water (500 ppm) and foliar application for spraying (1000 ppm). It was reported that the total plant weight and plant length increased in both applications compared to the control group (Choudhary et al., 2020). In another study with chickpeas, two different nitrogen applications, 20 and 100 kg ha^{-1}, were applied to the plants. It was stated that the root and stem dry weight of the samples decreased compared to the control group in both nitrogen applications (Kurdali, 1996). While there are studies on only heat stress or only nitrogen supplementation in the literature, there are very limited studies on the combined effect of heat stress and thiourea. The only article to the researchers' knowledge about chickpeas is the work of Laurie and Stewart (1993). In this study, nitrate supplementation was applied to plant samples subjected to high-temperature stress for 40 days. It was reported that the total dry weight of the plant increased until the 15th day, but decreased afterward. At the end of the study, it was determined that the samples had the lowest plant growth rate. It was determined that the fresh-dry weight ratio of the stem and leaves decreased, while it increased in the root (Laurie and Stewart, 1993). In a study with wheat, the seeds were pretreated with 6 mmol thioureas. Then, salt (NaCl) stress was applied to the plants, and the effect of thiourea on salt stress was investigated. As a result, it was stated that thiourea increased stem length and stem fresh-dry weight of plant samples with salt stress (Baqer et al., 2020). In other studies, using corn and mung beans, it was determined that the root and stem length, root and stem fresh-dry weights of thiourea application to plants with salt stress increased compared to the control group (only NaCl applied samples) (Kaya et al., 2015; Perveen et al., 2016). Khanna et al. (2017) investigated the combined effect of thiourea and heat stress in their study and used corn plants grown at 40°C and applied 2-20 mM thiourea as a material. They found that thiourea application increased root and stem length, and root and stem dry weight in samples under high-temperature stress (Khanna et al., 2017). In the study of Akladious (2014) with sunflower, plant seeds were impregnated with 10 and 20 mM thiourea and left to germinate for 14 days. High-temperature stress (35 and 45°C) was applied to the plants at the end of germination. Root-stem length, root-stem fresh and dry weight and stem diameter were found to increased in both thiourea applications in samples exposed to high-temperature stress (Akladious, 2014). The negative effect of stress on the morphological characteristics of the plant decreased with the effect of thiourea as mentioned above. In other words, the application of thiourea to the plant under stress regulated the cell division and cell cycle of the plant and reduced the negative effect of stress on the morphological characteristics of the plant.

4.2. Changes in physiological parameters

The effects of stress on the electron transport system, photosystems, pigments, photosynthesis-related enzyme activities, gas exchange, and chlorophyll fluorescence in plants have been investigated in many studies. It has been reported that photosynthesis is adversely affected by both low and high-temperature stress (Ibrahimova et al., 2021; Lu et al., 2014). It was observed that both low and high-temperature stress increased the chlorophyll a, b, and total chlorophyll content according to the optimum temperature (Fig. 1). Unlike our results, it has been reported in the literature that high-temperature stress reduces the total chlorophyll content in studies with chickpeas, beans, corn and rice (Kaushal et al., 2011, 2013; Awasthi et al., 2017; Bhandari et al., 2020). It has been reported that the amount of chlorophyll a, b and total chlorophyll decreases with the effect of low temperature in studies conducted with chickpea plants with low-temperature stress (Turan and Ekmekçi, 2011). Thiourea application to plants with low-temperature stress did not cause a significant change in chlorophyll a, b, and total chlorophyll levels of plants. However, thiourea application to plants with high-temperature stress significantly increased chlorophyll a, b and total chlorophyll content at both concentrations compared to optimum temperature and thiourea application (Fig. 1). In studies with corn and mung beans, different concentrations of thiourea were applied to plants with salt stress. The results showed that thiourea application reduced the amount of chlorophyll a, b, and total chlorophyll in plants with salt stress (Kaya et al., 2015; Perveen et al., 2016). In the study of Akladious (2014) with sunflowers,

high-temperature stress and two different concentrations of thiourea were applied to the plants. As a result, the amount of chlorophyll a, b, and total chlorophyll increased (Akladious, 2014). No study has been found in the literature investigating the interactive effect of thiourea with heat stress in chickpea plants.

Heat stress at the cellular level (high and low temperature) causes damage such as membrane damage, denaturation of proteins, improper synthesis, and folding, and inactivation of enzymes in mitochondria and chloroplasts (Kaushal et al., 2013; Donbaloglu Bozca and Leblebici, 2022). This study found, that low-temperature stress significantly increased the total protein amount compared to the control temperature. Considering the interactive effect of low-temperature stress and thiourea application, it was determined that the application of 5 mM thiourea significantly reduced the amount of total protein compared to the control temperature (Fig. 2). In their study conducted with chickpeas to which they applied low-temperature stress, reported that low temperature decreased total protein. On the other hand, in another study conducted at low temperatures, it was stated that low-temperature stress increased the amount of total protein (Kazemi-Shahandashti et al., 2014). The current study revelaed that the total protein amount decreased significantly under high-temperature stress compared to the control temperature. Similarly, both thiourea concentrations applied in addition to high-temperature stress significantly reduced the total protein amount (Fig. 2). In the literature, no study has been found that explains the effect of thiourea, which is made with chickpea plant and applied with heat stress, on the total protein of chickpea plant. However, there are studies in which different plants are used as materials and the interactive effects of different stresses are studied. For example, in a study conducted with coffee plants, it was shown that nitrogen application decreased the total protein amount (Reis et al., 2009). Another study established that thiourea application increased the total protein level in mung beans exposed to salt stress (Perveen et al., 2016). Both high and low temperatures also affect protein metabolism closely. Although heat stress stimulates the production of stress-related proteins, it causes the inactivation and degradation of proteins by negatively affecting the synthesis and folding of proteins in the continuation of stress (Gulen and Eris, 2003; Kaushal et al., 2013; Donbaloglu Bozca and Leblebici, 2022). The decrease in total protein content can be attributed to this.

Abiotic stresses such as drought, salinity, and low and high temperature cause an increase in ROS formation in plants due to disruption of cellular homeostasis (Mittler, 2002). Increasing ROS is also cleared by enzymatic antioxidants such as SOD, CAT, and APX (Mittler, 2002). It was observed that SOD and APX activity decreased significantly at low-temperature stress compared to the control temperature. It was established that 10 mM thiourea concentration applied in addition to low-temperature stress further reduced SOD activity (Fig. 3 and 5). In studies conducted with chickpeas under low-temperature stress in the literature, it was found that low-temperature stress reduced APX activity and increased SOD activity (Turan and Ekmekçi 2011, 2014). In another study conducted with chickpeas, it was observed that low-temperature stress increased SOD and APX activity and decreased CAT activity (Arslan et al., 2018; Karami-Moalem et al., 2018). In other studies, in which chickpea was used as the experimental material, it was reported that low-temperature stress increased CAT and APX activity, but there was no change in SOD activity (Nazari et al., 2012; Yousefi et al., 2018). Contrary to these results, in this study, it was reported that only high-temperature stress significantly increased SOD, CAT, and APX activities compared to the control temperature (Fig. 3, 4, and 5). Several studies also supported these results, in the literature, it has been stated that SOD, CAT, and APX activities increase depending on the temperature increase in chickpea, bean, rice, and corn plants exposed to high-temperature stress (Awasthi et al., 2017; Kabay and Şensoy, 2017; Bhandari et al., 2020). Unlike this study, in a study conducted with chickpeas, high-temperature stress at 35, 40, and 45 °C was applied to the plants. It was determined that high-temperature stress increased SOD and APX activity at 35 and 40°C, and decreased at 45°C. It was determined that CAT activity increased at all temperatures compared to the control (Kaushal et al., 2011). It was determined that the thiourea application in addition to the low temperature significantly increased the APX activity at both 5 mM and 10 mM concentrations and did not affect the SOD and CAT activities. However, it was shown that 10 mM thiourea concentration applied in addition to high-temperature stress significantly decreased SOD activity, while 5 mM thiourea concentration significantly increased APX activity. Also it was suggested that the activity of CAT, another of the antioxidative enzymes, increased significantly at both thiourea concentrations (Fig. 3, 4 ve 5). While there are studies on only heat stress or only nitrogen supplementation in the literature, a very limited number of studies on the combined effect of heat stress and thiourea have been found (Awasthi et al., 2017; Kabay and Şensoy, 2017; Bhandari et al., 2020). The effect of thiourea was investigated in a study on wheat samples exposed to salt stress. As a result, it was stated that thiourea increased the SOD, CAT, and APX activities of plants with salt stress (Baqer et al., 2020). In another study conducted with wheat, all thiourea applications increased SOD and CAT activities while decreasing APX activity in both the drought-stressed and non-stressed plant groups (Hassanein et al., 2015). In the study of Khanna et al. (2017) with maize, the combined effect of thiourea and heat stress increased root and stem CAT and SOD activities and reduced APX activity (Khanna et al., 2017). In a study on sunflowers, it was reported that the interactive effect of thiourea application and high-temperature stress increased SOD and CAT activities (Akladious, 2014). The decrease in NR and GS activity in plants exposed to heat stress acts as a biochemical adaptation to conserve energy by stopping nitrate assimilation (Hayat et al., 2009; Akladious, 2014). Both high and low temperature stress causes excessive ROS accumulation in the plant. The increase in ROS causes an increase in ROS-scavenging enzyme activities in the plant (Rymen et al., 2007; Aslantaş et al., 2010; Wu et al., 2022). This may be the reason for the increase in CAT and APX enzyme activities in our study. However, it has been reported that applying external substances to plants under stress reduces the negative effects of stress (Wahid et al., 2007; Akladious, 2014). This can be explained by the fact that the application of thiourea at increasing concentration reduces the SOD enzyme activity.

In this study, both low-temperature stress and high-temperature stress significantly reduced GS activity

compared to optimum temperature. Application of thiourea in addition to heat stress significantly increased GS activity at 5 mM concentration at both low and high temperatures. Conversely, 10 mM thiourea application significantly reduced GS activity at both low and high temperatures (Fig. 6). The application of low-temperature stress alone increased the NR activity significantly compared to the optimum temperature. In addition to low-temperature stress, the application of thiourea at 5 mM and 10 mM concentrations significantly reduced NR activity. High-temperature stress, on the other hand, increased the NR activity according to the optimum temperature, while the application of 5 mM and 10 mM thiourea further increased the NR activity (Fig. 7). In a study with chickpeas, it was stated that NR activity in the leaves increased with nitrogen supplementation at high temperature (Laurıe and Stewart, 1993). Another study also reported that NR activity increased in sunflowers, which was applied in both high-temperature stress and thiourea (Akladious, 2014). In a study conducted with coffee, N application at three different concentrations (0, 150, and 300 kg ha^{-1}) was used and decreased NR and GS activities in fruit development were reported (Reis et al., 2009). The decrease in the activity of nitrate metabolism enzymes in plants exposed to heat stress is related to the inhibition of nitrate assimilation in the first stage, thus saving energy (Akladious, 2014). The fact that thiourea application to plants under stress causes a significant increase in both NR and GS activity can be explained by the fact that plants can overcome the negative effects of heat stress more effectively with thiourea application.

5. Conclusion

As a result, when the effects of thiourea application at low temperature and high temperature in chickpeas, which is a protein-rich legume, were compared. It was established that nitrogen supplementation was more effective in plant growth and tolerance of cold stress at low temperature stress than high temperature stress. Especially in the study at 15°C, it was determined that 5 mM, which was a low thiourea application, positively affected plant growth when both morphological and physiological parameters were taken into account. Thus, it is thought that more agricultural production can be made with low concentration nitrogen supplementation in areas with a lower temperature than the optimum temperature required by chickpeas, which is a cool climate grain. It has been revealed that both 5 and 10 mM thiourea applications at 35°C, which are high-temperature applications, does not affect the plant's tolerance to heat stress.

Conflict of interest

The authors have declared no conflict of interest.

Authors' contribution

SL contributed conception and design of the study. SL and FDB performed the experiments, analyzed the data, and wrote the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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A floristic study on some natural and cultural sites of Adana (Türkiye) province

Ahmet Zafer TEL[1]*, İshak ORTAÇ[2], Ergün ÖZUSLU[3]
[1]*Iğdır University, Agriculture Faculty, Department of Agricultural Biotechnology, Iğdır, Türkiye*
[2]*Adıyaman University, Institute of Science, Department of Biology, Adıyaman, Türkiye*
[3] *Gaziantep Islam Science and Technology University, Engineering And Natural Sciences Faculty, Gaziantep, Türkiye*
**ergun.ozuslu@gibtu.edu.tr, [1]zafer.tel@igdir.edu.tr, [2]orto_19891989@yahoo.com.tr*

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Adana (Türkiye) ilinin bazı doğal ve kültürel sit alanları üzerine floristik bir çalışma

Abstract: This study was carried out between 2014-2016 to investigate the plant cover of seven localities (Ağyatan, Tuzla and Yumurtalık Lagoons, Yer Köprü Natural Formation, Akyatan Lagoon, Şekerpınar, Tatarlı Village Ancient Period Ruins) having cultural and natural site within the borders of Adana province in Eastern Mediterranean Region (Turkiye). As a result 219 taxa belong to 70 families and 180 genera were identified. Among them, 18 were determined in Ağyatan Lagoon, 41 in Tuzla Lagoon, 83 in Yumurtalık Lagoon, 13 in Akyatan Lagoon, 55 in Yer Köprü Natural Formation, 56 in Şekerpınar and 48 in Tatarlı Village Ancient Period Ruins. Eight of these taxa are endemic, and seven are in the critical species category.

Key words: Natural Site, Cultural Site, Flora, Adana, Turkey

Özet: Bu çalışma 2014-2016 yılları arasında Türkiye'nin Doğu Akdeniz Bölgesinde yer alan Adana ili sınırları içinde bulunan ve doğal ve kültürel sit özelliği taşıyan 7 lokalitenin (Ağyatan, Tuzla ve Yumurtalık Lagünleri, Yer Köprü Doğal Oluşumu, Akyatan Gölü, Şekerpınarı ve Tatarlı Köyü Antik Dönem Kalıntıları) bitki örtüsünü araştırmak amacıyla gerçekleştirilmiştir. Sonuçta 70 familya ve 180 cinse ait 219 takson tespit edilmiştir. Bunlardan 18 tanesi Ağyatan Lagününde, 41 tanesi Tuzla Lagününde, 83 tanesi Yumurtalık Lagününde, 13 tanesi Akyatan Lagününde, 55 tanesi Yer Köprü Doğal Oluşumunda, 56 tanesi Şekerpınar'ında 56 ve 48 tanesi de Tatarlı Köyü Antik Dönem Kalıntılarında tespit edilmiştir. Bu taksonların 8'i endemik, 7'si kritik tür kategorisindedir.

Anahtar Kelimeler: Doğal Sit, Kültürel Sit, Flora, Adana, Türkiye

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1. Introduction

Turkey has a rich plant diversity. The country has a crucial flora region in terms of geographic location, characteristics of plant geography, climate and edaphic differences, endemic plant diversity, and floristic area of the World (Davis, 1965-1985; Davis and Hedge, 1975). This study aims to investigate the floristic feature of natural and cultural sites located in Adana province, a part of the eastern Mediterranean region of Turkey. The research includes the site areas Ağyatan Lagoon, Tuzla Lagoon, Yumurtalık Lagoon, Yer Köprü Natural Formation, Akyatan Lagoon, Şekerpınar and Tatarlı Village Antic Ruins.

Phytogeographically Adana province is located in the East Mediterranean region. The research area is located in C5 according to Davis' grid-square system and have a rich and unique place in terms of plant diversity. This is due to the fact that Turkey has three different climates, landforms and rich plant diversity (Davis, 1965-1985; Davis and Hedge, 1975).

The first studies on the Flora of Turkey were carried out by Tournefort in the 1700s. Boissier carried out the first major study on the Flora of Turkiye between 1865-1888 and published "Flora Orientalis" (Boissier, 1867-1888). Turkish plants have mainly been investigated by P.H. Davis between 1965-1985 and published in 9 volumes with the name "Flora of Turkey and the East Aegean Islands". It has revealed our country's Flora with the contribution of many researchers under that day's conditions and became the primary source for floristic studies. After the publication of this Flora in 1965-1985, with the identification new taxa and carried out revisions have been publicated of the Flora of Turkey volume 10 in 1988 by Davis et al. (1988). Between 1988 and 2000, a study containing additional taxa for Flora of Turkey was published. During this period, many publications are related to the taxa in Turkey that were identified for the first time or brand new for science. (Güner et al., 2000). Later on, Güner et al. (2000) added the 11th volume to Flora of Turkey and Easgt Aegian Islands.

Meanwhile, many studies have been published regarding Turkey's Flora by researchers after the publication of additional volumes (Özhatay and Kültür, 2006; Özhatay, et al., 2009, 2011, 2013, 2015, 2017). This study again analyzes the number of taxa in Flora of Turkey, and 295 taxa (239 species and 56 subspecies) are added. In this way, the total number of taxa has reached 12301, and 163 species, eight subspecies, and 14 varieties are added as endemic. Ekim et al. (2000) and Erik and Tarikahya (2004) give the total endemic taxa of Turkiye as as 3963 (32.2%).

The list of vascular plants of Turkiye was published in 2012. According to this list, the total number of plant taxa of the Flora of Turkiye is given as 11707, 3649 of these taxa were defined as endemic and the endemism rate was determined as 31.82% (Güner et al., 2012). Today, the preparation of the Illustrated Flora of Turkiye continues and the first three volumes have been published (Güner et al., 2014; Güner et al., 2018; Güner et al., 2022).

Similar floristic studies were carried out in Yumurtalık Lagoon (Altınözlü, 2004), Akdağ and surroundings (Akıncı et al., 2018), Sarımsak Mountain and Korkun Valley (Paksoy and Savran, 2011), Kızıldağ Plateau and its surroundings (Keskin and Savran, 2020) and Karatepe-Aslantaş National Park, Harun Reşit Castle, Haruniye Thermal Springs and Şarlak Waterfall (Tel et al., 2019). In these studies, 234 taxa belonging to 186 genera, and 65 families are identified from Adana Yumurtalık Lagoon. Three endemic plant taxa are identified. The endemism rate is 3.1% (Altınözlü, 2004). Two hundred twenty-nine taxa belonging to 147 genera, and 45 families were identified from Burnaz Dunes, seven of which are endemic. Seven hundred thirty-nine taxa belonging to 362 genera, and 88 families were identified from Akdağ and its surroundings, 100 of which are endemic with an endemism rate of 13.6% (Akıncı, et al., 2018). Six hundred and fifty-two taxa belonging to 315 genera, and 78 families were identified from Sarımsak mountain and Korkun Valley. Among them 135 taxa are endemic with en endemism rate of 20.7% (Paksoy and Savran, 2011). Forty-nine taxa belonging to 110 genera, and 38 families were identified from Tatarlı Höyük with three endemic taxa and 2% endemism rate (Kavak et al., 2012). Five hundred seventy-four taxa belonging to 285 genera, and 75 families were identified from Kızıldağ Plateau and its surroundings. Among them 97 are endemic and the endemism rate is 16.8% (Keskin and Savran, 2020). One hundred sixty-four taxa belonging to 59 families, 139 genera, 135 species, 23 subspecies and six varieties, and were identified in the Karatepe-Aslantaş National Park, which is nearly a natural protected area (Tel et al., 2019).

Here we present the flora of the Ağyatan, Tuzla and Yumurtalık Lagoons, Akyatan Lagoon, the Yer Köprü Natural Formation, Şekerpınar and Tatarlı Village Ancient Period Ruins in Adana.

2. Material and Method

The investigation materials were 313 plant specimens collected from the research area between 2014-2016. During this period, 12 field studies were carried out. The voucher numbers of the collected plant specimens are given in Appendix-1. The plant specimens were dried in accordance with herbarium techniques and identified using the Flora of Turkey (Davis, 1965-1985; Güner et al., 2000). Some plant identifications were made by experts (Ahmet İLÇİM and Ergün ÖZUSLU). The collected and dried plant specimens have been deposited at the Herbarium of Adıyaman University. IUCN risk categories of threatened taxa were also traced from the relevant literature (Ekim et al., 2000, 2014; The IUCN Red List of Threatened Species [version 2021-1] (2021); Güner et al., 2012; John and Türk, 2017; Erdağ and Kürschner, 2017). The phytogeographic regions of the taxa are evaluated according to Davis (1965-1985) and Davis et al. (1988). The plant names are checked using Turkey Plants List: Vascular Plants (Güner et al., 2012), The International Plant Names Index (2022), Turkey Plant List (2022) and The Plant List (2022). The abbreviations used in the text and the floristic list are as follows: CR: Critically endangered; EN: Endangered; LC: Least concern; NT: Near threatened; VU: Vulnerable, DD: Data deficient. The floristic list is given in Appendix-1. Endemic and rare plants list have given alphabetically.

The study areas are numbered and shown in Figure 1, and the coordinate and general properties were given in Table 1 as [1] Ağyatan Lagoon, [2] Tuzla Lagoon, [3] Yumurtalık Lagoon, [4] Akyatan Lagoon, [5] Yer Köprü Natural Formation, [6] Şekerpınar Historical Bridge, [7] Tatarlı Village Ancient Period Ruins.

Figure 1: Research Areas (adopted from wikipedia)

3. Results

General characteristics of the research areas, investigated in this study, were given in Table 1. Among them, Ağyatan Lagoon is located in Karataş district of Adana province. It is a typical lagoon consisting of alluvium formed by delta sediments and dunes with the change of bed of Seyhan river. It is a first degree natural site. It is also protected as a wetland. Natural landscape elements are the sea, beach, dunes, Dalyan region, and Ağyatan lake. The area has some sand dunes between the lake and the sea, which has a flat land structure in terms of geomorphology. The surface area of it is 6.514.102,68 m^2. This lagoon shrinks significantly during the summer, and vast mud flats appear. The lake is connected to the sea by a canal from the southwest. In periods when lake water level are high, water flows from the lake to the sea through the canal and from the sea to the lake in the low periods. With the effect of rainfall and water carried by drainage channels in winter and spring, the lake water becomes sweeter. In summer, salinity increases due

Table 1. The study areas and general characteristics

Study area	Surface area (m^2)	Coordinates	Altitude (m)
Ağyatan Lagoon	6.514.102,68	36°39′37.67″ N - 35°17′06.25″ E	1
Tuzla Lagoon	17.460.549,73	36°42′32.88″ N - 35°03′20.56″ E	1
Yumurtalık Lagoon	90.208.440,02	36°41′38.59″ N - 35°33′14.37″ E	1
Akyatan Lagoon	83.796.264,3	36°38′23.81″ N - 35°15′57.17″ E	1
Yer Köprü Natural Formation	20.775,67	37°40′56.04″ N - 35°29′34.83″ E	771
Şekerpınar	31.671,49	37°28′18.48″ N - 34°51′42.63″ E	834
Tatarlı Village Antique Period Ruins	6.653,85	37°07′24.90″ N - 36°03′20.12″ E	37

to high evaporation and water ingress from the sea to the lake. The average annual rainfall of the research area is 769,9 mm. Average maximum and minimum temperatures respectively are 27,36 ºC and 10 ºC in August and May respectively. The climate diagram of Karataş district, where Ağyatan, Tuzla, Yumurtalık, and Akyatan lagoons take place, is given in Figure 2. The taxa identified from the natural site area are given in Appendix 1. Eighteen plant taxa have been identified in the Ağyatan Lagoon. Among these taxa, *Pancratium maritumum* L. (Sand Lily) is a rare taxon and is in the VU category.

Tuzla Lagoon is also located in Karataş district of Adana province. This area is a first degree natural protected area and wildlife development area. It is a wetland, and the area is under protection. The size of Tuzla Lagoon is 17.460.549,73 m^2. The area is located in the southern part of the Çukurova Delta, where the Seyhan and Ceyhan rivers flow into the Mediterranean. There is a Salt Lake close to the sea and an inner lake. Tuzla lake is filled with precipitation in winter and dries up in summer. It has a saltwater habitat. The area has a coastline of approximately 15.5 km and generally shows a natural landscape feature. It has a flat land structure geomorphologically. There are dunes between the lake and the sea. Identified taxa from Tuzla Lagoon are given in Appendix 1. Forty-two plant taxa have been identified in the area. There is one taxon that is critical species in the research area. This taxon is not endemic. *Pancratium maritimum* L. (Sand lily) is a rare taxon in the VU category.

