International Journal of Secondary Metabolite

IJSM
International Journal of Secondary Metabolite

e-ISSN: 2148-6905

http://dergipark.org.tr/en/pub/ijsm

IJSM

Volume: 8
Number: 1
2021
March

International Journal of Secondary Metabolite (IJSM)
is a peer-reviewed online journal

e-ISSN: 2148-6905

IJSM
International Journal of Secondary Metabolite

Volume 8 Issue 1 2021

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Frequency : 4 issues per year (March, June, September, December)
Online ISSN : 2148-6905
Website : https://dergipark.org.tr/en/pub/ijsm

International Journal of Secondary Metabolite (IJSM) is a peer-reviewed and academic online journal.
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International Journal of Secondary Metabolite

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International Journal of Secondary Metabolite
2021, Vol. 8, No. 1, 1–10

https://doi.org/10.21448/ijsm.765645

Published at http://dergipark.gov.tr/en/pub/ijsm Research Article

Variation of Phenolic and Pigment Composition Depending on Soil Type in Three Serpentinovag Plant Species

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Abstract: Serpentine soils are stressful for plant growth, due to nutrient deficiencies, especially Ca, low water-holding capacity, and high levels of heavy metals and Mg. Determination of biochemical differences of plants grown in serpentine and non-serpentine soil can contribute to understanding tolerance to serpentine soil. In this study, samples of three plant species (*Alyssum sibiricum* Willd., *Salvia absconditiflora* (Montbret & Aucher ex Bentham) Greuter & Burdet and *Centaurea urvillei* DC. subsp. *stepposa* Wagenitz) were compared in terms of the composition of phenolic compounds and chloroplast pigments. Higher amounts of phenolic compounds were measured in serpentine soil-grown forms of all three species. Total soluble phenolic content, in samples grown in serpentine and non-serpentine soil, respectively, *S. absconditiflora* 731.8 - 161.7, *C. urvillei* 121.0-49.7 and *A. sibiricum* 50.2- 47.3 µg g^{-1} DW). It was determined that the amount of chlorophyll a was higher in the serpentine soil grown forms of all three species while the amount of chlorophyll b was variable. In plants grown in serpentine soil, *β*-carotene values are higher than non-serpentine forms. Total xanthophyl values are also parallel with *β*-carotene findings, but lower. The findings in the present study show that antioxidant compounds such as phenolics and carotenoids may play a role in the stress tolerance of plants growing in serpentine habitats.

ARTICLE HISTORY

Received: July 07, 2020
Revised: November 10, 2020
Accepted: February 19, 2021

KEYWORDS

Carotenoid,
Chlorophyll,
Phenolic compounds,
Serpentine stress

1. INTRODUCTION

Serpentine soils are model systems for the study of evolution, ecology, and conservation (Harrison & Rajakaruna, 2011). Serpentine soils are stressful for plant growth, due to nutrient deficiencies, especially Ca, low water-holding capacity, and high levels of heavy metals and Mg (Kruckeberg, 1951). Serpentine soils as a product of ultramafic rocks formed of ferromagnesian silicates are extremely rich in terms of floristic diversity, particularly of endemic and rare taxa. High content of Mg and Fe and low Ca in serpentine soils are not suitable for plants growth and development thereon. Serpentine soils, although rich in heavy metals such as Ni, Co, and Cr, are poor in certain basic nutrients such as N, P, and K (Avcı, 2005).

Plants adapting to serpentine soil systems, those being essential serpentine plants, namely obligate ones, and not being able to go out of serpentine, are named as serpentinophyte, and

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ISSN-e: 2148-6905 /

those able to grow facultatively under different edaphic conditions, both serpentine and non-serpentine, as serpentinovag (Kurt et al., 2013). Arnold et al. (2016) genetically studied the serpentine soil adapted population of *Arabidopsis arenosa* (L.) Lawalrée to understand how plants recover from the combination of mineral deficiency, drought and toxic metal levels seen in serpentine habitats. Ultimately, they reported that genes that contributed to drought adaptation were selected.

The experimental results showed that serpentine plants do better on nonserpentine soils than on serpentine soils. Performance of putative soil-specific ecotypes of two plant species (*Achillea borealis* Bong. and *Gilia capitata* Sims.) when grown on nonserpentine and serpentine soils in the greenhouse (Kruckeberg, 1951). Kruckeberg (1951) has achieved three conclusions on plant endemism in serpentine soils: (1) plants are locally adapted to serpentine soils, forming distinct soil ecotypes; (2) soil ecotypes are the first stage in the evolutionary progression toward serpentine endemism; and (3) serpentine endemics are restricted from more fertile nonserpentine soils by competition (Anacker, 2014).