Yumurtalık Lagoon is located in Karataş and Yumurtalık districts of Adana province. It is a natural site and Turkey's eleventh Ramsar area. It is also a nature protection area with a surfarce area of 90.208.440,02 m^2. Yumurtalık Lagoon is a natural distribution area of the rare species (*Pinus halepensis* Mill.). The area contains tree, shrub, and a grass vegetation layers. It has a flat land structure geomorphologically, and has no elevation around it. Eighty-seven plant taxa have been identified in the area (Appendix 1). Two of the identified taxa in the area (*Echinops dumanii* C. Vural (CR) and *Pancratium maritimum* L. (Sand lily) (VU)) have critical importance. In a previous study, Altınözlü (2004) presented 234 species belonging to 186 genera and 65 families from the area. Among which 223 are naturally growing species while 11 are cultivated plants. The rate of endemism is low with 3 (1.3%) endemic species, *Polygonum praelongum* Coode & Cullen (EN), *Centaurea calcitrapa* ssp. *cilicica* (Boiss. & Balansa) Wagenitz (LC), *Tripleurospermum conoclinium* (Boiss. & Honey) Hayek (LC).

Akyatan Lagoon is another research area, located in Karataş district of Adana. The area is a first and second degree natural site area. The area is also a wildlife development area. Akyatan Lake has a special topography due to its alluvial structures. This lake is the largest lagoon lake formed by the Seyhan and Ceyhan rivers in the Çukurova delta. The area has a coastline of approximately 20 km. Identified flora taxa from Akyatan Lake Natural Protected Area are given in Appendix 1. Thirteen plant taxa have been identified in this area without any critical species.

Yer Köprü Natural Formation is located in Aladağ district of Adana province. The area is also a first degree natural site area. The area is also a wetland and is under protection. Yer Köprü Natural Formation covers a surface area of 20.775,67 m^2. The area and its surroundings are located in a valley. Altitude of the area is 500 m at the bottom of the valley. Identified flora taxa in the area are given in Appendix 1. Fifty-five taxa have been identified in the area. There are three endemic taxa in the field, which are *Mattiastrum calycinum* (Boiss. & Balansa) Brand (LC), *Alkanna hispida* Hub. - Mor. (EN) and *Centaurea chrysantha* Wagenitz - (EN).

Şekerpınar is located in Pozantı district of Adana province. The area is a second degree natural site area with a surface area of 31.671,49 m^2. In addition, the area is a wetland. There are cultivated plants around the facility area, and oak species (*Quercus* spp.) are sparsely spread in the upper parts. The area and its surroundings were transformed into a cultural landscape with an anthropogenic effect. Accommodation facilities, pipelines passing through the area, and road and railway transportation networks have caused changes in the natural landscape. The area has a geomorphologically active land structure between a valley with a steep slope. Though the area has a forest character, it has lost this characteristic to a great extent, due to anthropogenic effects. The area and its surroundings have an altitude of approximately 850-900 meters. Fifty-seven plant taxa were identified in Şekerpınar, three of which are endemic (Appendix 1).

Tatarlı Village Antique Period Ruins is located in the Ceyhan district of Adana. It is a first degree natural and archaeological site. It has a surface area of 6.653,85 m^2. The area contains tree, shrub, and a grass vegetation layer. This wetland is also a residential area. The area consists of flat plains with an altitude of about 40 meters. Identified taxa from the area are given in Appendix 1. Fourty-eight plant taxa were identified in the area. There are no critical species in the area. The climate diagram of the area is shown in Figure 3.

4. Discussions

This study was carried out in seven localities within Adana province, and 219 taxa belonging to 70 families and 180

genera were determined (Appendix 1). The allocation of taxa according to phytogeographical regions are follows: Mediterranean (including East Mediterranean) 76 (34,6%), Euro-Siberian 10 (4,5%), Irano-Turanian 11 (5%), multi-regional 11 (5%), unknown phytogeographic origin 76 (35%) (Table 2). The majority of the determined taxa are Mediterranean phytogeographical elements. This is a natural result of the fact that all of the investigated areas are in this region. The presence of Euro-Siberian and Irano-Turanian elements is due to the microclimates existing in the study area. Eight of the determined taxa are endemic, and four are in rare category (Table 3). The endemism rate is 3,65% and includes 8 endemic taxa in the research area. Phytogeographic distribution of the endemic taxa are as follows: 3 taxa Mediterranean, one taxon Irano-Turanian, and four taxa unknown. Endemic taxa were evaluated according to IUCN risk categories (Ekim et al., 2000; The IUCN Red List of Threatened Species (version 2021-1), 2021). 1 CR (Critically endangered), 2 VU (Vulnerable), 2 EN (Endangered) and 3 LC (Least concern).

Table 2. Distribution of the taxa detected in the study area according to phytogeographic regions

Phytogeographic region	# of taxa	Rate (%)
Mediterranean	76	34,6
Euro-Siberian	10	4,5
Irano-Turanian	11	5
Multi regional	46	20,9
Unknown	76	35
Total	219	100

Number of determined taxa, their distribution to the families (Table 4), phytogeographical distributions, endemism rate, and IUCN reda data categories were also evaluated speerately.

In Ağyatan Lagoon, 18 taxa were identified. Most crowded familis in the region are *Juncaceae*, *Asteraceae* and *Chenopodiaceae* each with two taxa. Two of the determined taxa are Mediterranean, two are East Mediterranean, one Irano-Turanian elements while three are multi-regional and ten taxa unknown. None of the determined taxa are endemic, but *Pancratium maritimum* L. taxon is in VU category (Table 5).

Forty-one taxa belong to 27 families, and 36 genera were identified in Tuzla Lagoon (Appendix 1). *Fabaceae* and Asteraceae were found to be the most crowded families in the region each with 5 taxa. They are followed by *Poaceae* with 3 taxa (Table 4). Eleven of the determined taxa are Mediterranean, five are East Mediterranean elements, while one of them is a cultivated plant, 7 taxa multi-regional, and 17 taxa unknown. This situation can be explained by the fact that the research area falls in the Mediterranean region phytogeographical region within the holoarctic floral kingdom. There are no endemic plants in our study area. Two of the them, *Pancratium maritimum* L. and *Zygophyllum album* L., are in VU category (Table 5).

Eighty three taxa were determined in Yumurtalık Lagoon (Appendix 1). In the study area, the wealthiest families regarding the number of taxa are *Fabaceae* (10), *Asteraceae* (8), and *Chenopodiaceae* (6). The first three families with the most genera and their ratios are given Table 4. Distribution of taxa in phytogeographical regions

Table 3. The number of families, taxa, endemic taxa and the rate of endemism in the study areas.

Study area	Family	Taxa	# of end. taxa	End. Rate (%)
Ağyatan Lagoon	14	18	-	0
Tuzla Lagoon	27	41	-	0
Yumurtalık Lagoon	39	83	1	2.4
Akyatan Lagoon	12	13	-	0
Yer Köprü Natural Formation	39	55	3	1.3
Şekerpınar	32	57	3	1.3
Tatarlı Village Antique Period Ruins	14	18	-	0

is as follows: 24 taxa are Mediterranean, 11 are East Mediterranean, four are Irano-Turanian, one is Euro-Siberian, 15 are multi-regional, and three taxa are unknown. The situation is a relevant result in terms of phytogeography. Among the determined taxa, one, *Echinops dumanii* C. Vural, is endemic, and three, *Pinus brutia* Ten., *Pancratium maritimum* L. and *Dianthus polycladus* Boiss, are in vulnerable "VU" category (Table 5). Altınözlü (2004) presented 234 taxa belonging to 65 families and 186 genera from Yumurtalık Lagoon Nature Protection Area with an endemism rate of 1.3%. The endemic taxa presented in Altınözlü (2004) are *Polygonum praelongum* Coode & Cullen (EN), *Centaurea calcitrapa* ssp. *cilicica* (Boiss. & Balansa) Wagenitz (LC), *Tripleurospermum conoclinium* (Boiss. & Bal) Hayek (LC). Current literature include a total of 7 taxa in Turkey Plants Red List from the region. Those non endemic ones are *Helianthemum stipulatum* (Forssk.) C.Chr. (VU), *Zygophyllum album* L. (VU), *Pancratium maritimum* L. (EN), and *Heliotropium ovalifolium* Forssk. (CR) (Ekim et al., 2000).

Thirteen taxa were identified in Akyatan Lagoon (Appendix 1). The most crowded families in the region are *Pinaceae*, *Poaceae* and *Verbanaceae* each with 2 taxa (Table 4). Among the determined taxa, 3 are Mediterranean elements, four are East Mediterranean elements while two taxa are multi-regional and four unknown. There is no Irano-Turanian element in the region. This results are also thought to be related with the phytogeography of the region. No endemic species were determined in the region, but *Pinus brutia* Ten. is in vulnerable “VU” category.

Fifty-five taxa belonging to 39 families, and 53 genera were identified in Yer Köprü Natural Formation (Appendix 1). Here the most crowded family was found to be *Asteraceae* with 7 taxa. It is followed by *Lamiaceae* and *Oleaceae* each with 3 taxa (Table 5). The phytogeographic distribution of the taxa determined in Yer Köprü Natural Formation is as follows: 14 taxa are Mediterranean, 10 are East

Table 4. Comparison of families with the most taxa and genus among the study areas

Study area		The most taxa containing family			The most genera containing family		
		Family	**# of taxa**	**Rate (%)**	**Family**	**# of genera**	**Rate (%)**
1	Ağyatan Lagoon	*Juncaceae*	2	11,11	*Asteraceae*	2	11,76
		Asteraceae	2	11,11	*Boraginaceae*	2	11,76
		Chenopodiaceae	2	11,11	*Chenopodiaceae*	2	11,76
		Others	12	66,66	Others	11	64,70
2	Tuzla Lagoon	*Fabaceae*	5	11,90	*Fabaceae*	4	11,11
		Asteraceae	5	11,90	*Asteraceae*	4	11,11
		Poaceae	3	7,14	*Poaceae*	3	8,33
		Others	28	69,04	Others	25	69,44
3	Yumurtalık Lagoon	*Fabaceae*	10	11,62	*Fabaceae*	9	12,16
		Asteraceae	8	9,30	*Asteraceae*	7	9,45
		Chenopodiaceae	6	6,97	*Chenopodiaceae*	6	8,10
		Others	63	72,09	Others	52	70,27
4	Akyatan Lagoon	*Pinaceae*	2	15,38	*Pinaceae*	2	16,66
		Poaceae	2	15,38	*Poaceae*	2	16,66
		Verbanaceae	2	15,38	*Verbanaceae*	2	16,66
		Others	7	53,84	Others	6	50,00
5	Yer Köprü Natural Formation	*Asteraceae*	7	12,72	*Asteraceae*	7	13,20
		Lamiaceae	3	5,45	*Lamiaceae*	3	5,66
		Oleaceae	3	5,45	*Oleaceae*	3	5,66
		Others	42	76,36	Others	40	75,47
6	Şekerpınar	*Lamiaceae*	7	12,28	*Lamiaceae*	4	7,84
		Asteraceae	5	8,77	*Asteraceae*	5	9,80
		Liliaceae	3	5,26	*Liliaceae*	3	5,88
		Others	42	73,68	Others	39	76,47
7	Tatarlı Village Antique Period Ruins	*Asteraceae*	6	12,50	*Asteraceae*	6	12,24
		Brassicaceae	4	8,33	*Brassicaceae*	4	8,16
		Scrophulariaceae	4	8,33	*Scrophulariaceae*	3	6,12
		Others	34	70.83	Others	36	73.46

Table 5. Endemic and non-endemic (rare) taxa in the study area

Family	Taxa	Phytogeographic region	Endemism	IUCN category
Asteraceae	*Echinops dumanii* C. Vural	Med. Elm.	End.	CR
Asteraceae	*Centaurea chrysantha* Wagenitz	-	End.	EN
Boraginaceae	*Alkanna hispida* Hub.-Mor.	E. Med. Elm.	End.	EN
Boraginaceae	*Paracaryum calycinum* Boiss. & Balansa	Ir.-Tur. Elm	End.	LC
Lamiaceae	*Salvia aucheri* Benth.	-	End.	VU
Lamiaceae	*Salvia cilicica* Boiss. & Kotschy	-	End.	VU
Lamiaceae	*Stachys rupestris* Montbret & Aucher ex Benth.	E. Med. Elm	End.	LC
Amaryllidaceae	*Pancratium maritimum* L.	Med. Elm.	-	EN
Zygophyllaceae	*Zygophyllum album* L.	-	-	VU
Pinaceae	*Pinus halepensis* Mill.	Med Elm.	-	VU
Caryophyllaceae	*Dianthus polycladus* Boiss.	E. Med. Elm.	-	VU

Mediterranean, 2 are Irano-Turanian, 4 are Euro-Siberian, 9 taxa are multi-regional, and 16 taxa are unknown. Three of the taxa were determined to be endemic, (*Centaurea chrysantha* Wagenitz, *Alkanna hispida* Hub.-Mor., *Mattiastrum calycinum* (Boiss. & Balansa) Brand. Non-endemic *Pinus brutia* Ten., which is in VU category also exist in the region (Table 5).

Fifty-six taxa were determined in Şekerpınar (Appendix 1), four of which are endemic, *Salvia cilicica* Boiss. & Kotschy (VU), *Salvia aucheri* Bentham var. *aucheri* (VU), *Teucrium chamaedrys* L. subsp. *tauricolum* Rech. fil. (LC), *Stachys rupestris* Montbret & Aucher ex Bentham (LC). In the region *Lamiaceae* was the most crowded family and represented with 7 taxa. Second crowded family was *Asteraceae* with 5 taxa, and the third crowded one is *Liliaceae* with 3 taxa (Table 4). Phytogeographically, six of the determined taxa are Mediterranean elements, 13 are Eastern Mediterranean, four are Irano-Turanian, two are Euro-Siberian, 12 taxa are multi-regional and 20 taxa are unknown.

Forty-eight taxa belonging to 34 families and 49 genera were identified in Tatarlı Village Antique Period Ruins. Here, *Asteraceae* is the richest family with 6 taxa in terms existing taxa number. It is followed by *Brassicaceae* and *Scrophulariaceae* families each with 4 taxa (Table 4). Distribution of taxa in phytogeographical regions are as follows: 8 taxa are Mediterranean, 4 taxa are East Mediterranean, 5 taxa are Euro-Siberian, 13 taxa are multi-regional and 18 taxa unknown. This distribution is consistent with the phytogeography of the area. *Pinus brutia* Ten., also exist in this region as being in VU category (Table 5).

The distribution ratio of phytogeographic elements of the study area is compared with the results of other investigations in nearby areas. Except Sarımsak Mountain and Korkun Valley, in all of the research areas, Mediterranean elements are the most crowded ones (Altınözlü, 2004; Akıncı et al., 2018; Paksoy, & Savran, 2011; Kavak et al., 2012; T.C. Ministry of Forestry and Water Affairs, 2013; Keskin & Savran, 2020; Tel et al., 2019). This situation can be explained by the fact that these areas are all in the Mediterranean region (Table 6).

The distribution of the determined taxa, as a whole, to the families were also compared with the findings of the neighbouring studies (Table 7). In general, in all of the research areas Asteraceae were found to be the most crowded family except those carried out in Yumurtalık Lagoon, Karatepe-Aslantaş National Park where Fabaceae family was found as the first family in terms of taxa number. This situation is an expected result according to Davis (1965-1985).

Conflict of Interest

The authors have declared no conflict of interest.

Authors' Contribution

The authors contributed equally.

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Table 6. Distribution rates of taxa in the study area and close regions according to phytogeographic regions (%)

Study area	Med.	Eu.-Sib.	Ir.-Tur.	Wide
This study	34,6	10	11	20.9
Adana Yumurtalık Lagoon (Altınözlü, 2004)	17.4	2.6	2.6	77.1
Akdağ and ıts Surroundings (Akıncı et al., 2018)	19.4	5.1	16.9	58.6
Sarımsak Mountain and Korkun Valley (Paksoy and Savran, 2011)	18.9	3.45	24.6	53.05
Kızıldağ Plateau and Its Surroundings (Keskin and Savran, 2020)	18.8	4.1	17.2	59.9
Karatepe-Aslantaş National Park (Tel et al., 2019)	38.4	6.1	4.3	51.2

Table 7. Families containing the most taxa in studies conducted in the research area and its close regions

Study area	*Asteraceae*	*Fabaceae*	*Lamiaceae*	*Brassicaceae*
This study	24	19	14	8
Adana Yumurtalık Lagoon (Altınözlü, 2004)	26	41	7	6
Akdağ and ıts Surroundings (Akıncı et al., 2018)	97	72	53	61
Sarımsak Mountain and Korkun Valley (Paksoy and Savran, 2011)	82	70	46	51
Kızıldağ Plateau and Its Surroundings (Keskin and Savran, 2020)	67	55	44	54
Karatepe-Aslantaş National Park (Tel et al., 2019)	13	26	13	7

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Appendix 1. Taxa list of study area

	Family	Taxon	Phytogeographic region	IUCN category	Speread situation	Area	Collection date	Collection No
1	*Adiantaceae*	*Adiantum capillus-veneris* L.	Widespread	-	*Wide*	**5,7**	08.05.2016	Ortaç 1000
2	*Aspleniaceae*	*Ceterach officinarum* DC.		-	*Wide*	**6**	03.04.2016	Ortaç 1001
3	*Ephadraceae*	*Ephedra foeminea* Forssk.		-	*Wide*	**6**	03.04.2016	Ortaç 1008
4	*Cupresaceae*	*Cupressus sempervirens* L.		-	*Regional*	**5,6**	03.04.2016	Ortaç 1004
5	*Cupresaceae*	*Juniperus drupacea* Lab.		-	*Bölgesel*	**6**	03.04.2016	Ortaç 1005
6	*Cupresaceae*	*Juniperus oxycedrus*L.	Widespread	-	*Wide*	**6**	03.04.2016	Ortaç 1007
7	*Pinaceae*	*Pinus brutia* Ten.	E. Med. Reg.	*VU*	*Regional*	**3,4,5,6,7**	07.04.2016	Ortaç 1011
8	*Pinaceae*	*Pinus halepensis* Mill.	E. Med. Reg.	*VU*	*Narrow*	**3**	08.05.2016	Ortaç 1012
9	*Pinaceae*	*Pinus nigra* Arn. subsp. *pallasiana* (Lamb.) Holmboe		-	*Wide*	**6**	03.04.2016	Ortaç 1013
10	*Pinaceae*	*Pinus pinea* L.	Widespread	-	*Regional*	**4**	07.04.2016	Ortaç 1014
11	*Amaryllidaceae*	*Pancratium maritimum* L.	Mediterranean	*EN*	*Regional*	**1,2,3**	22.07.2016	Ortaç 1424
12	*Anacardiaceae*	*Pistacia palaestina* Boiss.	E. Med. Reg.	-	*Regional*	**2,3,5,6**	07.04.2016	Ortaç 1017

13	*Apiaceae*	*Eryngium campestre* L. var. *virens* Link	Widespread	-	*Regional*	**6**	03.04.2016	Ortaç 1021
14	*Apiaceae*	*Eryngium maritimum* L.		-	*Wide*	**2**	07.04.2016	Ortaç 1023
15	*Apiaceae*	*Turgenia latifolia* (L.) Hoffm.	Widespread	-	*Wide*	**2**	07.04.2016	Ortaç 1025
16	*Apocynaceae*	*Nerium oleander* L.	Mediterranean	-	*Regional*	**3,5**	08.05.2016	Ortaç 1026
17	*Apocynaceae*	*Trachomitum venetum* (L.) Woodson subsp. *sarmatiense* (Woodson) Avetisian	E. Med. Reg.	-	*Wide*	**2,3,4**	07.04.2016	Ortaç 1027
18	*Araceae*	*Arum dioscoridis* Sm.	E. Med. Reg.	-	*Wide*	**6,7**	03.04.2016	Ortaç 1425
19	*Araliaceae*	*Hedera helix* L.		-	*Wide*	**5,6,7**	03.04.2016	Ortaç 1028
20	*Asclepiaceae*	*Cionura erecta* (L.) Griseb.	E. Med. Reg.	-	*Wide*	**2,4**	07.04.2016	Ortaç 1031
21	*Asclepiaceae*	*Vincetoxicum canescens*(Willd.) Decne.		-	*Wide*	**5**	22.07.2016	Ortaç 1032
22	*Asteraceae*	*Anthemis tinctoria* L.	Widespread	-	*Wide*	**5**	22.07.2016	Ortaç 1033
23	*Asteraceae*	*Artemisia absinthium L.*		-	*Wide*	**6**	03.04.2016	Ortaç 1034
24	*Asteraceae*	*Bellis perennis* L.	Eu.- Sibirian	-	*Wide*	**7**	08.05.2016	Ortaç 1035
25	*Asteraceae*	*Carduus pycnocephalus* L. subsp. *albidus* (Bieb.) Kazmi	Widespread	-	*Wide*	**5**	22.07.2016	Ortaç 1037
26	*Asteraceae*	*Centaurea calcitrapa* ssp. *cilicica* (Boiss. & Balansa) Wagenitz		-	*Regional*	**3**	08.05.2016	Arazi Gözlemi
27	*Asteraceae*	*Centaurea chrysantha* Wagenitz		*End./EN*	*Regional*	**5**	22.07.2016	Ortaç 1039
28	*Asteraceae*	*Centaurea iberica* Trevir. ex Spreng.	Widespread	-	*Wide*	**7**	08.05.2016	Ortaç 1040
29	*Asteraceae*	*Centaurea urvillei* DC. subsp. *armata* Wagenitz	E. Med. Reg.	-	*Wide*	**6**	03.04.2016	Ortaç 1044
30	*Asteraceae*	*Cichorium intybus* L.	Widespread		*Wide*	**6**	03.04.2016	Ortaç 1046
31	*Asteraceae*	*Echinops dumanii* C. Vural	Mediterranean	*End/CR*	*Narrow*	**3**	08.05.2016	Ortaç 1052
32	*Asteraceae*	*Echinops orientalis* Trautv.	İr.-Tur. Elm.,	-	*Wide*	**1**	08.05.2016	Ortaç 1053
33	*Asteraceae*	*Inula crithmoides* L.		-	*Regional*	**1,2,3**	08.05.2016	Ortaç 1055
34	*Asteraceae*	*Inula heterolepis* Boiss.	Mediterranean	-	*Wide*	**5**	22.07.2016	Ortaç 1057
35	*Asteraceae*	*Inula viscosa* (L.) Aiton	Mediterranean	-	*Wide*	**2,3**	07.04.2016	Ortaç 1058
36	*Asteraceae*	*Phagnalon graecum* Boiss.	E. Med. Reg.	-	*Wide*	**5**	22.07.2016	Ortaç 1066
37	*Asteraceae*	*Picnomon acarna* (L.) Cass.	Mediterranean	-	*Wide*	**5**	22.07.2016	Ortaç 1067
38	*Astraceae*	*Senecio vernalis* Waldst & Kit	Widespread	-	*Wide*	**2,6**	07.04.2016	Ortaç 1070
39	*Asteraceae*	*Silybum marianum* (L.) Gaertner (Schouw) D. Löve & P. Dansereau	Mediterranean	-	*Wide*	**3,7**	08.05.2016	Ortaç 1073
40	*Asteraceae*	*Sonchus oleraceus* L.		-	*Wide*	**2**	07.04.2016	Ortaç 1074
41	*Asteraceae*	*Tragopogon longirostris* Bisch ex Schultz Bip.		-	*Wide*	**5**	22.07.2016	Ortaç 1076
42	*Asteraceae*	*Xanthium spinosum* L.			*Wide*	**6,7**	03.04.2016	Ortaç 1078
43	*Asteraceae*	*Xanthium strumarium* L. subsp. *cavanillesii* (Schouw) D. Löve & P. Dansereau	Widespread	-	*Wide*	**2,3**	07.04.2016	Ortaç 1079
44	*Boraginaceae*	*Alkanna hispida* Hub.-Mor.	E. Med. Reg.	*End./EN*	*Regional*	**5**	22.07.2016	Ortaç 1084
45	*Boraginaceae*	*Anchusa aggregata* Lehm.	Mediterranean	-	*Wide*	**3**	08.05.2016	Ortaç 1087
46	*Boraginaceae*	*Anchusa azurea* Mill.	Widespread		*Wide*	**6**	03.04.2016	Ortaç 1088
47	*Boraginaceae*	*Cynoglossum creticum* Mill.		-	*Wide*	**7**	08.05.2016	Ortaç 1090
48	*Boraginaceae*	*Echium angustifolium* Mill.	E. Med. Reg.	-	*Wide*	**4**	07.04.2016	Ortaç 1092
49	*Boraginaceae*	*Echium parviflorum* Moench	Mediterranean	-	*Wide*	**2**	07.04.2016	Ortaç 1093
50	*Boraginaceae*	*Heliotropium europaeum L.*	Mediterranean	-	*Wide*	7	08.05.2016	Ortaç 1094
51	*Boraginaceae*	*Nonea obtusifolia* (Willd.) DC.	E. Med. Reg.	-	*Wide*	**1**	08.05.2016	Ortaç 1095
52	*Boraginaceae*	*Onosma alborosea* Fisch. & C.A.Mey.	Ir.-Turanian		*Wide*	**6**	03.04.2016	Ortaç 1096
53	*Boraginaceae*	*Onosma rascheyanum* Boiss.	Ir.-Turanian		*Wide*	**6**	03.04.2016	Ortaç 1097
54	*Boraginaceae*	*Mattiastrum calycinum* (Boiss. & Balansa) Brand	Ir.-Turanian	*End./LC*	*Regional*	**5**	22.07.2016	Ortaç 1098
55	*Brassicaceae*	*Arabis caucasica* Willd subsp. *brevifolia* (DC.) Cullen	E. Med. Reg.		*Wide*	**6**	03.04.2016	Ortaç 1101
56	*Brassicaceae*	*Capsella bursa-pastoris* (L.) Medik.	Widespread	-	*Wide*	**7**	08.05.2016	Ortaç 1106
57	*Brassicaceae*	*Cakile maritima* Scop.		-	*Regional*	**1,3**	08.05.2016	Ortaç 1105
58	*Brassicaceae*	*Diplotaxis tenuifolia* (L.) DC.			*Wide*	**6**	03.04.2016	Ortaç 1109
59	*Brassicaceae*	*Maresia nana* (DC.) Batt.		-	*Wide*	**2**	07.04.2016	Ortaç 1114
60	*Brassicaceae*	*Nasturtium officinale* R.Br	Widespread	-	*Wide*	**7**	08.05.2016	Ortaç 1115
61	*Brassicaceae*	*Neslia apiculata* Fisch.	Widespread		*Wide*	**7**	08.05.2016	Ortaç 1116

62	*Brassicaceae*	*Raphanus raphanistrum* L.	Widespread	-	*Wide*	**3**	08.05.2016	Ortaç 1117
63	*Campanulaceae*	*Legousia falcata* (Ten.) Fritsch	Mediterranean		*Wide*	**7**	08.05.2016	Ortaç 1126
64	*Campanulaceae*	*Michauxia campanuloides* L'Herit ex Aiton	E. Med. Reg.	-	*Wide*	**5,6**	03.04.2016	Ortaç 1127
65	*Capparaceae*	*Capparis spinosa* L.		-	*Wide*	**5,7**	08.05.2016	Ortaç 1129
66	*Caryophyllaceae*	*Dianthus polycladus* Boiss.	E. Med. Reg.	*VU*	*Wide*	**3,6**	08.05.2016	Ortaç 1132
67	*Caryophllaceae*	*Silene colorota* Poiret		-	*Wide*	**3**	08.05.2016	Ortaç 1138
68	*Caryophllaceae*	*Silene viscosa* (L) Pers		-	*Wide*	**3**	08.05.2016	Ortaç 1139
69	*Chenopodiaceae*	*Arthrocnemum fruticosum* (L.) Moq.		-	*Regional*	**3**	08.05.2016	Ortaç 1142
70	*Chenopodiaceae*	*Chenopodium album* L.		-	*Wide*	**1,3,7**	08.05.2016	Ortaç 1144
71	*Chenopodiaceae*	*Halimione portulacoides* (L.) Aellen		-	*Regional*	**3**	08.05.2016	Ortaç 1145
72	*Chenopodiaceae*	*Halocnemum strobilaceum* (Pall.) M.Bieb.		-	*Regional*	**3**	08.05.2016	Ortaç 1146
73	*Chenopodiaceae*	*Petrosimonia brachiata* (Pall.) Bunge		-	*Wide*	**3**	08.05.2016	Ortaç 1147
74	*Chenopodiaceae*	*Salsola soda* L.		-	*Regional*	**2,3**	07.04.2016	Ortaç 1151
75	*Chenopodiaceae*	*Salicornia europaea* L.		-	*Regional*	**1**	08.05.2016	Ortaç 1148
76	*Cistaceae*	*Cistus creticus* L.	Mediterranean	-	*Regional*	**3,5,7**	08.05.2016	Ortaç 1152
77	*Cistaceae*	*Cistus salviifolius* L.	Widespread	-	*Regional*	**3**	08.05.2016	Ortaç 1153
78	*Cistaceae*	*Helianthemum nummularium* (L.) Mill.				**3**	08.05.2016	Ortaç 1155
79	*Convolvulaceae*	*Calystegia sepium* (L.) R.Br.	Widespread	-	*Wide*	**3,7**	08.05.2016	Ortaç 1157
80	*Convolvulaceae*	*Convolvulus lanatus* Vahl	Sa. Sin. Reg.	-	*Regional*	**3**	08.05.2016	Ortaç 1159
81	*Convolvulaceae*	*Ipomoea stolonifera* (Cyr.) J.F.Gmelin	Mediterranean	-	*Regional*	**1,2,3**	08.05.2016	Ortaç 1160
82	*Cornaceae*	*Cornus sanguinea* L. subsp. *cilicica* (Wangerin) D.F.Chamb.	E. Med. Reg.		*Wide*	**6**	03.04.2016	Ortaç 1161
83	*Corylaceae*	*Ostrya carpinifolia* Scop.	Mediterranean	-	*Wide*	**5,6**	03.04.2016	Ortaç 1163
84	*Crassulaceae*	*Umbilicus erectus* DC.			*Wide*	**5,6,7**	03.04.2016	Ortaç 1165
85	*Crassulaceae*	*Sedum album* L.	Widespread		*Wide*	**6**	03.04.2016	Ortaç 1164
86	*Cucurbitaceae*	*Bryonia cretica* L.	E. Med. Reg.		*Wide*	**7**	08.05.2016	Ortaç 1166
87	*Cyperaceae*	*Bolboschoenus maritimus* (L.) Palla	Widespread	-	*Wide*	**3**	08.05.2016	Ortaç 1426
88	*Cyperaceae*	*Cyperus capitatus* Vandelli		-	*Wide*	**1,2,3**	22.07.2016	Ortaç 1427
89	*Dipsacaceae*	*Scabiosa rotata* M.Bieb.	Ir.-Turanian	-	*Wide*	**3**	08.05.2016	Ortaç 1169
90	*Ericaceae*	*Arbutus andrachne* L.		-	*Regional*	**5**	22.07.2016	Ortaç 1171
91	*Ericaceae*	*Erica manipuliflora* Salisb.	E. Med. Reg.	-	*Wide*	**3**	08.05.2016	Ortaç 1173
92	*Euphorbiaceae*	*Euphorbia aleppica* L.	Widespread	-	*Wide*	**2,6**	08.05.2016	Ortaç 1174
93	*Euphorbiaceae*	*Euphorbia helioscopia* L.	Widespread	-	*Wide*	**1**	08.05.2016	Ortaç 1175
94	*Euphorbiaceae*	*Euphorbia paralias* L.	Mediterranean		*Wide*	**2**	08.05.2016	Ortaç 1176
95	*Euphorbiaceae*	*Euphorbia peplus* L.	Widespread	-	*Regional*	**3**	08.05.2016	Ortaç 1178
96	*Euphorbiaceae*	*Mercurialis ovata* Sternb. & Hoppe	Eu.- Sibirian	-	*Wide*	**5**	22.07.2016	Ortaç 1179
97	*Fabaceae*	*Acacia nilotica* (L.) Willd. ex Delile subsp. *kraussiana* (Benth.) Brenan	Cultivar		*Wide*	**2**	08.05.2016	Ortaç 1181
98	*Fabaceae*	*Alhagi pseudalhagi* (Bieb.) Desv.	Ir.-Turanian	-	*Wide*	**3**	08.05.2016	Ortaç 1182
99	*Fabaceae*	*Anthyllis vulneraria* L. subsp. *boissieri* (Sag.) Bornm.	Widespread	-	*Regional*	**5**	22.07.2016	Ortaç 1184
100	*Fabaceae*	*Calicitome villosa* (Poiret) Link	Mediterranean	-	*Regional*	**5**	22.07.2016	Ortaç 1189
101	*Fabaceae*	*Cercis sliquastrum* L. subsp. *hebecarpa* (Bornm.) Yalt.		-	*Regional*	**5**	22.07.2016	Ortaç 1191
102	*Fabaceae*	*Coronilla emerus* L. subsp. *emeroides* (Boiss. & Spruner) Uhrova			*Wide*	**6**	03.04.2016	Ortaç 1195
103	*Fabaceae*	*Dorycnium hirsutum* (L.) Ser.	Ir.-Turanian	-	*Wide*	**3**	08.05.2016	Ortaç 1198
104	*Fabaceae*	*Glycyrrhiza glabra* L. var. *glandulifera* (Waldst. & Kit.) Boiss.	Widespread		*Wide*	**2,3**	08.05.2016	Ortaç 1204
105	*Fabaceae*	*Lotus edulis* L.	Mediterranean		*Wide*	**2,3**	08.05.2016	Ortaç 1214
106	*Fabaceae*	*Lotus corniculatus* L.	Widespread	-	*Wide*	**3**	08.05.2016	Ortaç 1212
107	*Fabaceae*	*Lotus halophilus* Boiss. & Spruner			*Regional*	**2**	08.05.2016	Ortaç 1215
108	*Fabaceae*	*Medicago marina* L.			*Wide*	**2**	08.05.2016	Ortaç 1217
109	*Fabaceae*	*Melilotus alba* Desr.	Widespread	-	*Wide*	**1,3**	08.05.2016	Ortaç 1220
110	*Fabaceae*	*Ononis viscosa* L. subsp. *breviflora* (DC.) Nyman		-	*Wide*	**3**	22.07.2016	Ortaç 1226
111	*Fabaceae*	*Robinia pseudoacacia* L.			*Wide*	**6**	03.04.2016	Ortaç 1230
112	*Fabaceae*	*Trifolium campestre* Schreb.	Widespread	-	*Wide*	**3**	22.07.2016	Ortaç 1232

113	*Fabaceae*	*Trifolium purpureum* Lois.	Widespread		*Wide*	**7**	08.05.2016	Ortaç 1233
114	*Fabaceae*	*Trigonella spicata* Sibth. & Sm.	E. Med. Reg.	-	*Wide*	**3**	22.07.2016	Ortaç 1237
115	*Fabaceae*	*Vicia sativa* L.		-	*Wide*	**3**	22.07.2016	Ortaç 1242
116	*Fagaceae*	*Quercus coccifera* L.	Mediterranean	-	*Regional*	**3,5,6**	22.07.2016	Ortaç 1246
117	*Gentianaceae*	*Centaurium pulchellum* (Sw.) Druce	Widespread	-	*Wide*	**1,3**	08.05.2016	Ortaç 1248
118	*Geraniaceae*	*Erodium gruinum* L.	E. Med. Reg.		*Wide*	**2**	08.05.2016	Ortaç 1250
119	*Geraniaceae*	*Erodium malacoides* (L) L'Herit	Mediterranean		*Wide*	**2**	08.05.2016	Ortaç 1251
120	*Geraniaceae*	*Geranium dissectum* L.			*Wide*	**7**	08.05.2016	Ortaç 1253
121	*Geraniaceae*	*Geranium molle* L.		-	*Wide*	**5**	22.07.2016	Ortaç 1256
122	*Geraniaceae*	*Geranium purpureum* Vill.		-	*Wide*	**5**	22.07.2016	Ortaç 1257
123	*Hypericaceae*	*Hypericum hircinum* L.	Mediterranean	-	*Regional*	**5**	22.07.2016	Ortaç 1258
124	*Hypericaceae*	*Hypericum origanifolium* Willd.		-	*Wide*	**5**	22.07.2016	Ortaç 1259
125	*Illecebraceae*	*Paronychia argentea* Lam.	Mediterranean	-	*Wide*	**3**	22.07.2016	Ortaç 1262
126	*Iridaceae*	*Crocus pallasii* Goldb.			*Wide*	**6**	03.04.2016	Ortaç 1428
127	*Iridaceae*	*Iris pseudacorus* L.			*Wide*	**7**	08.05.2016	Ortaç 1431
128	*Juncaceae*	*Juncus acutus* L.		-	*Regional*	**1,3**	22.07.2016	Ortaç 1433
129	*Juncaceae*	*Juncus heldreichianus* Marsson ex Parl.	E. Med. Reg.	-	*Wide*	**3**	22.07.2016	Ortaç 1434
130	*Juncaceae*	*Juncus inflexus* L.	Widespread	-	*Wide*	**3**	22.07.2016	Ortaç 1435
131	*Juncaceae*	*Juncus maritimus* Lam.		-	*Regional*	**1,2**	22.07.2016	Ortaç 1436
132	*Juncaceae*	*Juncus rigidus* Desf.			*Wide*	**2,3**	08.05.2016	Ortaç 1437
133	*Lamiaceae*	*Ballota nigra* L. subsp. *uncinata* (Fiori & Beg) Patzak	Mediterranean		*Wide*	**7**	22.07.2016	Ortaç 1266
134	*Lamiaceae*	*Lamium amplexicaule* L.	Eu.- Sibirian		*Wide*	**6**	27.10.2016	Ortaç 1268
135	*Lamiaceae*	*Lamium garganicum* subsp. *striatum* (Sm.) Hayek	Mediterranean		*Regional*	**6**	22.07.2016	Ortaç 1269
136	*Lamiaceae*	*Melissa officinalis* L. subsp. *inodora* (Bornm.) Bornm.	E. Med. Reg.		*Wide*	**7**	22.07.2016	Ortaç 1275
137	*Lamiaceae*	*Mentha longifolia* (L.) Hudson subsp. *typhoides* (Briq.) Harley	Widespread	-	*Wide*	**5,7**	22.07.2016	Ortaç 1276
138	*Lamiaceae*	*Micromeria myrtifolia* Boiss. & Hohen.	E. Med. Reg.	-	*Wide*	**5**	22.07.2016	Ortaç 1277
139	*Lamiaceae*	*Origanum onites* L.	E. Med. Reg.	-	*Wide*	**5**	22.07.2016	Ortaç 1281
140	*Lamiaceae*	*Thymbra spicata* L.	E. Med. Reg.	-	*Wide*	**3**	22.07.2016	Ortaç 1311
141	*Lamiaceae*	*Salvia aucheri* Benth.		*End./VU*	*Regional*	**6**	27.10.2016	Ortaç 1290
142	*Lamiaceae*	*Salvia cilicica* Boiss. & Kotschy		*End./VU*	*Regional*	**6**	27.10.2016	Ortaç 1292
143	*Lamiaceae*	*Salvia viridis* L.	Mediterranean		*Wide*	**2,4**	07.04.2016	Ortaç 1297
144	*Lamiaceae*	*Stachys rupestris* Montbret & Aucher ex Benth.	E. Med. Reg.	*End./LC*	*Regional*	**6**	27.10.2016	Ortaç 1307
145	*Lamiaceae*	*Teucrium chamaedrys* L. subsp. *tauricola* Rech. fil.	Mediterranean		*Regional*	**6**	27.10.2016	Ortaç 1308
146	*Lauraceae*	*Laurus nobilis* L.	Mediterranean	-	*Regional*	**5**	23.10.2016	Ortaç 1312
147	*Liliaceae*	*Asphodelus aestivus* Bro	Mediterranean	-	*Regional*	**3,5**	22.07.2016	Ortaç 1443
148	*Liliaceae*	*Asphodeline taurica* (Pallas) Kunth	E. Med. Reg.		*Regional*	**6**	27.10.2016	Ortaç 1442
149	*Liliaceae*	*Hyacinthus orientalis* L.	E. Med. Reg.		*Wide*	**6**	27.10.2016	Ortaç 1445
150	*Liliaceae*	*Muscari neglectum* Guss.	Widespread		*Wide*	**6**	27.10.2016	Ortaç 1450
151	*Liliaceae*	*Prospero autumnale* (L.) Speta	Mediterranean	-	*Regional*	**3**	22.07.2016	Ortaç 1453
152	*Liliaceae*	*Smilax aspera* L.		-	*Wide*	**3**	22.07.2016	Ortaç 1454
153	*Liliaceae*	*Drimia maritima* (L.) Stearn	Mediterranean	-	*Wide*	**5**	23.10.2016	Ortaç 1456
154	*Malvaceae*	*Malva sylvestris* L.			*Wide*	**7**	22.07.2016	Ortaç 1316
155	*Moraceae*	*Ficus carica* L. subsp. *carica*			*Wide*	**6,7**	27.10.2016	Ortaç 1317
156	*Moraceae*	*Ficus carica* L. subsp. *rupestris* (Hausskn.) Browicz	Ir.-Turanian	-	*Wide*	**5**	23.10.2016	Ortaç 1318
157	*Moraceae*	*Morus alba* L.	Widespread		*Wide*	**7**	22.07.2016	Ortaç 1319
158	*Mrytaceae*	*Eucalyptus camaldulensis* Dehnh.			*Wide*	**7**	22.07.2016	Ortaç 1321
159	*Myrtaceae*	*Myrtus communis* L.			*Bölgesel*	**2,3,4**	07.04.2016	Ortaç 1322
160	*Mrytaceae*	*Punica granatum* L.		-	*Wide*	**5,7**	22.07.2016	Ortaç 1323
161	*Oleaceae*	*Jasminum fruticans* L.	Mediterranean	-	*Regional*	**5,6**	27.10.2016	Ortaç 1327
162	*Oleaceae*	*Olea europaea* L.	Mediterranean	-	*Regional*	**3,5**	22.07.2016	Ortaç 1328
163	*Oleaceae*	*Phillyrea latifolia* L.	Mediterranean	-	*Regional*	**3,5,6**	22.07.2016	Ortaç 1330

164	*Onograceae*	*Epilobium hirsutum* L.	Widespread		*Wide*	**7**	22.07.2016	Ortaç 1332
165	*Orchidaceae*	*Orchis anatolica* Boiss.	E. Med. Reg.	-	*Regional*	**3**	22.07.2016	Ortaç 1459
166	*Orchidaceae*	*Orchis laxiflora* Lam.	Mediterranean	-	*Regional*	**3**	22.07.2016	Ortaç 1460
167	*Papaveraceae*	*Glaucium corniculatum* (L.) Rudolph	Widespread		*Wide*	**6**	27.10.2016	Ortaç 1334
168	*Platanaceae*	*Platanus orientalis* L.	Widespread	-	*Wide*	**5,6,7**	27.10.2016	Ortaç 1343
169	*Plantaginaceae*	*Plantago major* L.			*Wide*	**7**	22.07.2016	Ortaç 1341
170	*Plantaginaceae*	*Plantago maritima* L.	Widespread		*Regional*	**2,3**	08.05.2016	Ortaç 1342
171	*Plumbaginaceae*	*Limonium angustifolium* (Tausch) Turrill	Mediterranean	-	*Regional*	**3**	22.07.2016	Ortaç 1344
172	*Poaceae*	*Elymus elongatus* (Host) Runemark			*Wide*	**2,3**	08.05.2016	Ortaç 1468
173	*Poaceae*	*MelicaeligulataBoiss.*	Mediterranean			**7**	22.07.2016	Ortaç 1472
174	*Poaceae*	*Phragmites australis* (Cav.) Trin. ex Steudel			*Wide*	**2,3,4**	08.05.2016	Ortaç 1474
175	*Poaceae*	*Piptatherum miliaceum* (L.) Cosson subsp. *thomasii* (Duby) Freitag		-	*Wide*	**3**	22.07.2016	Ortaç 1475
176	*Poaceae*	*Poa bulbosa* L.	Widespread	-	*Wide*	**5**	23.10.2016	Ortaç 1476
177	*Poaceae*	*Saccharum ravennae* (L.) Murray			*Wide*	**2,3,4**	08.05.2016	Ortaç 1477
178	*Poligonaceae*	*Polygonum equisetiforme* Sibth & Sm.			*Wide*	**2,4**	07.04.2016	Ortaç 1345
179	*Poligonaceae*	*Polygonum maritimum* L.			*Wide*	**1,2,3**	22.07.2016	Ortaç 1346
180	*Poligonaceae*	*Polygonum praelongum* Coode & Cullen	Widespread	-	*Wide*	**3**	22.07.2016	Ortaç 1347
181	*Poligonaceae*	*Rumex crispus* L.		-	*Wide*	**1,3**	22.07.2016	Ortaç 1348
182	*Primulaceae*	*Anagallis arvensis* L.		-	*Wide*	**3,7**	22.07.2016	Ortaç 1351
183	*Ranunculaceae*	*Anemone coronaria* L.	Mediterranean		*Wide*	**6**	27.10.2016	Ortaç 1359
184	*Ranunculaceae*	*Ranunculus muricatus* L.			*Wide*	**7**	22.07.2016	Ortaç 1367
185	*Ranunculaceae*	*Paliurus spina-christi* Mill.			*Regional*	**3,5,6,7**	22.07.2016	Ortaç 1369
186	*Rhamnaceae*	*Rhamnus oleoides* L. subsp. *graecus* (Boiss.& Reut.) Holmboe	E. Med. Reg.		*Wide*	**2,3**	08.05.2016	Ortaç 1371
187	*Rosaceae*	*Cotoneaster nummularia* Fisch. & C.A.Mey.			*Wide*	**6**	27.10.2016	Ortaç 1373
188	*Rosaceae*	*Crataegus aronia* (L.) Bosc. ex DC.			*Wide*	**6**	27.10.2016	Ortaç 1374
189	*Rosaceae*	*Prunus spinosa* L.	Eu.- Sibirian	-	*Wide*	**5**	23.10.2016	Ortaç 1378
190	*Rosaceae*	*Pyracantha coccinea* Roemer		-	*Wide*	**3**	22.07.2016	Ortaç 1379
191	*Rosaceae*	*Rubus sanctus* Schreber	Widespread		*Wide*	**2,3,5,7**	08.05.2016	Ortaç 1381
192	*Rosaceae*	*Sanguisorba minor* Scop. subsp. *muricata* (Spach) Briq.	Widespread		*Wide*	**6**	27.10.2016	Ortaç 1382
193	*Rosaceae*	*Sarcopoterium spinosum* (L.) Spach	E. Med. Reg.	-	*Regional*	**1**	22.07.2016	Ortaç 1383
194	*Salicaceae*	*Populus alba* L.	Eu.- Sibirian		*Wide*	**7**	22.07.2016	Ortaç 1387
195	*Salicaceae*	*Salix alba L.*	Eu.- Sibirian			**3,5**	22.07.2016	Ortaç 1390
196	*Santalaceae*	*Osyris alba* L.	Mediterranean	-	*Wide*	**3**	22.07.2016	Ortaç 1391
197	*Schrophulariaceae*	*Anarrhinum orientale* Bentham	Ir.-Turanian		*Wide*	**6**	27.10.2016	Ortaç 1392
198	*Scrophulariaceae*	*Kickxia lanigera* (Desf.) Hand.-Mazz.	Mediterranean		*Wide*	**7**	22.07.2016	Ortaç 1393
199	*Schrophulariaceae*	*Linaria chalepensis* (L.) Mill.	E. Med. Reg.	-	*Wide*	**5**	23.10.2016	Ortaç 1394
200	*Schrophulariaceae*	*Scrophularia nodosa* L.	Eu.- Sibirian		*Wide*	**6**	27.10.2016	Ortaç 1395
201	*Schrophulariaceae*	*Scrophularia umbrosa* Dum.	Eu.- Sibirian			**7**	22.07.2016	Ortaç 1397
202	*Schrophulariaceae*	*Veronica cymbalaria* Bodard	Mediterranean		*Wide*	**6,7**	27.10.2016	Ortaç 1401
203	*Scrophulariaceae*	*Verbascum sinuatum* L. var. *sinuatum*	Mediterranean	-	*Wide*	**3**	22.07.2016	Ortaç 1399
204	*Simaroubaceae*	*Ailanthus altissima* (Miller) Swingle			*Wide*	**7**	22.07.2016	Ortaç 1403
205	*Solanaceae*	*Solanum nigrum* L.			*Wide*	**7**	22.07.2016	Ortaç 1405
206	*Stryraceae*	*Styrax officinalis* L.		-	*Regional*	**5,6**	27.10.2016	Ortaç 1406
207	*Tamaricaceae*	*Tamarix tetrandra* Pallas ex Bieb. emend. Willd.		-	*Regional*	**3**	22.07.2016	Ortaç 1408
208	*Tamaricaceae*	*Tamarix smyrnensis* Bunge		-	*Wide*	**1,2,3,6**	22.07.2016	Ortaç 1407
209	*Thmeliaceae*	*Daphne sericea* Vahl	E. Med. Reg.	-	*Regional*	**3,5**	22.07.2016	Ortaç 1410
210	*Thymeliaceae*	*Thymelaea hirsuta* (L.) Endl.	Mediterranean		*Wide*	**2,4**	08.05.2016	Ortaç 1411
211	*Ulmaceae*	*Ulmus glabra* Hudson	Eu.- Sibirian		*Wide*	**7**	22.07.2016	Ortaç 1413
212	*Urticaceae*	*Parietaria judaica* L.	Widespread	-	*Wide*	**5,7**	22.07.2016	Ortaç 1414
213	*Urticaceae*	*Urtica dioica* L.	Eu.- Sibirian .	-	*Wide*	**5**	23.10.2016	Ortaç 1415
214	*Valerianaceae*	*Valeriana dioscoridis* Sm.	E. Med. Reg.	-	*Wide*	**5**	23.10.2016	Ortaç 1416
215	*Verbanaceae*	*Verbena officinalis* L.	Widespread			**7**	22.07.2016	Ortaç 1418