Secondary metabolites are commonly found in plants adapted to special ecosystems and different soil types. Adaptation to special conditions changes not only the morphological properties of plants, but also their biochemical composition (Cheynier et al., 2013). The accumulation and diversity of phenolic substances is an important factor in stress tolerance. Phenolic compounds provide tolerance to oxidative stress occurring under stress conditions due to their strong antioxidant properties (Agati et al., 2013). There are various studies manifesting accumulation of phenolic substances in plants under stress. Even though there is a linear relation between amount of phenolic substances and antioxidant capacity, antioxidant capacities of phenolic compounds vary. Caffeic acid and *p*-coumaric acid have especially higher antioxidant activities. Antioxidant capacity is related to side groups of phenolic ring (Kim & Lee, 2004; Wojdylo et al., 2007).

One of the common stress symptoms in plants is the change in the amount of chloroplast pigments. Environmental stress factors such as drought, high temperature, lack of plant nutrients and heavy metal pollution cause chlorophyll loss (Öncel et al., 2000). Carotenoids, on the other hand, are more resistant to stress conditions with their antioxidant properties (Ramel et al., 2012). Carotenoid amounts can remain constant or increase under stress conditions. As a result of stress conditions affecting the two photosystems differently, the chlorophyll a / b ratio may vary. Increasing carotenoid / chlorophyll ratios may be a stress indicator for plants (Oncel et al., 2004).

The elements of the antioxidant defense system are particularly emphasized because environmental stress conditions frequently trigger oxidative stress. In addition to total antioxidant capacity, basic antioxidant compounds such as phenolic compounds, carotenoids and chlorophyll content important indicators of oxidative stress, have been analyzed. Our research findings could contribute to understanding whether there are any differences in antioxidant substance compositions of plants under stressful conditions of serpentine soils. Samples of three serpentinovag plant species that spread naturally in the serpentine regions of Central Anatolia were taken from altitudes of 850-1400 m. The steppe vegetation is dominant in this region of Central Anatolia. A characteristic continental climate prevails in the region, with rather cold winter and hot summer periods. Freezing in winter and drying in summer creates conditions not suitable for plants. The harsh climate conditions combined with the high metal levels of serpentine soils and the negativities caused by the insufficiency of plant nutrients make plant life difficult. The steppe plants that have adapted to living in the region are drought tolerant plants originating from the Iran-Turan biogeographical region. Data from the Ankara meteorological station show that the semi-arid continental climate is effective in the higher parts of the region and the Mediterranean climate in the lower parts (Akman, 2010).

The genus *Alyssum* L. consists of 230 species that are native to Europe, Asia and Northern Africa. Species richness and diversity are confined to the Mediterranean and Turkey, and only a few species are distributed in North Africa, Central Asia, Siberia and North America (Li et al., 2014). *Alyssum sibiricum* Willd. It is a species that spreads naturally in Thrace, North, West and Central Anatolia, the Balkans, Crimea and the Caucasus, especially in slopes, steppe and open areas. *Salvia* L., with about 980 species and a nearly cosmopolitan distribution, is the largest genus in the angiosperm family Lamiaceae. The genus is represented by at least 500 species in Middle and South America, about 250 species in Southwest Asia and the Mediterranean region, and about 100 species in East Asia (Guo-Xiong et al., 2018). In Turkey, it is represented by more than 100 taxa. *Salvia absconditiflora* (Montbret & Aucher ex Benth.) Greuter & Burdet. It is a species that spreads on rocky limestone slopes, dry steppe, fallow fields, roadsides. The genus *Centaurea* L. spreads around the world with 750 species in Asia, North Africa, America and Europe (Nagaresh & Rahiminejad, 2018). Turkey also has 255 taxa of the genus *Centaurea* and endemism rate is 66.8%. *Centaurea urvillei* DC. subsp. *stepposa* Wagenitz spreads in Rocky slopes, macchie, open *Pinus* forests.

This study was planned to determine the role of phenolic and isoprenoid metabolism in serpentine soils adaptation of plants grown in natural environments. In this way, information can be provided on the biochemical regulation required for tolerance to stress conditions caused by serpentine soil properties. It is aimed to compare the three plant species grown in serpentine soils with the populations grown in non-serpentine soils. For this purpose, analysis of phenolic compounds and plastid pigments was carried out in the samples of three serpentinovag species growing in serpentine and non-serpentine soil. While HPLC procedure was followed in the analysis of phenolic compounds, TLC and spectrophotometric methods were used in the analysis of isoprenoids.