216	*Verbanaceae*	*Vitex agnus-castus* L.	Mediterranean		*Wide*	**2,3,4**	08.05.2016	Ortaç 1419
217	*Verbanaceae*	*Phyla nodiflora* (L.) Greene	Widespread	-	*Wide*	**4**	08.05.2016	Ortaç 1417
218	*Vitaceae*	*Ampelopsis orientale* (Lam.) Planchon			*Regional*	**5**	23.10.2016	Ortaç 1420
219	*Zygophyllaceae*	*Zygophyllum album* L.		VU		**2,3**	08.05.2016	Ortaç 1423

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Investigation of the antifungal activity of lichen (*Usnea longissima*) extracts against *Fusarium graminearum*

Asmar TAGHİYEVA[1], Ayşe Feyza TUFAN DÜLGER[2]*, Emre YÖRÜK[3], Tubanur ASLAN ENGİN[4]

[1]*Haliç University, Graduate Institute of Education, Molecular Biology and Genetics Program, İstanbul, Türkiye*
[2]*Ondokuz Mayıs University, Agriculture, Department of Agricultural Biotechnology, Samsun, Türkiye*
[3]*Istanbul Yeni Yüzyıl University, Faculty of Faculty Sciences and Literature, Department of Molecular Biology and Genetics, Istanbul, Türkiye*
[4]*Yalova University, Health Sciences Faculty, Department of Physiotherapy and Rehabilitation, Yalova, Türkiye*
**feyzadulger@omu.edu.tr, [1]esmer.tva @gmail.com, [3]emre.yoruk@yeniyuzyil.edu.tr, [4]tubanur.engin@yalova.edu.tr*

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Liken (*Usnea longissima*) ekstraktlarının *Fusarium graminearum*'a karşı antifungal aktivitesinin araştırılması

Abstract: The aim of this study was to investigate the antifungal effects and molecular changes caused by *Usnea longissima* Ach. extracts against *Fusarium greaminearum*. In agar well diffusion assay, the zone of inhibition increased as the concentration increased in both of methanol and acetone extracts (1, 10, 20 and 50 mg/ml). In terms of bioactivities, 1 mg/ml was active, while other concentrations were very active. At the molecular level, changes caused by 50 mg/ml methanol extract was analyzed by qPCR with terms of *cat*, *mst20*, and *tri5* genes, which are associated with antioxidation, apoptosis, and trichothecene production, respectively. Transcript levels of *tri5* decreased (0.29 fold) while *cat* (2.41 fold) and *mst20* (1.48 fold) increased. Findings from this study showed that *U. longissima* extracts could be natural antifungal agent against worldwide phytopathogen *F. graminearum*.

Key words: Agar well diffusion assay, *Fusarium graminearum*, gene expression, *Usnea longissima*

Özet: Bu çalışmanın amacı, *Usnea longissima* ekstraktlarının *Fusarium greaminearum*'a karşı neden olduğu antifungal etkileri ve moleküler değişiklikleri araştırmaktır. Agar kuyu difüzyon testinde, hem metanol hem de aseton özütlerinde (1, 10, 20 ve 50 mg/ml) konsantrasyon arttıkça inhibisyon bölgesi arttı. Biyoaktiviteler açısından bakıldığında, 1 mg/ml konsatrasyon aktifken, diğer konsantrasyonlar çok aktifti. Moleküler düzeyde, 50 mg/ml metanol özütünün neden olduğu değişiklikler, sırasıyla antioksidasyon, apoptoz ve trikotesen üretimi ile ilişkili *cat*, *mst20* ve *tri5* genleri açısından qPCR ile analiz edildi. *tri5*'in transkript seviyeleri azalırken (0.29 kat), *cat* (2.41 kat) ve *mst20* (1.48 kat) arttı. Bu çalışmadan elde edilen bulgular, *U. longissima* ekstraktlarının dünya çapında fitopatojen *F. graminearum*'a karşı doğal antifungal ajan olabileceğini göstermektedir.

Anahtar Kelimeler: Agar kuyu difüzyon testi, *Fusarium graminearum*, gen ekspresyonu, *Usnea longissima*

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1. Introduction

Fusarium graminearum Schwabe is the major causal agent of the Fusarium head blight of small grain cereals worldwide. The disease cause yield loss and decrease in product quality (Parry et al., 1995). In addition, contamination with mycotoxins such as deoxynivalenol, nivalenol and zearalenone during disease cause economic and health problems (Snijders, 1990; Desjardin, 2007; Osborne and Stein, 2007). Fungicides are generally used to prevent diseases. However, fungicides may cause environmental pollution as well as pathogenic fungi may develop resistance to fungicides. In the prevention of diseases, biological agents derived from different organisms can be considered as an alternative method by reducing the chemical input to the environment and being economical (Yuen and Schoneweis, 2007; Mielniczuk and Skwaryło-Bednarz, 2020). There are several examples of biological agents used in agriculture with their pesticide and insecticide activities (Yee and Toscano, 1998; Koch, 1999; Warrior et al.,1999; Lacey et al., 2011).

Lichens are an ecosystem complex that arise from a symbiotic association of an algae or cyanobacteria with fungi (Hawksworth and Grube, 2020). They are used for different purposes such as folk medicine, air pollution monitoring, dyeing clothes, cosmetics, and perfumery (Upreti et al., 2005; Joulain and Tabacchi, 2009; Aslan et al., 2013; Sharma and Mohammad, 2020). Lichens produce diverse range of secondary metabolites associated with various biological activities such as antibiotic, antibacterial, antifungal, antiviral, antiproliferative and cytotoxic effects (Shukla et al., 2010; Brisdelli et al., 2013; Furmanek et al., 2022). Although they have various activities, the agrochemical potential of lichens is not fully known.

Usnea longissima Ach. is a fruticose lichen produces unique secondary metabolites and has medicinal applications for centuries in Indian tradition (Halıcı et al., 2015; Reddy et al., 2019). There are limited studies on the antifungal effects of *U. longissima* extracts (Goel et al., 2011; Devashree et al., 2019; Yadav et al., 2021). However, there is no study on the effect of the *U. longissima* against world wide phytopathogen *F. graminearum*. The objective

of this study was to examine the in vitro antifungal effects and molecular changes caused by extracts of *U. longissima* against *F. graminearum*. In this context, methanol and acetone extracts were tested at concentrations of 1, 10, 20 and 50 mg/ml against *F. graminearum*. After seven days, the effects of treatments on fungal growth were determined by agar well diffusion assay and transcriptomic changes in *cat*, *mst20*, and *tri5* genes were investigated via qPCR.

2. Materials and Method

2.1. Preparation of *U. longissima* extracts

Two different organic solvent used for preparation of methanol and acetone extracts. Finely ground of dried *U. longissima* (10 g) were extracted in the relevant solvent (100 ml) according to Tiwari et al. (2011a). The dry extracts were stored at -20 °C. The extracts were dissolved in 5% dimethyl sulphoxide (DMSO) for further experiments.

2.2. Culture conditions of *F. graminearum*

F. graminearum PH-1 was grown in ½ strength potato dextrose agar (PDA) media at 25 ºC for 7 days. Then, 8-10 fungal discs were placed in carboxymethyl cellulose (CMC) media for macroconidia formation and incubated for 7 days at 28 °C, 100 rpm (Nalam et al., 2016). Concentrations of macroconidia were measured using a hemacytometer and standardized to $1x10^6$ macroconidia/mL.

2.3. Agar well diffusion assay

50 µl of a macroconidial suspension was spread on PDA medium. 5 mm of wells were punched from agar plates. Concentrations of 1, 10, 20 and 50 mg/ml extracts (60 µl) were loaded into the wells. Solvents were used as negative controls. The plates were kept at 25°C for 7 days. The zones of inhibition were measured using a ruler. Bioactivities of extracts were assessed as follows: very active, > 19 mm zone of inhibition; active, 13-19 mm zone of inhibition; partially active, 10-12 mm zone of inhibition; and inactive, < 10 mm zone of inhibition (Quinto and Santos, 2005). Experiments were performed with 3 technical and 3 biological replicates.

2.4. Total RNA isolation, cDNA synthesis and real time polymerase chain reaction (qPCR)

Total RNAs were extracted from *F. graminearum* PH-1 treated with methanol extract at a concentration of 50 mg/ml according to the manufacturer's protocol (NucleoSpin RNA Mini Kit, Macherey Nagel). The quantity and quality of the total RNAs were determined by a spectrophotometer and agarose gel electrophoresis.

Synthesis of cDNAs were performed from 1 µg of total RNA according to the manufacturer's protocol (ProtoScript® First Strand cDNA Synthesis BioLABs). Synthesized cDNAs were diluted to 20 ng/µl for further experiment.

Changes in the transcript levels of *cat*, *mst20*, and *tri5* genes were measured using qPCR. The reaction consisted of a total volume of 10 µL containing 5 µL of 1X Sybr Green I mix, 0.4 µM of each primers (Gazdağlı et al., 2018) and 2 µL of cDNA. Conditions were 2 min at 95°C, followed by 40 cycles of 5 s at 95 °C, 10 s at 58 °C, and 10 s at 72 °C. At the end of cycling, melting curve analysis was performed between 65 ºC - 95 ºC by increasing 0.5 ºC at 2-5 sec/step (Bio-Rad, CFX Connect RealTime System*). β-tubulin* was used as the internal control. Fold changes in gene expressions were determined by $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen, 2001). Experiments were performed in 3 technical and 3 biological replicates.

2.5. Statistical analysis

Gene expression analyses were performed using the one-way ANOVA and Tukey post-test using GraphPad Prism (Version 5.01) (*$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

3. Results

3.1. Effects of extracts against *F. graminearum*

Antifungal activities of methanol and acetone extracts against PH-1 isolate were evaluated by in vitro agar well diffusion assay. On the 7th day of the assay, inhibition zones were observed in the extracts while the PH-1 control group covered the entire petri dish (Fig. 1). The zone of inhibition (mm) increased with the increase in the extract concentrations. In terms of bioactivity of methanol extract, 1 mg/ml of extract (14.5 mm) were active while 10, 20 and 50 mg/ml of extracts were very active (22.5 mm, 27.5 mm and 30 mm, respectively). Similarly, the acetone extracts were found active at 1 mg/ml (16.5 mm) while very active at 10, 20 and 50 mg/ml of concentrations (21 mm, 22.5 mm and 26.5 mm, respectively) (Table 1).

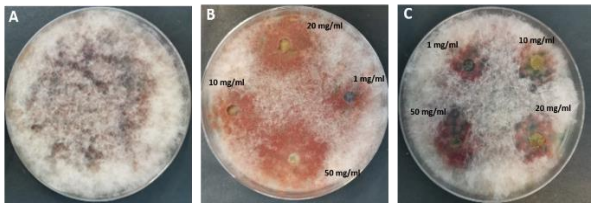

Figure 1. Agar well diffusion assay. (A) *F. graminearum* PH-1 control, (B) methanol and (C) acetone extracts of *U. longissima* at 1, 10, 20 and 50 mg/ml concentrations against PH-1.

Table 1. Zone of inhibition (mm) measurements in determining the bioactivities of methanol and acetone extracts of *U. longissima* against PH-1 through agar well diffusion assay. Error bars represent ± standard errors (SE) of three replicates.

Extract	Concentration	Inhibition zone (mm)
Methanol	1 mg/ml	14.5±0.7
	10 mg/ml	22.5±3.5
	20 mg/ml	27.5±3.5
	50 mg/ml	30±0
Acetone	1 mg/ml	16.5±2.1
	10 mg/ml	21.0±1.4
	20 mg/ml	22.5±3.5
	50 mg/ml	6.5±2.1

3.2. Gene expression analysis

To determine the molecular effect of 50 mg/ml methanol extract on PH-1 transcript levels of *cat*, *mst20*, and *tri5* genes were examined. A significant decrease was observed in the *tri5* gene, which is associated with trichothecene biosynthesis (0.29 fold, **). Significant increases were detected in the antioxidant-related *cat* gene (2.41 fold, ***) and the apoptosis-related *mst20* gene (1.48 fold, *) (Fig. 2).

4. Discussions

In this study, we firstly tested the *in vitro* antifungal effects of 1, 10, 20 and 50 mg/ml of methanol and acetone extracts of *U. longissima* against phytopathogenic *F. graminearum*.

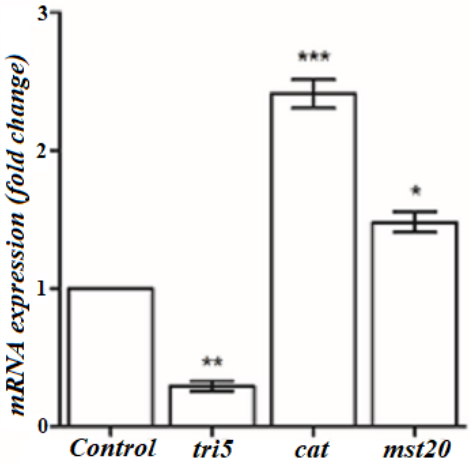

Figure 2. Fold changes of *tri5*, *cat* and *mst20* genes in PH-1 treated with 50 mg/ml methanol extract. Error bars represent ± standard errors (SE) of three replicates.

There are some reports testing the effect of lichen extracts on *Fusarium* Link. species. In a study by Tiwari et al. (2011a), it was determined that acetone and methanol extracts of *Bulbothrix setschwanensis* (Zahlbr.) Hale, *Everniastrum nepalense* (Taylor) Hale ex Sipman, *Heterodermia diademata* (Taylor) D.D. Awasthi, *Parmelaria thomsonii* (Stirt.) D.D. Awasthi lichens were effective against *F. oxysporum* Schltdl., *F. solani* (Mart.) Sacc. and *F. roseum* Link. In a study in which the antifungal activities of acetone, methanol and chloroform extracts of *Parmotrema tinctorum* (Despr. ex Nyl.) Hale were tested on ten plant pathogenic fungi, it was shown that the methanol extract was the most effective of all studied plant pathogens (Tiwari et al., 2011b). On the other hand, there are also some studies of lichen extracts in which inhibitory effects against *Fusarium* species were not observed. In a study with acetone extracts of Ramalina, no inhibitory activity was observed against *F. oxysporum*, *F. solani* and *F. verticillioides* (Sacc.) Nirenberg (Gazo et al., 2019). Similarly, acetone extracts of *Evernia prunastri* (L.) Ach., *Hypogymnia physodes* (L.) Nyl. and *Cladonia portentosa* (Dufour) Coem. lichen species were found to have no effect against *F. solani* (Halama and Van, 2004). There are limited studies on the antifungal, antimalarial, anti-insect, antimicrobial, antiquorum sensing, antioxidant, anti-urease, anti-elastase and antitumor effects of *U. longissima* lichen extracts (Goel et al., 2011; Yıldırım et al., 2012; Aydin et al., 2018, Dandapat and Paul, 2019; Devashree et al., 2019; Pamenta et al., 2020; Yadav et al., 2021; Bharti et al., 2022). In a study by Goel et al (2011), hexane, dichloromethane, ethyl acetate, methanol and aqueous extracts of four lichen species including *U. longissima* were tested on 9 pathogenic fungi, including *F. oxysporum*, and especially hexane and dichloromethane extracts were found to be the most active. However, there was no study on the effect of the lichen extracts against world wide phytopathogen *F. graminearum*. To our knowledge, we firstly reported that *U. longissima* extracts could exhibit a strong inhibitor activity against *F. graminearum*.

At the molecular level, we analyzed the alterations caused by the methanol extract in terms of antioxidant machinery (*cat*), apoptosis (*mst20*) and trichotocene production (*tri5*) in *F. graminearum*. We found that methanol extract induced antioxidant mechanism and apoptosis while suppressing trichothecene biosynthesis. Similarly, there are some reports of increased *cat* and *mst20* levels and decreased *tri5* level in determining the antifungal activity of the compounds (Gazdağlı et al., 2018; Shao et al., 2021; Teker et al., 2021; Xiu et al., 2021; Yörük et al., 2022).

Findings from this study showed that *U. longissima* extracts could be natural antifungal agent against worldwide phytopathogen *F. graminearum*. Considering the limited studies in the literature based on the antifungal effects of lichens, this study is unique and important in terms of contributing to research on biocontrol agent. To our knowledge, this is the first report that *U. longissima* extract inhibits fungal growth and DON biosynthesis in *F. graminearum*.

Conflict of Interest

Authors have declared no conflict of interest.

Authors' Contributions

The authors contributed equally.

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Macrofungal biodiversity of Kop Mount (Bayburt-Erzurum)

Tolga POLAT[1], Ali KELEŞ[2*]
[1]*İnönü Vocational and Technical Anatolian High School, Eskişehir, Türkiye*
[2]*Yüzüncü Yıl University, Education Faculty, Department of Science and Mathematics Education, Van, Türkiye*
alikeles61@yahoo.com, polat105@yahoo.com

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Kop Dağı (Bayburt-Erzurum)'nın makromantar biyoçeşitliliği

Abstract: The study was mainly based on macrofungi samples collected from Kop Mount within the boundaries of Bayburt and Erzurum province between 2010 and 2011. Together with some previously presented taxa, a total of 88 macrofungi species belonging to 63 genera, 30 families, eight orders within classes *Agaricomycetes*, *Leotiomycetes* and *Pezizomycetes* have been compiled. The list of the determined taxa were presented together with their habitats, substrates, collection localities and personel voucher numbers.

Key words: Biodiversity, macrofungi, mycota, Turkiye

Özet: Çalışma temel olarak 2010 ve 2011 yıllarında Bayburt ve Erzurum sınırları içinde yer alan Kop Dağı'ndan toplanan makromantar örnekleri üzerinde gerçekleştirilmiştir. Önceden yayınlanmış bazı taksonlarla birlikte, *Agaricomycetes*, *Leotiomycetes* ve *Pezizomycetes* sınıfları içinde yer alan sekiz takım, 30 familya ve 63 cinse ait 88 tür derlenmiştir. Belirlenen taksonlar, habitatları, substratları, toplanma yerleri ve toplayıcı numaraları ile birlikte listelenmiştir.

Anahtar Kelimeler: Biyoçeşitlilik, makromantar, mikota, Türkiye

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1. Introduction

Fungi is a diverse kingdom and estimated to contain more than 1.5 million species (Hawksworth et al., 1995). Members of the kingdom can grow almost everywhere in the world. Some of them form fruiting bodies that can be seen by the naked eye, and are known as macrofungi. Wild edible mushrooms are used in many countries as delicious and nutritional foods and medicine (Saiqa et al. 2008). About 27.000 fungal species have been reported worldwide (Chang and Miles, 2004).

Many studies have also been carried out on the macromycetes of Türkiye. Most of these studies have been conducted during the last five decades. The latest checklist include about 2.500 macrofungi species (Sesli et al., 2020). New contributions were also made to this list either as regional lists (Çetinkaya et al., 2021; Çevik et al., 2021; Doğan et al., 2021; Kesici and Uzun, 2021; Oruç et al., 2021; Sadullahoğlu et al., 2021) or as new records (Acar et al., 2021; Kaplan et al., 2021; Kaygusuz et al., 2021; Sesli, 2021a,b).

Kop Mount is located at the intersection of the borders of three provinces, Bayburt, Erzincan and Erzurum, as a part of the eastern Black Sea Mountain system. It has an altitude of 2918 m. The region has an annual average temperature of 6.85°C, and a rainfall of 906.3 mm. Steppes are the main vegetation type in the region. Forest vegetation is resembled mainly by scarce *Quercus* L. sp. and *Pinus* L. sp. populations. *Salix* L. and *Populus* L. populations also exist in the region.

Some studies were carried out within the boundaries of Bayburt (Uzun et al., 2004), Erzincan (Keleş and Demirel, 2010; Allı, 2011) and Erzurum (Demirel et al., 2003; Keleş et al., 2017; Sadullahoğlu et al., 2021). Edible taxa determined in the region were also presented in 10th Congress of Turkey Edible Mushrooms, and published as a special issue (Keleş et al., 2016).

The study aims to determine the macrofungal biodiversity of the region and make a contribution to the mycobiota of Türkiye.

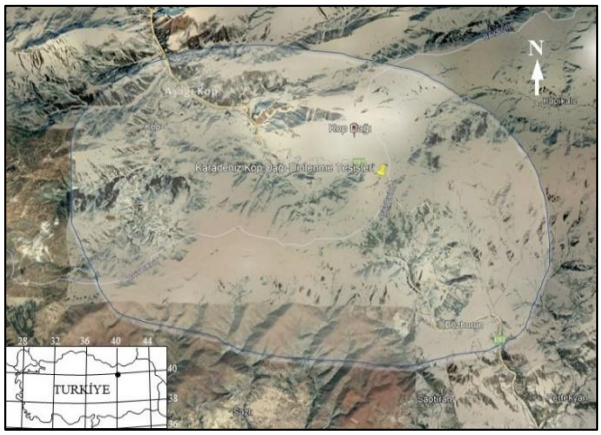

Figure 1. Macrofungi collection region (adopted from Google Earth)

2. Material and Method

Macrofungi samples were collected from Kop Mount within the boundaries of Bayburt and Erzurum provinces during field studies performed between 2010 and 2011. Fruit bodies were photographed at their natural habitats and notes were taken about their morphology, ecology and geography. Then the fruit bodies were collected and put in paper boxes. Then they were transferred to the fungarium.

The samples were dried in an air conditioned room and kept as fungarium materials in polyethylene bags. Further investigations were carried out in the fungarium on dried samples. Microscopic investigations were performed under a compound microscope. The specimens were identified with the help of the relevant literature (Philips, 1981; Moser, 1983; Breitenbach and Kränzlin, 1984, 1986, 1991, 1995, 2000; Buczacki, 1989; Bresinsky and Besl, 1990; Hansen and Knudsen, 1992, 1997; Jordan, 1995; Pegler et al., 1995; Abbott and Currah, 1997; Bessette et al., 1997; Dähncke, 2004; Hausknecht, 2009). The determined macrofungi samples are kept in the fungarium of Biology Department, Science Faculty, Van Yüzüncü Yıl Üniversity(VANF).

3. Results

The determined taxa are listed in alphabetical order. Kirk et al. (2008) and Index Fungorum (accessed on 20 August 2022) were followed for the systematics of taxa. Previously reported taxon was given with the citation.

Fungi R.T. Moore

Ascomycota Caval.-Sm.

Leotiomycetes O.E. Erikss. & Winka

Helotiales Nannf.

Helotiaceae Rehm

1. ***Hymenoscyphus calyculus*** (Fr.) W. Phillips: Erzurum, eastern foothills of Kop Mount around stone pit, on decaying *Salix* sp. branches, 39°59'435"N, 40°31'969"E, 1536 m, 28.10.2010, P.72.

Pezizomycetes O.E.Erikss. & Winka

Pezizales J. Schröt.

Helvellaceae Fr.

2. ***Helvella acetabulum*** (L.) Quél.: (Keleş et al., 2016).

3. ***Helvella lacunosa*** Afzel.: (Keleş et al., 2016).

4. ***Paxina queletii*** (Bres.) Stangl: (Keleş et al., 2016).

Morchellaceae Rchb.

5. ***Morchella elata*** Fr.: (Keleş et al., 2016).

6. ***Morchella esculenta*** (L.) Pers.: (Keleş et al., 2016).

7. ***Morchella semilibera*** DC.: (Keleş et al., 2016).

Pezizaceae Dumort.

8. ***Peziza repanda*** Wahlenb. ex Fr.: Erzurum, Bozburun village, on *Salix* sp. stump, 39°58'056"N, 40°34'154"E, 1660 m, 04.06.2011, P.221.

Pyronemataceae Corda

9. ***Cheilymenia granulata*** (Bull.) J. Moravec: Erzurum, Bozburun village, under *Quercus* L. sp., 39°59'516"N, 40°32'676"E, 1850 m, 04.06.2011, P.231.

10. ***Geopora arenicola*** (Lév.) Kers: Erzurum, eastern foothills of Kop Mount, under Salix sp., 39°59'516"N, 40°32'676"E, 1850 m, 04.06.2011, P.243.

Basidiomycota R.T. Moore

Agaricomycetes Doweld

Agaricales Underw.

Agaricaceae Chevall.

11. ***Agaricus arvensis*** Schaeff.: (Keleş et al., 2016).

12. ***Agaricus bitorquis*** (Quél.) Sacc.: (Keleş et al., 2016).

13. ***Agaricus comtulus*** Fr.: (Keleş et al., 2016).

14. ***Agaricus heimii*** Bon: (Keleş et al., 2016).

15. ***Agaricus pampeanus*** Speg.: (Keleş et al., 2016).

16. ***Coprinus comatus*** (O.F. Müll.) Pers.: (Keleş et al., 2016).

17. ***Cystolepiota seminuda*** (Lasch) Bon: Bayburt, western foothills of Kop Mount, under coniferous trees, 39°59'541"N, 40°32'658"E, 2078 m, 28.10.2010, P.106.

18. ***Lepiota clypeolaria*** (Bull.) P. Kumm.: Bayburt, western foothills of Kop Mount, under coniferous trees, 40°01'884"N, 40°25'245"E, 2117 m, 04.06.2011, P.274.

19. ***Lepiota cristata*** (Bolton) P. Kumm.: Erzurum, eastern foothills of Kop Mount around stone pit, under *Populus* sp., 39°59'435”N, 40°31'969”E, 1536 m, 28.10.2010, P. 63.

20. ***Leucoagaricus leucothites*** (Vittad.) Wasser: (Keleş et al., 2016).

21. ***Macrolepiota excoriata*** (Schaeff.) Wasser: (Keleş et al., 2016).

22. ***Tulostoma brumale*** Pers.: Erzurum, southern foothills of Kop Mount, among grass, 40°03'262”N, 40°26'528”E, 1838 m, 31.10.2010, P.167

Bolbitiaceae Singer

23. ***Conocybe aporos*** Kits van Wav.: Erzurum, eastern foothills of Kop Mount, among grass under *Salix* sp., 39°57'705”N, 40°34'635”E, 1612 m, 06.05.2011, P.180,

24. ***Conocybe pulchella*** (Velen.) Hauskn. & Svrček: Bayburt, Aşağı Kop village, roadside, 40°04'050”N, 40°25'970”E, 1841 m, 04.06.2011, P.296.

Crepidotaceae Singer

25. ***Crepidotus caspari*** Velen.: Erzurum, Kop Dağı eteği, daş ocağı cıvarı, on dead *Salix* sp. branches, 39°59'435”N, 40°31'969”E, 1539 m, 28.10.2010, P.71.

Hygrophoraceae Lotsy

26. ***Arrhenia obscurata*** (D.A. Reid) Redhead, Lutzoni, Moncalvo & Vilgalys: Bayburt, western foothills of Kop Mount, under coniferous trees, 39°59'541”N, 40°32'658”E, 2078 m, 28.10.2010, P. 118.

27. ***Hygrocybe calciphila*** Arnolds: (Keleş et al., 2017)

28. ***Hygrophorus gliocyclus*** Fr.: (Keleş et al., 2016).

Hymenogastraceae Vittad.

29. ***Galerina sideroides*** (Bull.) Kühner: Erzurum, eastern foothills of Kop Mount, around stone pit, around *Salix* sp. stump, 39°59'435”N, 40°31'969”E, 1536 m, 28.10.2010, P.58.

30. ***Hebeloma laterinum*** (Batsch) Vesterh.: Erzurum, eastern foothills of Kop Mount, under deciduous trees, 40°03'262”N, 40°26'528”E, 1838 m, 31.10.2010, P.161.

31. ***Psilocybe coronilla*** (Bull.) Noordel.: (Keleş et al., 2016).

Incertae sedis

32. ***Clitocybe rivulosa*** (Pers.) P. Kumm.: Erzurum, eastern foothills of Kop Mount, under shrubs, 39°57'707”N, 40°34'633”E, 1625 m, 04.06.2011, P.212.

33. ***Crucibulum laeve*** (Huds.) Kambly: Bayburt, western foothills of Kop Mount, on remains of coniferous trees, 39°59'541”N, 40°32'658”E, 2078 m, 28.10.2010, P.115.

34. ***Lepista nuda*** (Bull.) Cooke: (Keleş et al., 2016).

35. ***Lepista personata*** (Fr.) Cooke: (Keleş et al., 2016).

36. ***Melanoleuca cognata*** (Fr.) Konrad & Maubl.: (Keleş et al., 2016).

37. ***Melanoleuca exscissa*** (Fr.) Singer: Bayburt, Aşağı Kop village, under *Salix* sp., 40°03'618”N, 40°26'460”E, 1866 m, 29.10.2010, P.99.; under coniferous trees, 39°59'541”N, 40°32'658”E, 2078 m, 28.10.2010, P.301; Erzurum, Bozburun village, under *Salix* sp., 38°30'359”N, 43°23'159”E, 1615 m, 09.05.2010, P.8.

38. ***Melanoleuca stridula*** (Fr.) Singer: (Keleş et al., 2016).

Inocybaceae Jülich

39. ***Inocybe dulcamara*** (Pers.) P. Kumm.: Bayburt, Aşağı Kop village, under *Populus* sp., 40°03'618”N, 40°26'460”E, 1866 m, 04.06.2011, P.258,

40. ***Inocybe hystrix*** (Fr.) P. Karst.: Erzurum, eastern foothills of Kop Mount, around *Quercus* sp., 39°59'477”N, 40°32'642”E, 1840 m, 09.05.2010, P.27.

41. ***Pleurocybella porrigens*** (Pers.) Singer: Erzurum, eastern slopes around peak of Kop Mount, on dead branches, 40°01'335”N, 40°32'370”E, 2335 m, 08.10.2010, P.48.

Lycoperdaceae F. Berchtold & J. Presl

42. ***Apioperdon pyriforme*** (Schaeff.) Vizzini: (Keleş et al., 2016)..

43. ***Bovista pila*** Berk. & M.A. Curtis: Erzurum, Bozburun village, Hasbek place, meadow, 39°59'442”N, 40°31'978”E, 1880 m, 01.06.2011, P.315,

44. ***Bovistella utriformis*** (Bull.) Demoulin & Rebriev: (Keleş et al., 2016).

45. ***Lycoperdon perlatum*** Pers.: (Keleş et al., 2016).

Marasmiaceae Roze ex Kühner

46. ***Marasmius rotula*** (Scop.) Fr.: Bayburt, Aşağı Kop village, on remains of *Salix* sp. leaves, 40°03'618”N, 40°26'460”E, 1866 m, 29.10.2010, P.102.

Mycenaceae Overeem

47. ***Mycena epipterygia*** (Scop.) Gray: Bayburt, western slopes of Kop Mount, among needle litter under coniferous trees, 40°01'780”N, 40°24'595”E, 2090 m, 29.10.2010, P.122.

48. ***Mycena mirata*** (Peck) Sacc.: Bayburt, western slopes of Kop Mount, under coniferous trees, 39°59'541”N, 40°32'658”E, 2078 m, 28.10.2010, P.110.

Omphalotaceae Bresinsky

49. ***Gymnopus fusipes*** (Bull.) Gray: Bayburt, western slopes of Kop Mount, around hotels, under *Quercus* sp., 40°03'263”N, 40°26'546”E, 2018 m, 30.10.2010, P.149.

Pleurotaceae Kühner

50. ***Pleurotus eryngii*** (DC.) Quél.: (Keleş et al., 2016).

51. ***Pleurotus ostreatus*** (Jacq.) P. Kumm.: (Keleş et al., 2016).

52. ***Pleurotus populinus*** O. Hilber & O.K. Mill.: (Keleş et al., 2016).

Pluteaceae Kotl. & Pouzar

53. ***Volvopluteus gloiocephalus*** (DC.) Vizzini, Contu & Justo: (Keleş et al., 2016).

Psathyrellaceae Vilgalys, Moncalvo & Redhead

54. ***Candolleomyces candolleanus*** (Fr.) D. Wächt. & A. Melzer: (Keleş et al., 2016).

55. ***Coprinellus disseminatus*** (Pers.) J.E. Lange: (Keleş et al., 2016).

56. ***Coprinellus micaceus*** (Bull.) Vilgalys, Hopple & Jacq. Johnson: (Keleş et al., 2016).

57. ***Coprinellus xanthothrix*** (Romagn.) Vilgalys, Hopple & Jacq. Johnson: Erzurum, eastern foothills of Kop Mount, among decaying wood chips, 39°59'627”N, 40°32'702”E, 1807 m, 07.10.2010, P.36.; 40°03'262”N, 40°26'528”E, 1838 m, 31.10.2010, P.171.

58. ***Coprinopsis atramentaria*** (Bull.) Redhead, Vilgalys & Moncalvo: Bayburt, Aşağı Kop village, around *Salix* sp. stump, 40°03'618”N, 40°26'460”E, 1866 m, 04.06.2011, P.223; Erzurum, eastern foothills of Kop Mount, around stone pit, around *Salix* sp. stump, 39°59'435”N, 40°31'969”E, 1536 m, 28.10.2010, P.53; Bozburun village, 39°58'043”N, 40°34'154”E, 1623 m, 28.10.2010, P.78.

59. ***Coprinopsis nivea*** (Pers.) Redhead, Vilgalys & Moncalvo: Erzurum, eastern foothills of Kop Mount, on cow dung, 39°57'707”N, 40°34'633”E, 1625 m, 04.06.2011, P.207.

60. ***Parasola plicatilis*** (Curtis) Redhead, Vilgalys & Hopple: (Keleş et al., 2016).

61. ***Tulosesus impatiens*** (Fr.) D. Wächt. & A. Melzer: Bayburt, western foothills of Kop Mount, under Quercus sp., 40°03'263”N, 40°26'546”E, 2018 m, 30.10.2010, P.144.

Strophariaceae Singer & A.H. Sm.

62. ***Agrocybe dura*** (Bolton) Singer: (Keleş et al., 2016).

63. ***Hypholoma acutum*** (Sacc.) E. Horak: Bayburt, Aşağı Kop village, around *Salix* sp. stump, 40°04'049”N, 40°25'978”E, 1840 m, 08.10.2010, P.42.

64. ***Pholiota aurivella*** (Batsch) P. Kumm.: (Keleş et al., 2016).

65. ***Pholiota populnea*** (Pers.) Kuyper & Tjall.-Beuk.: Bayburt, western slopes of Kop Mount, on *Populus* sp. trunk, 40°01'780”N, 40°24'595”E, 2090 m, 29.10.2010, P.140; Erzurum, Bozburun village, Hasbek place, on *Populus* sp. trunk, 39°59'437”N, 40°31'970”E, 1901 m, 30.10.2010, P.49.

Tricholomataceae Lotsy

66. ***Tricholoma atrosquamosum*** Sacc.: (Keleş et al., 2016).

67. ***Tricholoma portentosum*** (Fr.) Quél.: (Keleş et al., 2016).

Tubariaceae Vizzini

68. ***Cyclocybe cylindracea*** (DC.) Vizzini & Angelini: (Keleş et al., 2016).

69. ***Tubaria furfuracea*** (Pers.) Gillet: (Keleş et al., 2016).

Boletales E.-J. Girbert

Boletaceae Chevall.

70. ***Boletus edulis*** Bull.: (Keleş et al., 2016).

71. ***Butyriboletus appendiculatus*** (Schaeff.) D. Arora & J.L. Frank: (Keleş et al., 2016).

72. ***Rubroboletus satanas*** (Lenz) Kuan Zhao & Zhu L. Yang: Erzurum, eastern foothills of Kop Mount, under *Quercus* sp., 39°59'627"N, 40°32'702"E, 1807 m, 15.08.2010, P.31.

Paxillaceae Lotsy

73. ***Paxillus involutus*** (Batsch) Fr.: Bayburt, Yukarı Kop village, under *Populus* sp., 40°02'938"N, 40°25'650"E, 1922 m, 04.06.2011, P.294.

Sclerodermataceae Corda

74. ***Pisolithus arhizus*** (Scop.) Rauschert: Erzurum, eastern foothills of Kop Mount, under *Quercus* sp., 39°59'542"N, 40°32'657"E, 1856 m, 28.10.2010, P.87.

Suillaceae Besl & Bresinsky

75. ***Suillus bovinus*** (L.) Roussel: (Keleş et al., 2016).

76. ***Suillus luteus*** (L.) Roussel: (Keleş et al., 2016).

Geastrales K. Hosaka & Castellano

Geastraceae Corda

77. ***Geastrum coronatum*** Pers.: Erzurum, eastern foothills of Kop Mount, around stone pit, under coniferous trees, 39°25'870"N, 40°05'221"E, 1920 m, 08.10.2010, P.47.

78. ***Geastrum quadrifidum*** Pers.: Bayburt, western slopes of Kop Mount, around hotels, under *Malus* L. sp., 40°03'263"N, 40°26'546"E, 2018 m, 30.10.2010, P.141.

Hymenochaetales Oberw.

Hymenochaetaceae Donk

79. ***Phellinus igniarius*** (L.) Quél.: Bayburt, Aşağı Kop village, on *Salix* sp. stump, 40°04'049"N, 40°25'978"E, 1840 m, 08.10.2010, P.50; Erzurum, Bozburun village, 38°30'359"N, 43°23'159"E, 1615 m, 09.05.2010, P.34.

Polyporales Gäum.

Polyporaceae Fr. ex Corda

80. ***Cerioporus squamosus*** (Huds.) Quél.: (Keleş et al., 2016).

81. ***Ganoderma resinaceum*** Boud.: (Uzun et al., 2004).

82. ***Lentinus brumalis*** (Pers.) Zmitr.: Bayburt, western slopes of Kop Mount, around hotels, on decaying *Quercus* sp. branches, 40°03'263"N, 40°26'546"E, 2018 m, 30.10.2010, P.143; Erzurum, eastern foothills of Kop Mount, 39°59'477"N, 40°32'642"E, 1840 m, 09.05.2010, P.25.

83. ***Pycnoporus cinnabarinus*** (Jacq.) P. Karst.: Bayburt, western slopes of Kop Mount, around hotels, on decaying *Quercus* sp. stump, 40°03'263"N, 40°26'546"E, 2018 m, 30.10.2010, P.146; Erzurum, eastern foothills of Kop Mount, 39°59'477"N, 40°32'642"E, 1840 m, 09.05.2010, P.24.

84. ***Trametes hirsuta*** (Wulfen) Lloyd: Bayburt, eastern foothills of Kop Mount, on *Quercus* sp. stump, 40°01'780"N, 40°24'595"E, 2090 m, 29.10.2010, P.126.

85. ***Trametes trogii*** Berk.: Erzurum, southern foothills of Kop Mount, on *Salix* sp. stump, 39°57'707"N, 40°34'633"E, 1625 m, 04.06.2011, P.205.

Russulales K. Hosaka & Castellano

Russulaceae Lotsy

86. ***Lactarius acerrimus*** Britzelm.: Erzurum, eastern foothills of Kop Mount, eastern foothills of Kop Mount, under *Quercus* sp. 39°59'477"N, 40°32'642"E, 1840 m, 15.08.2010, P.32.

87. ***Lactarius deliciosus*** (L.) Gray: (Keleş et al., 2016).

88. ***Russula delica*** Fr.: (Keleş et al., 2016).

4. Discussions

Eighty eight macrofungi species belonging to 63 genera, 30 families, eight orders and three classes were compiled from Kop Mount. Ten of the determined taxa belong to *Ascomycota* (*Leotiomycetes* 1, *Pezizomycetes* 9) while 78 belong to *Basidiomycota* (*Agaricomycetes* 78). Fourty three of the determined taxa are new for the region (Demirel et al., 2003; Uzun et al., 2004; Keleş, 2010; Allı, 2011; Keleş et al., 2016; Sadullahoğlu et al., 2021).

The taxa, determined in the region, are distributed in eight orders (Fig. 2) and 30 families. *Agaricaceae* was found to be the most crowded family in the region with 12 taxa. It is followed by *Psathyrellaceae* and *Polyporaceae* with eight and six taxa respectively. *Lycoperdaceae* and *Strophariaceae* were found to be represented by four taxa each. Seven (*Boletaceae, Helvellaceae, Hygrophoraceae, Hymenogastraceae, Morchellaceae, Pleurotaceae, Russulaceae*) of the families are represented with three taxa, and eight (*Bolbitiaceae, Geastraceae, Inocybaceae, Mycenaceae, Pyronemataceae, Suillaceae, Tricholomataceae, Tubariaceae*) of them are represented with two taxa. The rest of nine families are represented with only one taxon in the region (Fig. 3).

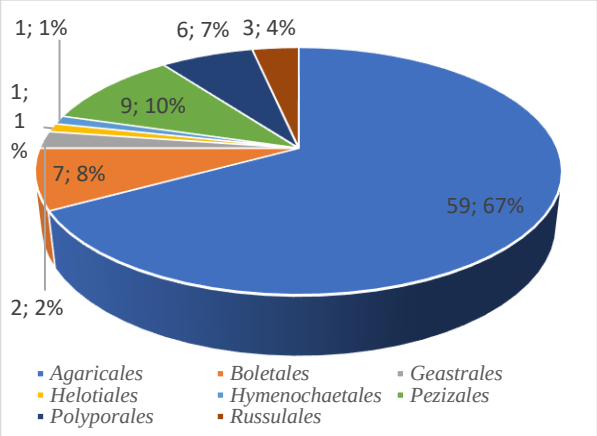

Figure 2. Ditribution of the determined taxa within orders.

The compiled taxa are distributed in 63 genera. The most crowded one is *Agaricus*. *Coprinellus, Melanoleuca, Morchella* and *Pleurotus* follow it with 3 taxa. *Conocybe, Coprinopsis, Geastrum, Helvella, Inocybe, Lactarius, Lepiota, Lepista, Mycena, Pholiota, Suillus, Trametes* and *Tricholoma* come next each with 2 taxa, while the rest of the genera are represented with only one taxon in the region. Fourty four (%55.00) of the determined taxa are edible. Among them *Pleurotus eryngii* and *Agaricus* sp. are collected and consumed in the region (Keleş et al., 2016).

Table 1. Similarity percentages of neighbouring studies with Kop Mount.

Neighbouring study	# of identical taxa	Total taxa	Similarity (%)
Ağrı (Demirel et al., 2002)	22	44	50.00
Bayburt (Uzun et al., 2004)	23	51	45.10
Erzincan (Keleş and Demirel, 2010)	40	193	20.73
Erzurum (Demirel et al., 2003)	23	114	20.18
Gümüşhane (Uzun et al., 2006)	22	105	20.95
Kemaliye (Allı, 2011)	25	106	23.58
Zigana Mount (Akata et al., 2016)	19	182	10.44

The rest of the taxa are regarded as inedible or poisonous by local public. Thirty eight (%47.50) of them are inedible and six (%7.50) of them (*Coprinopsis atramentaria, Inocybe dulcamara, Inocybe hystrix, Lepiota cristata, Paxillus involutus, Rubroboletus satanas*) are more or less poisonous.

The determined taxa were compared with those presented by the studies carried out in neighbouring regions and some similarities were observed. These studies and the similarity percentages are given in Table 1.

Conflict of Interest

Authors have declared no conflict of interest.

Authors' Contribution

The authors contributed equally.

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Phenolic and flavonoid amounts and antioxidant capacity of *Lavandula officinalis* (lavender) callus grown in different growth regulator combinations

Abayhan BURAN*, Aykut TOPDEMİR[2]
[1,2]*Fırat University, Engineering Faculty, Department of Bioengineering, Elazığ, Türkiye*
**a.buran@firat.edu.tr*, [2]*atopdemir@firat.edu.tr*

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Farklı büyüme düzenleyici kombinasyonlarda yetiştirilen *Lavandula officinalis* (lavanta) kalluslarının fenolik ve flavonoid miktarları ve antioksidan kapasitesi

Abstract: In this study, it was aimed to determine the total antioxidant capacity and phenolic and flavonoid amounts of *Lavandula officinalis* (lavender), which is an aromatic plant, callus extracts. Combinations of Naphthalene Acetic Acid (NAA), Benzylaminopurine (BAP), Kinetin (KIN) and 2,4-Dichlorophenoxy Acetic Acid (2,4-D) plant growth regulators at different concentrations were used in the growth medium. The specified analyzes were carried out for the extracts obtained from the callus of plants grown in different media. The callus were extracted with water and ethanol solvents. Total phenolic amount analyzes were accomplished using the Folin-Ciocalteu method. The spectrophotometric $AlCl_3$ method was used for the total flavonoid amount analysis and the antioxidant capacity of lavender callus extracts was measured considering the radical cation capture ability of 2,2′-azino-bis -3- ethylbenzthiazoline -6- sulphonicacid (ABTS). The highest antioxidant capacity (9.24 ± 0.14 mmol TEAC/g dry callus weight) was obtained from the callus of plants grown in medium containing 0.5 mg/L BAP + 0.5 mg/L 2,4-D combination; the highest amount of phenolic substance (35.74± 0.48 mg GAE/g dry callus weight) was obtained from the callus of plants grown in medium containing the combination of 0.5 mg/L BAP + 0.5 mg/L NAA; the highest amount of flavonoid substance (32.42 ± 0.46 mg QE/g dry callus weight) was obtained from the callus of plants grown in medium containing the combination of 0.5 mg/L BAP + 1 mg/L 2,4-D. The results are compared for the combination of plant growth regulators and the effects of the different growth medium ingredients were specified.

Key words: Antioxidant, callus, flavonoid, plant growth regulator, *Lavandula officinalis*, phenolic

Özet: Bu çalışmada, tıbbi ve aromatik bir bitki olan *Lavandula officinalis* (lavanta) kallus ekstraktlarının toplam antioksidan kapasitesinin ve fenolik ve flavonoid madde miktarlarının belirlenmesi amaçlanmıştır. Büyüme ortamında Naftalin Asetik Asit (NAA), Benzilaminopurin (BAP), Kinetin (KIN) ve 2,4-Diklorofenoksi Asetik Asit (2,4-D) bitki büyüme düzenleyicilerinin farklı konsantrasyonlardaki kombinasyonları kullanılmıştır. Farklı ortamlarda yetiştirilen bitkilerin kalluslarından elde edilen ekstraktlar için belirtilen analizler yapılmıştır. Kalluslar su ve etanol solventleri ile ekstrakte edilmiştir. Toplam fenolik miktarı analizleri Folin-Ciocalteu yöntemi kullanılarak yapılmıştır. Toplam flavonoid miktarı analizi için spektrofotometrik $AlCl_3$ yöntemi kullanılmış ve lavanta kallus ekstraktlarının antioksidan kapasitesi 2,2′-azino-bis -3-etilbenztiazolin -6- sülfonik asidin (ABTS) radikal katyon yakalama kabiliyeti dikkate alınarak ölçülmüştür. En yüksek antioksidan kapasite (9,24 ± 0,14 mmol TEAC/g kuru kallus ağırlığı) 0,5 mg/L BAP + 0,5 mg/L 2,4-D kombinasyonunu içeren ortamda yetiştirilen bitkilerin kalluslarından elde edilmiştir; en yüksek fenolik madde miktarı (35,74± 0,48 mg GAE/g kuru kallus ağırlığı) 0,5 mg/L BAP + 0,5 mg/L NAA kombinasyonunu içeren ortamda yetiştirilen bitkilerin kalluslarından; en yüksek flavonoid madde miktarı (32,42 ± 0,46 mg QE/g kuru kallus ağırlığı) 0,5 mg/L BAP + 1 mg/L 2,4-D kombinasyonunu içeren ortamda yetiştirilen bitkilerin kalluslarından elde edilmiştir. Sonuçlar, bitki büyüme düzenleyicilerinin kombinasyonu için karşılaştırılmış ve farklı yetiştirme ortamı bileşenlerinin etkileri belirlenmiştir.

Anahtar Kelimeler: Antioksidan, kallus, flavonoid, bitki büyüme düzenleyici, *Lavandula officinalis*, fenolik

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1. Introduction

It is known that medicinal and aromatic plants have been used to protect or improve human health. Throughout history, mankind has discovered the therapeutic properties of parts of plants such as roots, stems, leaves, flowers and fruits by chance. It is stated in Ninova (3000 BC) tablets that Mesopotamian civilizations such as Assyrians, Akats and Sumerians benefited from medicinal aromatic plants and animal therapeutic products for medicinal purposes (Genç & Kaçar, 2012). Medicinal aromatic plants and therapeutic drugs of animal origin have formed the basis of drugs used in modern pharmacy and medicine today. It is known that most of these drugs are of plant origin. With the developing technology, the chemical components of the plants and the effective substances they contain have been clarified and isolated purely. In this way, the foundations of the concept of "pure and standard medicine" were laid (Tanrıseven, 2013).

Lavender, which is among the medicinal and aromatic plants, is rich in essential oils. It is also commercially cultivated for the extraction of spices and essential oils. Lavender essential oils, with their antiseptic and anti-inflammatory properties, are used primarily in soaps, bubble baths, shampoos, etc. It is also used in perfumes, massage oils, and creams, including bath products (Moon et al., 2006). The lavender plant is a perennial and semi-

bush plant of the Mediterranean geography. It is a dicot plant whose roots can reach up to 1 m depending on the environmental conditions. The stem is hairy or bare. The lavender body has a gray-green color and gives many side branches (Koç, 2002).

Lavandula officinalis, which is considered as an important medicinal and aromatic plant, is included in the Lamiaceae family. It contains 150 bioactive components (El Sherif at al., 2020). The essential oils of the lavender plant, which is one of the most produced essential oils in the world, are used extensively in the pharmaceutical industry, perfumery and cosmetics industry. The dried flowers and leaves of the lavender plant are generally used in the pharmaceutical industry. The Lamiaceae family includes approximately 224 genera and 5600 species worldwide (Hickey and King, 1997), and Turkey is an important geography for the Lamiaceae family. Turkey has an important place in the world in terms of geography and species diversity. It is a great opportunity for the country to benefit from this richness where medicinal and aromatic plants (lavender, rosemary, basil etc.) are widely spread.

From a biotechnological point of view, the basic system that is considered in plant tissue processes and genetic improvements is the regeneration of the plant. In the plant regeneration process, tissue culture and callus formation are observed. Callus formation and further biotechnological developments depend on the plant resources and growth medium content in the culture. Hormones, generally called plant growth regulators, are added to the growth medium. Different results can be obtained with callus obtained with plant growth regulators added in different concentrations and combinations in callus formation or in later stages.

In this study, it was aimed to determine the total amount of antioxidant capacity and phenolic and flavonoid amounts in lavender, which is a medicinal and aromatic plant, by extracting the calluses obtained from media containing various plant growth regulator combinations with water and ethanol solvents.

2. Materials and method

In this study, *L. officinalis* (lavender) plant, which is a member of the Lamiaceae family, was used. Lavender seeds were obtained and identified at Firat University Plant Tissue Culture Laboratory and Greenhouse, Elazığ, Türkiye. Plants were grown from seed to grown-up plants under stable conditions at 22±2 ℃, 16-h d^{-1} photoperiod, 3000 lux light intensity. Node parts of the plant were used to obtain callus. Node explants were obtained on the day they were taken into the medium.

2.1. Method

Lavender explants were collected with the help of a scalpel. The explants were washed with tap water. They were kept in 70% ethanol for 30 seconds and kept in 50% sodium hypochlorite solution for 5 minutes. 5% commercial bleach was used as sodium hypochlorite. Then, the bleach was removed by washing 3 times with sterile distilled water.

Murashige and Skoog (MS) medium was used as the growth medium. MS medium meets almost all the basic nutritional needs for plant tissue culture. It also contains vitamins and minerals, macro and micro elements, which are the basic needs of plants.

In this study, combinations of Naphthalene Acetic Acid (NAA), Benzylaminopurine (BAP), Kinetin (KIN) and 2,4-Dichlorophenoxy Acetic Acid (2,4-D) plant growth regulators at different concentrations were used in addition to the MS medium. These combinations are shown in Table 1 and Table 2.

Table 1. Plant growth regulators in combination with BAP

Medium Code	2,4-D(mg/L)	BAP(mg/L)	NAA(mg/L)	KIN(mg/L)
0,5B-0,5N	-	0.5	0.5	-
0,5B-1N	-	0.5	1	-
0,5B-2N	-	0.5	2	-
0,5B-0,5D	0.5	0.5	-	-
0,5B-1D	1	0.5	-	-
0,5B-2D	2	0.5	-	-

Table 2. Plant growth regulators in combination with KIN

Medium Code	2,4-D(mg/L)	BAP(mg/L)	NAA(mg/L)	KIN(mg/L)
0,5K-0,5N	-	-	0.5	0.5
0,5K-1N	-	-	1	0.5
0,5K-2N	-	-	2	0.5
0,5K-0,5D	0.5	-	-	0.5
0,5K-1D	1	-	-	0.5
0,5K-2D	2	-	-	0.5

Growth medium was prepared with 4.4 g/L MS and 30 g/L sucrose. 7.8 g/L agar was added after the pH was stabilized in the range of 5.6-5.8. In case of any confusion, the coding process was applied to the prepared media as in Table 1 and Table 2. Explants were planted in the growth medium that was sterilized. The cultivated explants were kept at +24 ± 2 ℃ for 16 hours of light and 8 hours of dark.

The callus obtained were left to dry for 48 hours at 55 °C. Dry callus were crushed into small pieces with the help of a pestle. From the obtained dry callus, 0.1 g was weighed for each sample. For each 0.1 g mass of dry callus, 4 mL of water and ethanol solvents were added separately. Callus with added solvents were kept at +4 ℃ for 72 hours and were used for analysis after filtering by filter paper (Whatman No:1).

All experiments were done in 3 repetitions. The obtained results were tabulated and their standard deviations were calculated. The results are given with their standard deviations.

2.1.1. Analysis of total phenolic amount

In this study, total phenolic content analyzes were accomplished using the Folin-Ciocalteu method (Singleton & Rossi, 1965). For the analyses, 1.5 mL of 10-fold diluted Folin-Ciocalteu reagent was mixed with 300 µL of lavender callus extract. After a waiting period of about 2 minutes, 1.2 mL of 7.5% Na_2CO_3 solution was added and mixed thoroughly. Then, the absorbance values of the mixtures, which were kept in the dark for 90 minutes, were measured against pure water at a wavelength of 765 nm. The total amount of phenolics was calculated with the equation obtained from the gallic acid calibration curve and given as gallic acid equivalent (GAE) per g of dry callus weight

(d.c.w.). The absorbance values of gallic acid measured at different concentrations (0.025-0.05-0.1-0.2-0.4 mg/mL) were used for the calibration chart.

2.1.2. Analysis of total flavonoid amount

The spectrophotometric $AlCl_3$ method was used in the analysis of the total flavonoid content of lavender callus extracts (Lamaison et al., 1990). While preparing 2% $AlCl_3$ solution glacial acetic acid/methanol (50%, v/v) was used as solvent. In this analysis, 1 mL of lavender callus extracts and 1 mL of 2% $AlCl_3$ solution were mixed and kept at room conditions for 10 minutes. The absorbance values of the samples were then measured against the blank (2% $AlCl_3$) at a wavelength of 394 nm. The equation obtained from the quercetin calibration curve for the total amount of flavonoid substance was used and the results were given as quercetin equivalent (QE) per g of d.c.w. The absorbances of quercetin at different concentrations (0.025-0.05-0.1-0.2-0.4 mg/mL) were used for the calibration plot.

2.1.3. Determination of total antioxidant capacity

The antioxidant capacity of lavender callus extracts was measured considering the radical cation capture ability of 2,2′-azino-bis -3- ethylbenzthiazoline -6- sulphonicacid (ABTS). ABTS•+ radical is formed as a result of the oxidation of ABTS with persulfate.

The discoloration of the ABTS solution mixed with the plant extract indicates the presence of antioxidant activity. With the effect of this antioxidant presence, the ABTS•+ cation was fragmented and the dark blue color became lighter (Miller et al., 1995).

In this method, ABTS stock solution (7 mM) was diluted with phosphate buffer until its absorbance was approximately 0.7 and kept away from light as much as possible. Before the absorbance readings, 100 µL of lavender callus extract and 1900 µL of ABTS solution were mixed and kept at room conditions. At the end of the 6th minute, absorbance values were read against phosphate buffer at 734 nm wavelength. The results were given as trolox equivalent antioxidant capacity (TEAC) per g of d.c.w. using the line equation in the trolox calibration curve. The absorbances of trolox at different concentrations (0.05-0.1-0.2-0.3-0.4 mmol/L) were used for the calibration curve.

2.2. Statistical analysis

Statistical analyses of the data were performed by one way ANOVA and post hoc multiple comparisons (SPSS 21, Statistical Software, IBM). A probability value of $p<0.05$ was considered to denote a statistically significant difference. All determinations were performed in three replicates. Data were presented as mean values ±Standard Deviations (SD).

3. Results

The total antioxidant capacity, total phenolic and flavonoid substance amounts of the samples obtained by extracting the callus of the lavender plant with water and ethanol were obtained as a result of the analysis. Obtained results are shown on graphs and the differences between solvents and plant growth regulators are compared. In callus cultures of the study, the best callus formation was observed in growth medium containing 0.5 BAP + 1 mg/L 2,4-D.

3.1. Total phenolic content

While calculating the total content of phenolics, the line equation obtained from the gallic acid calibration graph (Fig. 1) was used. The absorbances measured during the analysis were substituted in the equation and the results were obtained as mg GAE/g.

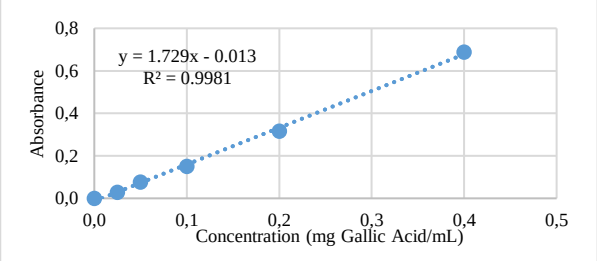

Figure 1. Gallic acid calibration curve

The results obtained from the samples using water and ethanol as solvents are given in graphs (Fig. 2 and Fig. 3). According to the results, the highest phenolic substance (35.74± 0.48 mg GAE/g) was obtained from 0.5 mg/L BAP + 0.5 mg/L NAA water extracts. The lowest amount of phenolic substance (24.22 ± 1.03 mg GAE/g) was obtained from the ethanol extract of the sample taken from the growth medium containing the combination of 0.5 mg/L KIN + 2 mg/L 2,4-D.

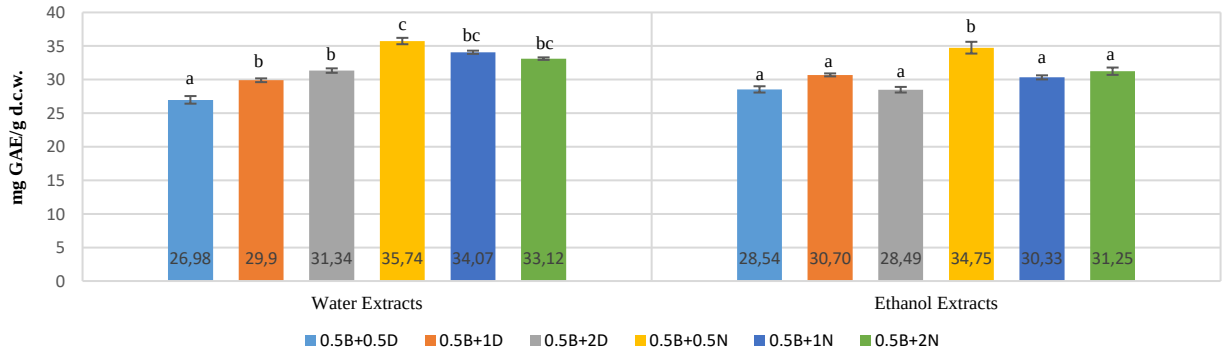

Figure 2. Total Phenolic Contents (mg GAE/g d.c.w.) of *L. officinalis* callus extracts according to grown different plant growth regulators (B: BAP, N:NAA, D: 2,4-D.) combinations (X mg/L+X mg/L). The values with different letters within column designate statistically significant differences, $p < 0.05$ by ANOVA. Error bars represent ± Standard Deviations (SD).

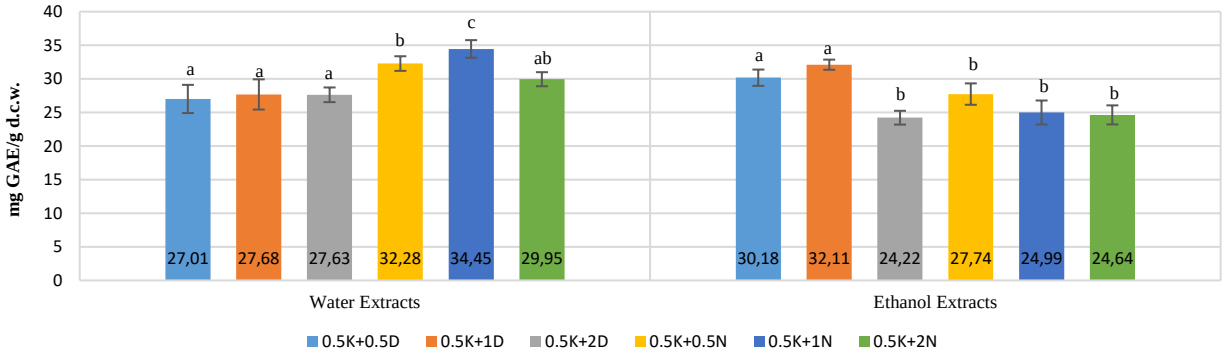

Figure 3. Total Phenolic Contents (mg GAE/g d.c.w.) of *L. officinalis* callus extracts according to grown different plant growth regulators (K: KIN, N:NAA, D: 2,4-D.) combinations (X mg/L+X mg/L). The values with different letters within column designate statistically significant differences, $p < 0.05$ by ANOVA. Error bars represent ± Standard Deviations (SD).

3.2. Total flavonoid substance content

The line equation obtained in the quercetin calibration graph (Fig. 4) was used to calculate the total content of flavonoid substance.

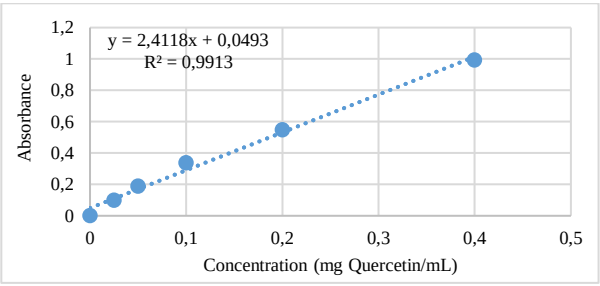

Figure 4. Quercetin calibration curve

The absorbance values measured during the analysis were substituted in the equation obtained from the graph, and the results were obtained as mg QE/g.

The results obtained from the samples using water and ethanol solvents are given in graphs (Fig. 5 and Fig. 6). According to the results, the highest amount of flavonoid content (32.42 ± 0.46 mg QE/g) was obtained from the water extract of the callus sample taken from the growth medium containing 0.5 mg/L BAP + 1 mg/L 2,4-D combination. The lowest amount of flavonoid content (11.64 ± 0.80 mg QE/g) was obtained from the ethanol extract of the callus sample taken from the growth medium containing the combination of 0.5 mg/L BAP + 2 mg/L NAA.

3.3. Total antioxidant capacity

The equation obtained in the trolox calibration graph (Fig. 7) was used to calculate the total antioxidant capacity. The absorbances measured during the analysis were substituted in the equation obtained from the graph, and the results were obtained as mmol/g TEAC.

The results obtained from the samples using water and ethanol solvents are given in a single graph (Fig. 8 and Fig. 9). According to the results, the highest antioxidant capacity (9.24 ± 0.14 mmol/g TEAC) was obtained from the water extract of the callus sample taken from the growth medium containing the combination of 0.5 mg/L BAP + 0.5 mg/L 2,4-D.

The lowest amount of antioxidant capacity (5.17 ± 0.41 mmol/g TEAC) was obtained from the ethanol extract of the callus sample taken from the growth medium with a combination of 0.5 mg/L KIN + 1 mg/L NAA.

4. Discussions

Growth media containing plant growth regulators at different concentrations affect callus development. The combination of different concentrations of these regulators also changes the development of the callus and the amount of total antioxidant, phenolic and flavonoid substances that the callus has.

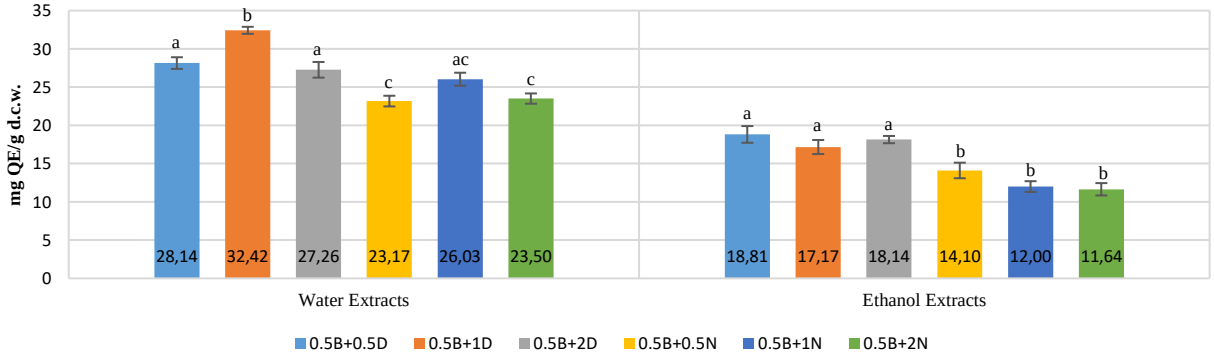

Figure 5. Total Flavonoid Contents (mg QE/g d.c.w.) of *L. officinalis* callus extracts according to grown different plant growth regulators (B: BAP, N:NAA, D: 2,4-D.) combinations (X mg/L+X mg/L). The values with different letters within column designate statistically significant differences, $p < 0.05$ by ANOVA. Error bars represent ± Standard Deviations (SD).

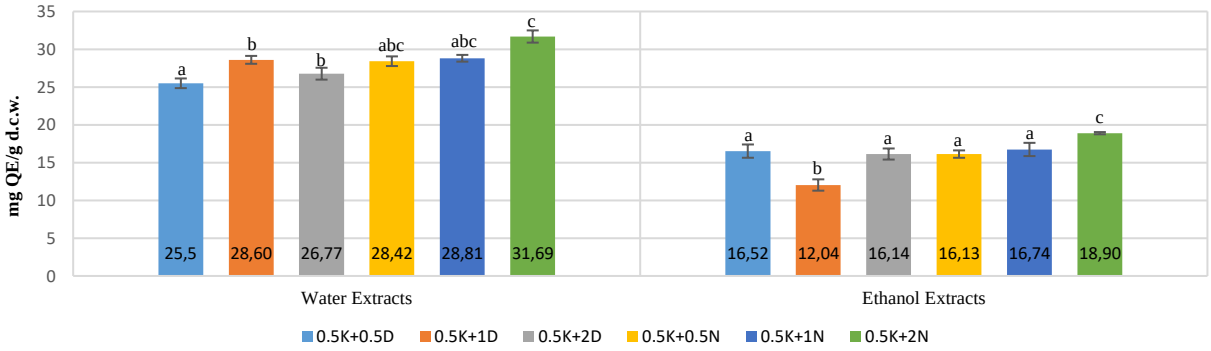

Figure 6. Total Flavonoid Contents (mg QE/g d.c.w.) of *L. officinalis* callus extracts according to grown different plant growth regulators (K. KIN, N:NAA, D: 2,4-D.) combinations (X mg/L+X mg/L). The values with different letters within column designate statistically significant differences, $p < 0.05$ by ANOVA. Error bars represent ± Standard Deviations (SD).

In callus cultures of this study, the best callus formation was observed in growth medium containing 0.5 BAP + 1 mg/L 2,4-D. On the other hand, Meric et al. (2019) observed callus formation from shoot explants of *Lavandula angustifolia* and used different growth regulators. They reported that the best callus development was obtained in MS medium containing 2 mg/L 2,4-D + 2 mg/L BAP.

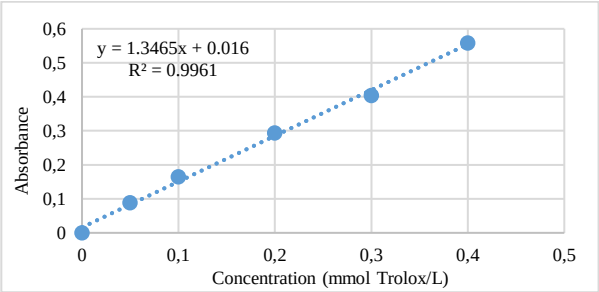

Figure 7. Trolox calibration curve

When the antioxidant activity studies of *L. officinalis* callus culture were examined, almost no study was found comparing growth medium combinations with antioxidant activity, phenolic and flavonoid content above the standards, as in this study. However, in studies with similar plants, studies comparing the relationships between plant growth regulator combinations and antioxidant activity were found. When the antioxidant activity studies with different types of callus are examined, it is seen that different results are generally obtained from the callus samples taken from different environments. The results obtained from callus extracts found in medicinal and aromatic plants generally prove that callus also shows antioxidant activity as in the total plant. It has also been reported in some studies that the presence of antioxidant activity in callus extracts was determined according to the materials used in natural antioxidant source research (Grzegorczyk et al., 2007; Yesil-Çeliktaş et al., 2007).

Kim et al., (2003) obtained 1.57 mg QE/g flavonoid substance in the water extract of the callus of *Stevia rebaudiana* plant. They found the amount of phenolic substance as 43.99 µg catechin equivalents/mg.

Kovacheva et al., (2006) in a study they conducted, found the amount of phenolics in ethanol extracts of *Lavandula vera* callus to be 42.3±4.2 mg GAE/g. They found the antioxidant activity to be 5.76 µm Trolox/g based on the trolox standard. In this study, 32.42 ± 0.46 mg QE/g was obtained in the water extract of *L. officinalis* callus obtained from the combination of 0.5 mg/L BAP + 1 mg/L

2,4-D. The amount of phenolic substance was found to be 35.74 ± 0.48 mg GAE/g based on the gallic acid standard. In a study by Kousalya and Bai (2016), they obtained callus from the explants of *Canscora decussata* plant with different growth regulators in the growth medium. As a result, the highest antioxidant capacity was obtained as 12.23 mmol/g TEAC in the callus obtained from the growth medium containing 1/2 MS + 0.5 mg/L NAA combination. In this study, the highest antioxidant capacity was obtained as 9.08 ± 0.13 mmol/g TEAC from the combination of 0.5 mg/L BAP + 0.5 mg/L 2,4-D.

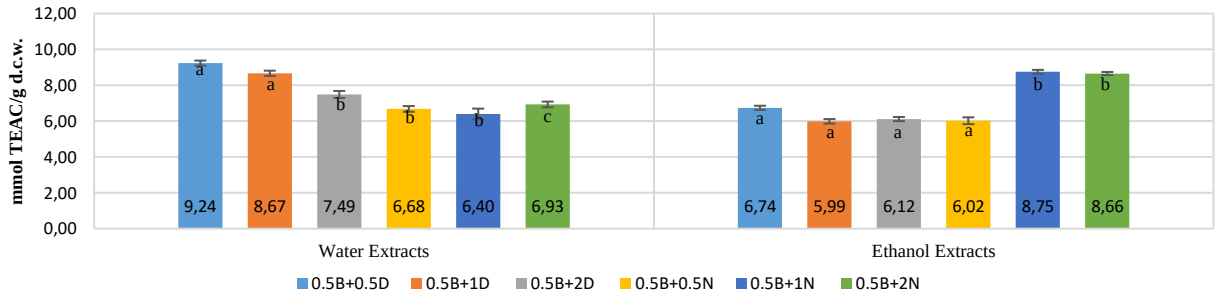

Figure 8. Total Antioxidant Capacity (mmol TEAC/g d.c.w) of *L. officinalis* callus extracts according to grown different plant growth regulators (B: BAP, N:NAA, D: 2,4-D.) combinations (X mg/L+X mg/L). The values with different letters within column designate statistically significant differences, $p < 0.05$ by ANOVA. Error bars represent ± Standard Deviations (SD).

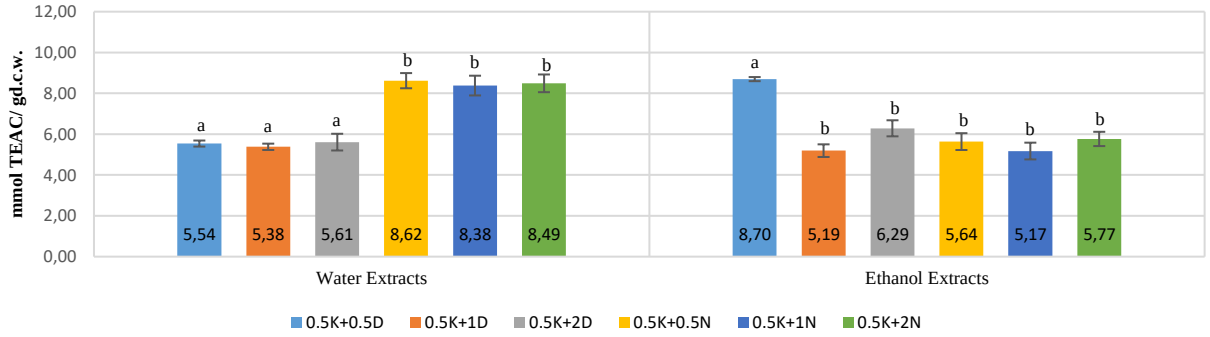

Figure 9. Total Antioxidant Capacity (mmol TEAC/g d.c.w) of *L. officinalis* callus extracts according to grown different plant growth regulators (K: KIN, N:NAA, D: 2,4-D.) combinations (X mg/L+X mg/L). The values with different letters within column designate statistically significant differences, $p < 0.05$ by ANOVA. Error bars represent ± Standard Deviations (SD).

Yoon et al. (2006) conducted a study investigating the antioxidant activities of *Rosmarinus officinalis* and *Lavandula spica* L. callus. They observed that the antioxidant activity of the plants changed according to the growth medium. They reported the highest effect in the lavender plant as 0.1 mg/L 2,4-D extract (37.6±0.9 µg/mL RC50).

Topdemir (2018), in her thesis study with *Melissa officinalis* L., examined the amount of highest antioxidant capacity of *M. officinalis* callus. Accordingly, it was reported that the combination of 1 mg/L 2,4-D + 1 mg/L PIC (Picloram) + 0.5 mg/L KIN plant growth regulator was obtained as 9.15±0.74 mmol/g TEAC from the water extract. When the ethanol extracts of callus were examined, it was reported that the highest antioxidant capacity was obtained as 14.61± 1.14 mmol/g TEAC from the plant growth regulator combination of 1.5 mg/L 2,4-D + 1 mg/L PIC + 0.5 mg/L KIN.

In a different thesis study with the callus of the basil (*Ocimum basilicum* L.) plant, the highest antioxidant content was 0.5 mg/L KIN – 0.5 mg/L 2,4-D plant growth regulator combination was 5.74±0.20 mmol/g TEAC from the ethanol extract of callus. was obtained. The amount of flavonoid substance was obtained as 3.01±0.34 mg quercetin/g from the ethanol extract of callus obtained from 0.5 mg/L BAP – 0.25 mg/L 2,4-D plant growth regulator combination. The highest total phenolic content was obtained from the callus ethanol extract obtained from the combination of 0.5 mg/L KIN – 0.5 mg/L NAA plant growth regulator as 2.55±0.11 mg GAE/g (Topdemir et al., 2019a,b).

5. Conclusion

When the results of the study are considered, it is seen that the extracts obtained with both solvents, water and ethanol,

have antioxidant activity. There are different uses of plant growth regulator in plant tissue culture. As a result, antioxidant activity and phenolic and flavonoid content of *L. officinalis* callus extracts obtained from growth media containing different plant growth regulator combinations were revealed.

Considering the results, it is seen that the callus show antioxidant capacity and contain phenolic and flavonoid substances. According to the general results of lavender calluses, it is seen that higher yields are obtained from the extracts using water as a solvent, especially considering the total antioxidant capacity and total flavonoid content. Growth medium and conditions should be considered in plant tissue engineering studies and in plants to be grown for commercial purposes. Plant cell suspensions can be used commercially for the production of secondary metabolites. For this purpose, more efficient productions can be obtained by transferring from flasks or petri dishes to bioreactors. It is possible to obtain higher results by optimizing the growth conditions of these callus and by more efficient extraction methods.

Conflict of Interest

Authors have declared no conflict of interest.

Authors' Contributions

The authors contributed equally.

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Investigations on anatomical and morphological characteristics of some *Crocus* L. taxa around Abant Lake

Emel USLU[1]*, Mehmet Tekin BABAÇ[2], Yasin BAKIŞ[3]
[1]*Bolu Abant İzzet Baysal University, Science and Arts Faculty, Department of Biology, Bolu, Türkiye*
[2]*Nezahat Gökyiğit Botanical Garden (NGBB), İstanbul, Turkiye*
[3]*Tulane University, Epista LLC, Louisiana, United States of America*
**uslu_e@ibu.edu.tr,[2]mtbabac@gmail.com,[3]yasinbakis@gmail.com*

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Abant gölü çevresindeki bazı *Crocus* L. taksonlarının anatomik ve morfolojik özellikleri üzerine araştırmalar

Abstract: In this study; the two *Crocus* L. taxa endemic to Bolu province, *Crocus abantensis* T. Baytop et Mathew and *Crocus*× *paulineae* Pasche & Kerndorff (hybrid) together with *C. ancyrensis* (Herbert) Maw subsp. *ancyrensis*, and *C. olivieri* J.Gay, were used to reveal their detailed leaf anatomical features. In view of these characteristics, it was aimed to determine the true parents of the hybrid and possible other hybrid taxa distributed in the south-southeast coasts Abant Lake. These two *Crocus* taxa, which are endemic to this region, have no previous anatomical studies. In this sense, deficiencies related to *Crocus* taxonomy have been completed. The main differences of *C.* × *paulineae* from the other taxa; having the thickest cuticle (3.80 µm), the longest parenchyma (20.93 µm) cell in the mesophyll, and having papillae like structure on the keel corners of cuticle. This structure was also observed in *C. olivieri* over the cuticle at the corners of the keel. The other important differences was the number of small vascular bundles among the studied taxa. The chromosomal number of hybrid was also given for the first time.

Key words: Abant Lake, *Crocus*, anatomy, endemic, Bolu

Özet: Bu çalışmada; Bolu ili için endemik iki *Crocus* L. taksonu, *Crocus abantensis* T. Baytop et Mathew ve *Crocus*× *paulineae* Pasche & Kerndorff (melez), ile birlikte *C. ancyrensis* (Herbert) Maw subsp. *ancyrensis* ve *C. olivieri* J.Gay, Bull. ayrıntılı yaprak anatomik özelliklerini ortaya çıkarmak için kullanıldı. Bu özellikler ışığında Abant Gölü'nün Güney-Güneydoğu kıyısında yayılış gösteren hibrit ve olası diğer hibrit taksonların gerçek ebeveynlerinin belirlenmesi amaçlanmıştır. Bu bölgeye endemik olan bu iki *Crocus* taksonun daha önce anatomik çalışması yoktur. Bu anlamda *Crocus* taksonomisi ile ilgili eksiklikleri tamamlanmıştır. *C.* × *paulineae*'nın diğer taksonlardan temel farklılıkları; mezofildeki en kalın kütikül (3.80 µm), en uzun parankima (20.93 µm) hücresine ve kütikülün karina köşelerinde papilla benzeri bir yapıya sahip olmasıdır. Bu yapı *C. olivieri* de de gözlenmiştir. Diğer önemli farklılıklar, incelenen taksonlar arasındaki küçük damar demetlerinin sayısıdır. Hibritin kromozom sayısı da ilk kez verilmiştir.

Anahtar Kelimeler: Abant Gölü, çiğdem, anatomi, endemik, Bolu

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1. Introduction

Turkey is rich in floristic terms with the increasing number of new species and even compared to Eastern Europe and West Asia and North Africa. A total of 9753 natural species have been recorded and 3035 of them are endemic for Turkey (Güner et al., 2012). Therefore, endemic species percentage is 31.12% (Mathew, 1984; Güner et al., 2012). It is much more important to increase the number of taxonomically important studies that will add value to the wealth beside the number of wealth of Turkish Flora. Bulbous plants of Turkey constitute an important part of this richness, such as about 800 taxa (Mathew, 1984; Ekim et al., 2000; Çolak, 2005). *Crocus* genus has been reported to be around 200 species in recent years, increasing significantly in the world (Harpke et al., 2014; Ruksans, 2017).

Turkey's Flora comprises a total of 103 *Crocus* L. species, including a natural hybrid among 235 *Crocus* species in the world (Mathew, 1984; Özhatay, 2002; Alavi-Kia et al., 2008; Kerndorff et al., 2012; Yüzbaşıoğlu et al., 2015; Erol and Çiftçi, 2022). Tuberous plants are grown in temperate climate regions of the world. *Crocus* species have been used as ornamental plants in European countries such as England and Germany for quite long time (Bowles, 1954; Mathew, 2000; Goode, 2005). New hybrids are grown from natural species. These beautiful flowers are also economically valuable in the ornamental plants market.

There are many systematic studies on this genus in literature (Pasche, 1994; Kerndorff and Pasche, 1994; 1996a,b; 1997; 2004; Mathew, 1982; Coşkun et al., 2010; Uslu et al., 2012). Coşkun et al. (2010) studied the phylogenetic relationship between 15 *Crocus* taxa using morphological and anatomical characters. In recent years, more studies have been conducted in the form of new taxa definitions (Candan and Özhatay, 2013; Harpke et al., 2014; Erol et al., 2014 and 2015; Yüzbaşıoğlu and Celep, 2016). One of these new taxa is *C. ancyrensis* Maw. subsp. *Güneri* Yüzb., was identified from Amasya region (Yüzbaşıoğlu and Celep, 2016), therefore; *C. ancyrensis* in the Abant region, according to taxonomic rules it will be accepted as subsp. *ancyrensis* in this study.

It is observed that most of the studies in the world have been done on *C. sativus* species (Negbi et al., 1989; Rios et al.,

1996; Bhargava, 2011). Rudall and Mathew (1990) have studied *Crocus* in terms of leaf anatomy, and many researchers now refer to this work in leaf studies. Mathew (2002) reviewed the morphological characteristics of the *Crocus* genus. Akan and Eker (2004) studied, the morphological and anatomical features of *C. pallasii* subsp. *turcicus* B.Mathew and *C. cancellatus* subsp. *damascenus* (Herb.) B.Mathew. Özdemir et al. (2004) have made anatomical and morphological studies on two endemic species in Turkey, which *C. danfordia* Maw. and *C. fleischeri* Baker Kandemir (2011) also compared 14 *Crocus* taxa in terms of leaf anatomy. Anatomical studies on *Crocus olivieri* J.Gay, Bull. were made by Özdemir et al. (2011). In another study, morphological and leaf anatomy of two yellow-flowered endemic taxa of *Crocus* (*Crocus ancyrensis*, *Crocus siehenaus* Hort. ex B.L.Burtt) were studied (Candan, 2015). Raca et al. (2017) studied three *Crocus* taxa from Verni series in Serbia.

When the anatomical and morphological studies of *Crocus* genus are examined in the literature; it is observed that the two endemic *Crocus* for Bolu, *Crocus abantensis* T. Baytop et Mathew, and *Crocus* × *paulineae* Pasche & Kerndorff, taxa are not included. The other two taxa *C. olivieri* J.Gay, (Özdemir et al., 2011) and *C. ancyrensis* (Herbert) Maw subsp. *ancyrensis* (Candan, 2015) have been studied by various researchers. The study also includes chromosome numbers and karyotype of the hybrid, were presented for the first time. In addition, two other taxa spread around Abant Lake; *C. ancyrensis* subsp. *ancyrensis*, and *C. olivieri* were also included in the study. Because it was considered that it is necessary in order to reveal the parents of the endemic *C.* x *paulineae* and if any other hybrids.

2. Material and method

Four *Crocus* taxa, *Crocus abantensis*, *Crocus* × *paulineae*, *C. ancyrensis* subsp. *ancyrensis* and *C. olivieri*, were collected from the Abant Lake in February-March 2018 during the flowering of *Crocus*. The abbreviations (ABA, C×PAU, ANC, and OLV respectively) of taxa were used in the Tables and Figures. Collected samples were diagnosed according to the Flora of Turkey and East Aegen Islands (Mathew, 1984; Güner et al., 2012). Voucher specimens of the taxa were deposited in the Abant İzzet Baysal University, Department of Biology. For morphological studies, 15 plant specimens were collected from each taxa. For anatomical studies, 10 individuals were stored in 70% alcohol.

Anatomical sections were obtained from fully developed leaves of specimens stored in 70% alcohol. Transverse and superficial (top and bottom) parts of the leaves were sectioned manually. Due to the fact that there was not much difference in the anatomical features of the parts such as root and stem (scape) in the *Crocus*, the leaf structure was emphasized in the study. The sections were prepared by the Glycerine-Gelatine method (Jensen, 1962) and examined. The photos were taken with the DP71 digital camera, which is compatible with the Olympus BX51light microscope. In order to illuminate the tissues in the best way, different dye solutions (Bozdağ et al., 2016) have been applied. Safranin O was used alone or together with fast green.

For anatomical studies, nineteen characters previously used in various studies (Mathew, 1984; Erol and Küçüker, 2007; Kandemir et al., 2012), which gave good results, were selected and 10 repeats were made (Table 1). However; the characters that given in the table with "†" sign did not included in the analysis; as there was no difference in the studied taxa. In the superficial sections of the epithelial cells forming the abaxial and adaxial surfaces of the leaf, the length and width measurements of the cells were made using micrometric slide and ocular. Stoma index of leaf superficial sections was also calculated as 10 repeats for each taxa. Since there was no stoma in the adaxial surface epidermis of the leaf, this procedure was performed for the abaxial surfaces. The number of stomata in per mm^2 was calculated (Meidner and Mansifield, 1968). The counts on the microscope were estimated over the area calculation.

For karyotype studies, roots of *C.* × *paulineae* were fixed in ethanol-glacial acetic acid (3:1) at 4°C for 24 hours and stored in 70% ethanol at 4°C. Prior to staining, hydrolysis was done using 1 N HCL at 60°C for 15 minutes. Finally, they were stained with 2% aceto-orcein for 2 hours and squashes were made with 45% acetic acid.

The photographs were taken using an Olympus BX51 light microscope with camera DP71 attachment. Karyotype parameters were prepared from well-spread metaphase plates. The somatic chromosome number and karyotypic details were studied in at least five well-prepared metaphase plates, and the mean values were used in the analysis. Chromosome pairs were identified according to the nomenclature of Levan et al. (1964).

In Cluster Analysis, the distance matrix was calculated by measuring different micro-morphological and anatomical characters (Table 2, 3) from 15-10 individuals respectively and the proximity of taxa was examined. Ward's distance matrix was calculated by taking average values. Clustering analysis (UPGMA) was performed by using Past (Paleontological Statistics Software Package for Education and Data Analysis, Hammer et al., 2001) program in order to investigate the proximity of studied taxa (Figure 2). In order to explore the groupings of the studied taxa, Principal Component Analysis (PCA) was carried by individual taxa data (Figure 3).

3. Results

Leaf cross-section of studied taxa was made manually and examples were given in Figure 1. Among them *C. olivieri* was the largest one, in Figure 1D. Epidermis cells (abaxial) were observed as a single row and square shape in all the studied taxa. *Crocus olivieri* and *C.x paulinea* had single-row epidermis and micro-papilla protrusions over the cuticle at the corners of the keel (Figure 1-B,D).

In the leaves of *Crocus* genus; the parenchymatic cells in the middle of the leaf arms, melted and formed the air space called lacuna, rectangular in shape, triangular or in the form of a central space. Among the taxa studied, *C. abantensis* and *C. olivieri* this space was very clearly rectangular (Figure 1-A, D). In *C. ancyrensis* subsp. *ancyrensis*, it was; closer to the triangular structure, while *C.*×*paulineae* could not be detected in a very obvious shape (Figure 1-C, B). The maximum lacuna space length and width was found in *C. ancyrensis* subsp. *ancyrensis*, while the least lacuna space length and width was in *C.*×*paulineae* (Table 2). The longest arm length (155.87 µm) was in *C. olivieri* the shortest one was in *C. abantensis*, the other taxa were ranged in between them (Table 2).

Table 1. The list of character names and their codes that used in the study (†: The characters were not included analysis, due to have no differences among the taxa).

Morphological characters	Anatomical characters
Corm Length (CL)	Thickness of Cuticle (TUC)
Corm Width (CW)	Upper Epidermis Length (UEL)
Corm Diameter (CD)	Upper Epidermis Width (UEW)
Leaf Length (LL)	Lower Epidermis Length (LEL)
Cataphyll Length 1 (CTL1)	Lower Epidermis Width (LEW)
Cataphyll Length 2 (CTL2)	Number of Large Vascular Bundle (NLV)†
Cataphyll Length 3 (CTL3)	Number of Medium Vascular Bundle (NMV)
Cataphyll Width 1 (CTW1)	Number of Small Vascular Bundle (NSV)
Cataphyll Width 2 (CTW2)	Lacuna Space Length (LSL)
Cataphyll Width 3 (CTW3)	Lacuna Space Width (LSW)
Bracte Length (BRTL)	Number of Palisade Cell (NPC)†
Bracteol Length (BRLL)	Palisade Cell Length (PCL)
Perianth Tube Length (PTL)	Palisade Cell Width (PCW)
Perianth Inner Segment Length (PISL) †	Number of Sponge Cell (NSC) †
Perianth Inner Segment Width (PISW)	Narrowest Carina Base Length (NCL)
Perianth Outer Segment Length (POSL)	Carina Arm's Length (CAL)
Perianth Outer Segment Width (POSW)	Stoma Number of Lower Epidermis (STN) †
Anther Length (AL)	Numbers of epidermal Cell (NEC)
Filament Length (FL) †	Stoma Index: (STI=STN/STN+NEC) * 100
Style Length (STYL)	
Stigma Length (STGL)	
Scape Length (SCL)	
Ovarium Length (OVL)	
Ovarium Length/Scape Length (P3) †	
Perianth Inner S. Length/Width (P4)	
Style Length (STYL)	
Perianth Outer S. Length/Width (P5)	

Table 2. Mean values of the anatomical characters (long names are given in Table 1) and standard deviations of the studied taxa.

Characters	ABA	ANC	PAU	OLV
TUC (µm)	3.27 ± 0.80	3.33 ± 0.49	3.80 ± 0.41	2.60 ± 0.51
UEL (µm**)**	6.03 ± 0.85	5.33 ± 0.49	6.00 ± 0.65	5.77 ± 0.68
UEW (µm)	5.67 ± 0.45	5.87 ± 0.83	5.67 ± 0.72	5.00 ± 0.76
LEL (µm)	6.73 ± 1.10	5.73 ± 0.70	5.27 ± 0.46	5.73 ± 0.80
LEW (µm)	6.90 ± 1.11	5.93 ± 0.70	5.53 ± 0.52	6.60 ± 0.99
NMV	4.00 ± 0.00	4.00 ± 0.00	4.00 ± 0.00	6.20 ± 2.08
NSV	1.20 ± 0.41	4.53 ± 1.41	5.60 ± 0.51	7.13 ± 2.00
LSL (µm)	35.33 ± 2.44	40.93 ± 8.30	32.00 ± 3.96	34.40 ± 4.67
LSW (µm)	13.00 ± 2.17	25.60 ± 4.47	15.93 ± 1.87	17.73 ± 3.22
PCL (µm)	16.33 ± 3.98	17.27 ± 1.71	20.93 ± 2.46	18.67 ± 2.82
PCW (µm)	4.67 ± 0.49	3.97 ± 0.58	3.83 ± 0.24	4.97 ± 0.64
NCL (µm)	61.53 ± 1.92	58.47 ± 3.85	48.33 ± 4.37	43.73 ± 7.42
NSC	2.73 ± 0.46	3.07 ± 0.59	3.00 ± 0.00	2.73 ± 0.46
CAL (µm)	113.53 ± 4.78	127.40 ± 18.04	119.00 ± 6.60	155.87 ± 17.71
STN	63.80 ± 12.17	69.60 ± 11.59	68.87 ± 7.93	70.80 ± 13.23
NEC	128.47 ± 22.24	135.87 ± 17.15	118.93 ± 8.48	135.73 ± 19.15
SI (%)	33.25 ± 4.18	33.83 ± 3.10	36.61 ± 2.49	34.22 ± 3.71

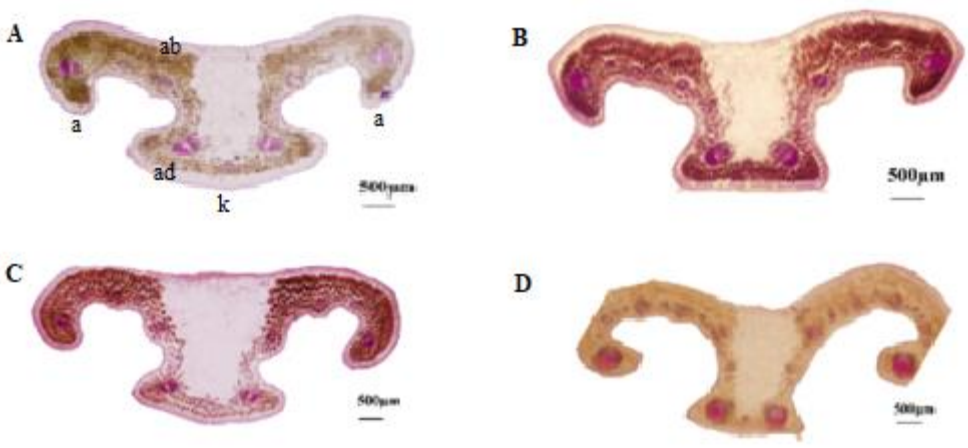

Figure 1. A- *Crocus abantensis* **B-** *Crocus* × *paulineae*,**C-** *C. ancyrensis* subsp. *ancyrensis*, **D-** *C. olivieri*. (a: arms; ab: abaxial epidermis; ad: adaxial epidermis; p: parenchyma also referred lacuna area; k: keel; m: mesophyle; v: vascular bundle).

Cuticle thickness was high (3.80 µm) in hybrid taxon, the least thickness (2.60 µm) was in *Crocus olivieri*. The number of palisade parenchyma cell line was 2, and the number of large vascular bundles was 4 in all the taxa; for this reason, they were not included in the Table 2. Large vascular bundles are located at the ends of the arms and keel corners (Figure 1 A-D). Small and medium vascular bundles are scattered between large ones. There was a difference in terms of number of small bundles, the least number (average 1.2) was in *C. abantensis.* On the other hand the average number of small bundles was; 5.60 in *C.*×*paulineae*, 7.13 in *C. olivieri* and, 4.53 in *C. ancyrensis* subsp. *ancyrensis* (Table 2).

Table 3. Mean values of the morphological characters (long names are given in Table 1) and standard deviations of the studied taxa.

	ABA	ANC	PAU	OLV
CL	0.68 ± 0.15	0.83 ± 0.16	0.61 ± 0.15	1.17 ± 0.41
CW	0.81 ± 0.22	0.86 ± 0.17	1.29 ± 0.37	1.13 ± 0.19
CD	2.67 ± 0.51	2.57 ± 0.40	3.59 ± 0.65	3.45 ± 0.69
LL	8.51 ± 1.39	11.21 ± 2.03	7.65 ± 1.26	14.47 ± 3.67
CTL1	1.93 ± 0.63	1.83 ± 0.46	2.75 ± 1.08	2.35 ± 0.64
CTL2	4.26 ± 1.42	3.67 ± 0.91	4.03 ± 1.06	5.85 ± 2.07
CTL3	5.63 ± 1.05	5.24 ± 1.09	6.49 ± 0.78	7.44 ± 2.53
CTW1	0.78 ± 0.19	0.71 ± 0.15	1.00 ± 0.21	1.09 ± 0.31
CTW2	0.79 ± 0.19	0.85 ± 0.23	2.55 ± 0.74	2.13 ± 0.70
CTW3	0.94 ± 0.20	0.85 ± 0.30	3.65 ± 1.66	4.55 ± 0.56
BRTL	5.61 ± 1.11	5.85 ± 1.14	3.52 ± 2.60	8.39 ± 2.19
BRLL	5.27 ± 0.98	4.01 ± 1.10	4.88 ± 0.68	8.89 ± 1.85
PTL	5.79 ± 1.18	5.71 ± 0.94	5.43 ± 0.77	7.53 ± 1.24
PISW	1.17 ± 0.26	0.71 ± 0.20	0.95 ± 0.12	0.96 ± 0.19
PISL	24.80 ± 0.27	23.13 ± 0.38	24.47 ± 0.38	24.40 ± 0.27
POSL	2.68 ± 0.18	2.59 ± 0.54	2.33 ± 0.29	2.42 ± 0.19
POSW	1.18 ± 0.18	0.72 ± 0.28	1.10 ± 0.22	0.82 ± 0.05
AL	1.19 ± 0.12	1.33 ± 0.37	0.97 ± 0.12	0.98 ± 0.14
STYL	6.79 ± 0.49	6.27 ± 0.95	6.09 ± 0.94	7.10 ± 0.93
FL	0.83 ± 0.20	0.75 ± 0.21	0.83 ± 0.18	0.75 ± 0.17
STGL	0.58 ± 0.14	0.51 ± 0.11	0.70 ± 0.23	0.61 ± 0.17
SCL	2.82 ± 0.48	3.71 ± 0.69	3.33 ± 0.92	4.17 ± 1.44
OVL	0.43 ± 0.12	0.58 ± 0.10	0.45 ± 0.07	0.55 ± 0.11
P1	5.77 ± 0.60	5.01 ± 1.38	6.35 ± 1.22	6.72 ± 1.46
P2	2.47 ± 0.41	1.73 ± 0.38	1.97 ± 0.66	1.85 ± 0.89
P3	0.19 ± 0.05	0.16 ± 0.05	0.15 ± 0.05	0.15 ± 0.06
P4	2.20 ± 0.41	3.46 ± 0.95	2.63 ± 0.41	2.69 ± 0.65
P5	2.34 ± 0.37	4.24 ± 2.03	2.39 ± 0.61	2.79 ± 0.26
P5	2.34 ± 0.37	4.24 ± 2.03	2.39 ± 0.61	2.79 ± 0.26

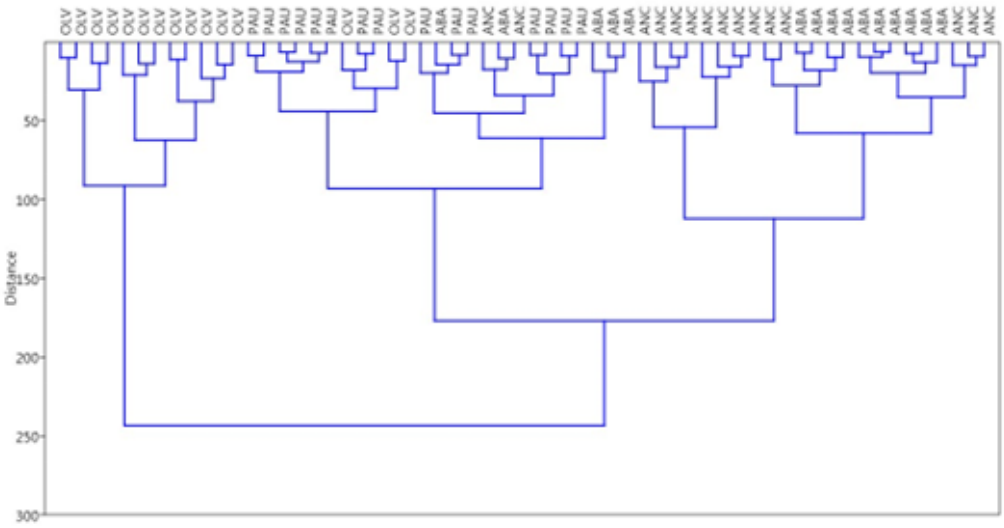

Figure 2. Graph of the taxa studied according to Ward's distance matrix by UPGMA method

In all the studied taxa; stomas were observed only in the lower surfaces of the leaves with the same plane as the epidermis cells. Stoma number (0.01 mm^2), and stoma indexes were calculated for each taxa (Table 2). Although there is no big difference between taxa; the lowest stoma average number was, 63.80, found in *C. abantensis*, and 69.60 in *C. ancyrensis* subsp. *ancyrensis*, 68.87 in hybrid taxon, and finally; 70.80 in *C. olivieri*. In terms of stoma index, all taxa were found very close to each other and the index was calculated between 33.25-36.61% (Table 2).

Among the morphological characters; corm diameter (CD: 3.59 cm), first cataphyll length (CTL1:2.75 cm), second cataphyll width (CTW2: 2.55 cm) and stigma length (STGL: 0.70 cm) values were, observed highest in hybrid taxon compared to other taxa (Table 3). Beside these, filament length, (0.83 cm) was also found high in both taxa *C. abantensis* and the hybrid. On the contrary to these characters, leaf length (LL: 7.65 cm), perianth tube length (PTL: 5.43 cm), anther length (AL: 0.97 cm), style length (STYL: 6.09 cm) and bracte length (BRTL: 3.52 cm); was the lowest value observed in the hybrid taxon (Table 3). Index type characters P1, P2, P3, P4, P5 were not found very useful for discriminating the taxa.

Crocus leaf has two arms and keel in the middle of these arms. The structure of arms; curved towards the keel, and differences are observed between the taxa such that the curl is at a narrow or wide angle (Table 4). The curved arms are usually extending parallel to the keel. Arm ends curved towards to keel with a narrow angle, and reach 2/3 of the keel; except *C. olivieri*. In this taxon, the arm ends usually reach the base of the keel (Figure 1-D; Table 4).

In the cluster analysis graph; *C.* × *paulineae*, *C. abantensis* and *C. ancyrensis* subsp. *ancyrensis* were creating a cluster together, *C. olivieri* formed the other cluster (Figure 2). Similarly in order to explore the groupings of the studied taxa, Principal Component Analysis (PCA) was carried by individual taxa data. The most important characters; their eigenvalues and percentages were given in Table 5. According to this graph, all the taxa were overlapped with each other (Figure 3).

Table 4. Comparison of leaf anatomical parts

	Leaf shape (general appearance)	**Mesophyll parenchyma**	**Epidermis (adaxial)**
Crocus×*paulineae*	The curved ends of the arms are curved at a narrow angle towards the keel and reach 2/3 of the keel. Wide keel base, slightly rounded corners, with a few papillae at corners	Spongy: elliptical Palisade: rectangular and two-rows	Single row and square shape, micro papillae at corners
Crocus abantensis	Curved; the arms extending parallel to the keel, reach 2/3 of the keel. Keel base is quite wide, and have rounded corners.	Spongy: oval-elliptical Palisade: rectangular and two-rows	Single row and square shape
Crocus ancyrensis subsp. *ancyrensis*	Curved arm tips are directed towards the keel at an acute angle and the arms reach 2/3 of the keel. The carina area is long and wide. Keel base is quite wide, with rounded corners.	Spongy: oval Palisade: rectangular and two-rows	Single row and square shape
Crocus olivieri	The curved arm ends are curled at a right or narrow angle and approach to the keel. The ends almost reach the base of the keel. Keel pointed corners, base wide. There are several protrusions on the cuticle.	Spongy: oval-elliptical Palisade: rectangular and two-rows	Single-row and square, micro papillae-like protrusions present in the cuticle

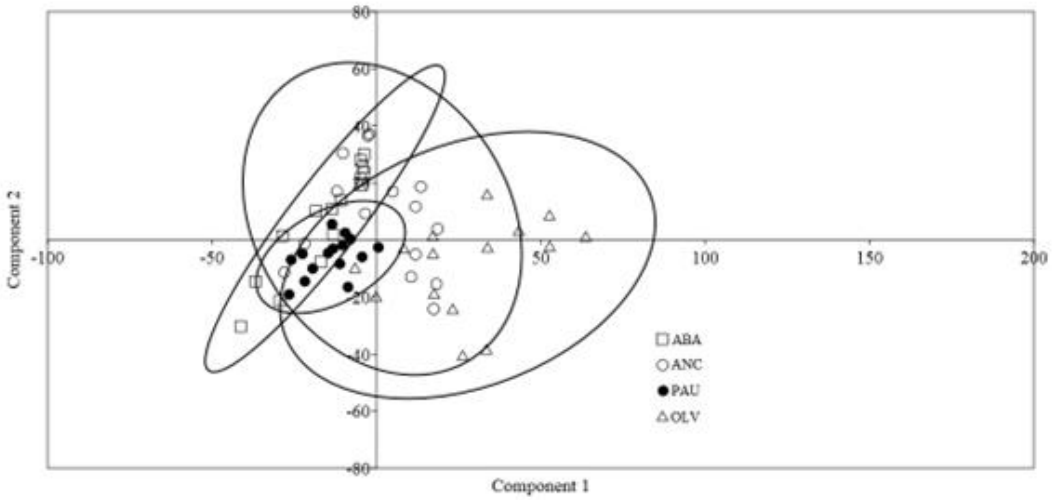

Figure 3. Groupings of the studied *Crocus* taxa by Principal Component Analysis (Cumulative percentage was about 62.1%).

Table 5. The most important characters; their eigenvalues and percentages for PCA graph.

PC	Eigenvalue	Variance (%)
1	20.1504	49.496
2	5.11277	12.559
3	3.06262	7.5228
4	2.6368	6.4769

The morphological characters of the studied taxa were compared in Table 6. Among these characters *C. x paulineae* and *C. olivieri* shared the similar structure for style, having 3 brached and bifurcate end. On the other hand *C. olivieri* has membranous tunica while the other taxa have reticulate fibrous structure (Table 6).

C. × *paulineae* chromosomal counts and its karyotype was prepared for the first time (Figure 4). There were 14 chromosomes; 2 pairs of them were sub-metacentric, and the rest were metacentric type chromosomes. Haploid component of its chromosomes was 24.79 µm and the length ranges were between 1.98-4.93 µm.

4. Discussions

Epidermis cells on leaf surface were observed as single row and square shape in all studied *Crocus* taxa. Only difference; *Crocus olivieri*, and *C. x paulineae* have a papilla-like structure on the cuticle above the corners of the keel (Figure 1-D, B). Similar structures were also observed in *C. caspius* Fisch. & Amp; C.A.Mey and *C. pallasii* Goldb. species (Rudall and Mathew, 1990). Likewise; these structures, were also found on their arms of *C. cansellatus* subspecies, Herb. ssp. *cancellatus*, ssp. *pamphylicus* B. Mathew and ssp. *damascenus* (Herb.) B. Mathew (Kandemir, 2011). Walls of the epidermis in stomatal regions (abaxial) for all the taxa are generally sinuous. The shape of transversal cross-section, in the widest part of the leaf, is highly relevant for taxonomy; as it is already assumed by Rudall and Mathew (1990). Structure of arms; keel, and this curl with narrow or wide angle, such differences between taxa were observed in this study as well (Table 4).

Among the taxa, *C. abantensis* and *C. olivieri* this space was clearly observed as a rectangle shape. *C. olivieri* was also reported to be rectangular by Özdemir et al. (2011); similarly this shape was rectangular some other *Crocus* taxa (Kandemir, 2009). In *C. ancyrensis* subsp. *ancyrensis*, this space was closer to the triangular shape, whereas in *C.x paulineae* there was not any obvious shape (Figure 1-B). Özdemir et al. (2006) in *C. flavus* subsp. *flavus* this shape was observed as triangular. The length of the arms and curl varies according to taxa; such as, *C. olivieri* has the longest arm (155.87µm), while *C. abantensis* has the least arm length (113.53 µm). Similarly, the arms of these two taxa approached to the keel at a narrower angle (Figure 1-A, D). Furthermore, in a study of Erol and Küçüker (2007), the

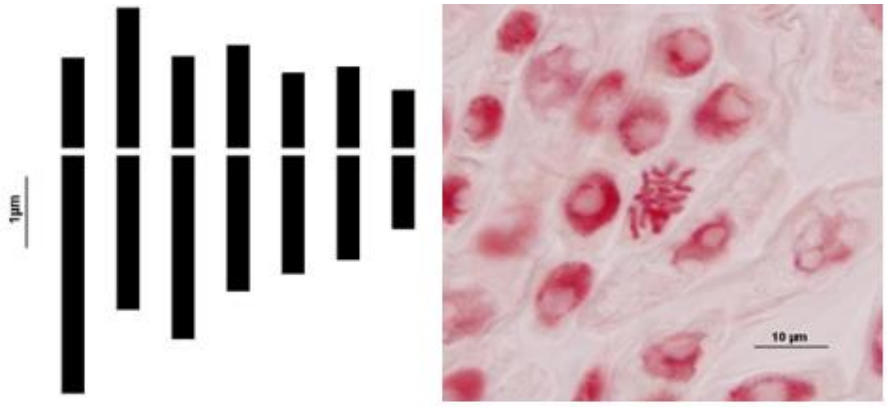

Figure 4. Karyotype and chromosome of *C. x paulineae*

arm length and curl variations were observed; among the taxa of *C. candidus* Clarke, *C. istanbulensis* (B.Mathew) Rukšāns. In another study, the length of arms and their curving degree differ even among the populations of *Crocus cf. heuffelianus* Herb. (Raca et al., 2019).

Rectangular shape palisade cells were found in the mesophyll, and almost round shape sponge parenchyma cells were present in all the taxa. The maximum length of palisade cell was, 20.93 µm which, observed in *C. x paulineae*. The second maximum length was, 18.67 µm, belong to the *C. olivieri* (Table 2). *Crocus ancyrensis* subsp. *ancyrensis* had 17.27 µm average length in this study, while maximum length was found 12.0 µm in the same taxon by Candan (2015). However, the length of palisade cell variations was between 37-72 µm in the *Crocus cf. heuffelianus* of different populations (Raca et al., 2019). The number of palisade cell was 2, sponge cell was 3-4 in the studied taxa; and in many studies (Özdemir et al., 2004; Satıl and Selvi, 2007; Kandemir, 2009; Raca et al., 2019). The vascular bundle was variable in terms of shape and number compared to the studied taxa. The number of medium and large vascular bundles was generally found to be 4 in all the taxa. Large vascular bundles were located at the ends of the arms with keel corners (Figure 1-A-D). Small and medium vascular bundles were scattered between them. Generally, there was a difference in terms of number of small vascular bundles among the taxa. The least average number was found in *C. abantensis* with the average number 1.20, while the highest number, 7.13 was seen in *C. olivieri* (Figure 1-A, D). The average number was 5.60 in hybrid taxa; 4.53 in *C. ancyrensis* (Figure 1-B,C). In similar studies, the number of large vascular bundles was four, and the number of small vascular bundles was varied at keel corners and arm ends (Kandemir, 2011; Erol and Küçüker, 2007; Satıl and Selvi, 2007; Raca et al., 2019).

Corm diameter was between 5-36 mm and leaf length between 5-79 mm among 15 *Crocus* taxa studied by Coşkun et al. (2010). In this study, corm diameter was found to be 2.57-3.59 cm, and leaf length 7.65-14.47 cm which was almost in the similar range as those *Crocus* data except leaf length was high in this study (Table 3). Leaf length can be related to the taxa. Similarly; anther length was 0.97-1.33 cm, filament length 0.75-0.83 cm, and style length 6.09-7.10 cm in this study. Anther length shorter or longer than 13 mm, filament length shorter or longer than 8.00 mm and style length shorter or longer than 15 mm was found in the study of Coşkun et al. (2010). In the study of Harpke et al. (2014) 9 different species was used and, filament length was found between 3.70-7.00 mm, anther length 7.00-14.00 mm and style length 5.00-11.50 mm. In this study periant outer segment length and width (POSL, POSW) was between 2.33-2.69 cm and 0.72-1.18 cm (Table 3) while, in Harpke et al. (2014) study, they were in the range of 21-30 mm and 5.00-10.50 mm respectively.

The plot of Cluster and Principal Component Analysis, was based on two purposes. First of all; the proximity of the studied taxa was examined; and secondly, *C. x paulineae* as the natural hybrid and its parents of this hybrid was investigated. In the literature, this taxon has not been studied much since, the diagnosis of the hybrid (Pasche and Kerndorff, 1999). The parents of this endemic natural hybrid was given as *C. abantensis* and *C. ancyrensis* subsp. *ancyrensis* by Pasche and Kerndorff (1999). However, another yellow-flowered taxon, *C. olivieri* flowering in the same period was also present in the same area. In order to identify whether this taxon might also have been another parent of the hybrid; these graphs were drawn. The result of cluster analysis and PCA of the studied taxa was given in Figure 2 and 3 respectively. There were 2 main groups in CA; hybrid and its parent's *C. abantensis* and *C. ancyrensis* subsp. *ancyrensis*. In the second group *C. olivieri* was joined to the first group (Figure 2). However; in the first group hybrid was joined to the parental groupings from outside, it did not form between *C. abantensis* and *C.*

Table 6. Comparison of leaf morphological parts.

***Crocus* taxa**	**Corm, Tunica, Leaf length, Periant tube Length**	**Periant inner and outer segment**	**Style and Filamanet**
Crocus×paulineae	Corm diameter is the biggest, corm length is the smallest one. Tunica densely reticulate fibrous type. There is no ring condition at tunica base. Leaf length is short according to the other taxa. Periant tube length is also shorter than the others.	Inner segments shape are broadly elliptical with brownish yellow color. Outer segments are elliptical shape having acute tips. Color is yellow with brown or purple spotted.	Style is 3 branched with bifurcate end, yellow or orange color. Filaments are 8 mm and yellow.
Crocus abantensis	Corm diameter is the second small taxon. Tunica densely reticulate fibrous. No ring formation at tunica base. Leaf length is the second short taxon. Periant tube length is longer than *C x paulineae*.	Inner segments shape are inverted ovoid with blue-liliac color. Outer segments are almost same with inner segments having obtuse or pointed tips. Color is blue-liliac.	Style is 3 branched with orange color. Filaments are about 8 mm and light yellow.
Crocus ancyrensis subsp. *ancyrensis*	Corm diameter is the smallest taxon. Tunica coarsely reticulate fibrous. No ring formation at tunica base. Leaf length is the second long taxon. Periant tube length is the second short taxon.	Inner segments shape are ovoid elliptic with broadly acute tips. Color is bright yellow. Outer segments shape are same with inner segments having acute tip. Color is bright yellow.	Style yellow or orange-red to orange, 3 branched. Filaments are 7.5 mm yellow.
Crocus olivieri	Corm diameter is the smallest taxon. Tunica coarsely reticulate fibrous. No ring formation at tunica base. Leaf length is the second long taxon. Periant tube length is the second short taxon.	Inner segments shape are narrowly elliptical with broadly acute tips. Color is yellow to golden yellow. Outer segments shape are almost same with inner segments. Color is golden yellow.	Style is 3 branched with bifurcate end, yellow to orange color. Filaments are 7.5 mm and dark yellow color.

ancyrensis subsp. *ancyrensis* (Figure 2). Similar groupings were also observed in PCA; *C x paulineae, C. abantensis, C. ancyrensis* subsp. *ancyrensis,* and *C. olivieri* were also overlapped with those taxa. (Figure 3). Therefore, according to these graphs both taxa *C. olivieri* and *C. acncyrensis* subsp. *ancyrensis* still have the possibility to be one of the parents of *C. x pauline*.

The study was aimed to show anatomical structure of Abant Lake *Crocus* taxa. There has been no records about hybrid taxon, *C.* × *paulineae* research. Especially; it is the first study for the natural hybrid and one of its parent *C. abantensis*, which were the two important and endemic species for Bolu. The main differences of hybrid from the other taxa; having the thickest cuticle (3.90 µm), the longest parenchyma (20. 80 µm) cell in the mesophyll, having papillae like structure on the keel corners of cuticle, sponge cell shape was oval-elliptic (Table 2-4). The last two characters; were in common with *C. olivieri*.

The chromosomal counts of the hybrid was 14 and its karyotype was also presented in this study for the first time (Figure 4). In the earlier study *C. ancyrensis* subsp. *ancyrensis* and *C. olivieri* chromosome counts were both 2n = 6 and all the chromosomes were subtelosentric type, and *C. abantensis* 2n = 8, which 2 pairs were submetacentric, the rest was metacentric (Table 7) (Uslu et al., 2012). According to chromosomal comparison hybrid taxon shows more chromosomal similarity to one of the its parents, *C. abantensis*. On the other hand the other parent, *C. ancyrensis* subsp *ancyrensis* shows similarity with *C. olivieri* (Table 7). This information also correlates that one of the parents of natural hybrid *C. ancyrensis* subsp. *ancyrensis*, shares the same chromosomal counts and characteristics with *C. olivieri*.

Table 7. Comparison of chromosome numbers, karyotypic descriptions and morphometric parameters of studied *Crocus* taxa

Taxon name	Chromosome Number	Karyotypic Description	Haploid Complement (µm)	L/S	I^C	A_1	A_2
C. abantensis	2n = 8	6 m + 2 sm	17.41	1.49	0.40	0.33	0.15
C. olivieri	2n =6	6 st	27.24	4.82	0.32	0.79	0.12
C. ancyrensis subsp. ancyrensis	2n =6	6 st	31.06	4.28	0.19	0.77	0.13
C. x paulinea	2n = 14	10m + 2 sm	24.79	1.59	0.39	0.77	0.29

Conflict of Interest

Authors have declared no conflict of interest.

Authors' Contributions

The authors contributed equally.

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