2. MATERIAL and METHODS

2.1. Material

Samples were taken from the different populations of the three plant species that were the subject of the study (Table 1), both in and without serpentine. Field studies were carried out in May and June in the Elmadağ and Imrahor walley, located in the south east of Ankara, in 2015 and 2016. The plants were identified using Flora of Turkey and The East Agean Islands, vol. 1, 5 and 7 (Davis, 1965, 1975, 1982) by Latif Kurt and plant samples are preserved in ANK herbarium. After the plants were brought to the laboratory, they were superficially cleaned and dried in a 60°C oven. For the analysis, only the aboveground parts of the plants consisting of leaf and herbaceous stem tissues were used. All analyzes were performed on samples from at least three different regions and average values are presented in the tables.

2.2. Analyses of Phenolic Compounds

Extraction of plant phenolic compounds and quantitative analysis with the help of HPLC were performed according to the method proposed by Caponio et al. (1999). 0.1 g sample taken from the above-ground parts of the plants was homogenized by breaking up in methanol. The homogenate was centrifuged at 10000 g for 10 minutes and the supernatant was filtered through 0.45 µm cellulose acetate filters. 20 µL volumes of the filtrates were injected into the HPLC column. Phenolic compounds contained in the standards and samples were separated by passing the reverse phase Supelco LC18 (size 250x4.6 mm and pore diameter 2.5 µm) through the column. As a mobile phase for separation, 2% acetic acid (A) and methanol (B) solutions with a flow rate of 0.8 mL min^{-1}. It was used with the gradient flow program determined by Caponio et al. (1999). The phenolic compound content in the samples was determined with a UV detector at 278 nm. A mixture containing a total of 15 different phenolic compounds was used as standard. (benzoic acid, chlorogenic acid, caffeic acid, catechin, epicatechin, gallic acid,

hesperidin, hydroxybenzoic acid, *p*-coumaric acid, quercetin, rosmarinic acid, sinapic acid, syringic acid, *t*-cinnamic acid, *t*-ferulic acid). Area measurements of the peaks obtained with the standard series were made and standard curves were created. The peaks of the phenolic compounds in the plant extract were compared with the standard peaks and their quantities were calculated.

Table 1. The plant species subject to the study, their families and their soil characteristics.

Code	Species	Family	Soil type	Locality, Collector and Number of plants
As (S)	*Alyssum sibiricum* Willd.	Brassicaceae	On Serpantin	Elmadağ Serpentine Series, 1350 m., Kurt, L., 12365
As (NS)	*Alyssum sibiricum* Willd.		Non Serpantin	Limestone slopes, Imrahor valley, 980 m., Kurt, L., 13427
Sa (S)	*Salvia absconditiflora* (Montbret & Aucher ex Bentham) Greuter & Burdet	Lamiaceae	On Serpantin	Elmadağ Serpentine Series, 1350 m., Kurt, L., 12370
Sa (NS)	*Salvia absconditiflora* (Montbret & Aucher ex Bentham) Greuter & Burdet		Non Serpantin	Limestone slopes, Imrahor valley, 980 m., Kurt, L., 13452
Cu (S)	*Centaurea urvillei* DC. subsp. *stepposa* Wagenitz	Asteraceae	On Serpantin	Elmadağ Serpentine Series, 1350 m., Kurt, L., 12342
Cu (NS)	*Centaurea urvillei* DC. subsp. *stepposa* Wagenitz		Non Serpantin	Limestone slopes, Imrahor valley, 980 m., Kurt, L., 13471

2.3. Analyses of Chlorophylls and Carotenoids

Chlorophyll analyzes were made from 0.2 g tissues from dried plant leaves and herbaceous stem samples. The pH was adjusted to 7.8 for extraction and a cooled 80% acetone solution was used. Samples were crushed with 2x4 mL solvent in pre-cooled mortars and transferred to centrifuge tubes. After centrifugation at 5000 g for 5 minutes, absorbance measurements were made with spectrophotometer at 664 and 647 nm. Chlorophyll contents were calculated with equations determined by Porra et al. (1989). Analysis of carotenoids was performed by absorbance measurement in the spectrophotometer followed by separation by thin layer chromatography determined by Moore (1974). 0.2 g of dry leaves and herbaceous stem tissue were homogenized by breaking into 5 mL of cold acetone. The homogenate glass was filtered through fiber discs and taken into the rotary evaporator flasks and the solvent was completely evaporated at 50˚C. The residue was dissolved in 1 mL chloroform and taken into eppendorf tubes. 50 µL portions of extracts and standards were applied to silica gel sheets (20 × 20, 0.25 mm thickness). Hexane, diethyl ether, acetone, (60:30:20, v: v: v) mixture was used as mobile phase. The carotene and xanthophyll stains separated on the sheet were scraped separately and taken into the centrifuge tubes. It was shaken for 10 minutes by adding 5 mL of acetone, centrifuged for 5 minutes at 5000 g and absorbances at 450 nm were measured in the spectrophotometer. The amount of carotenoids was calculated with the help of curves drawn by *β*-carotene and lutein standards.

2.4. Statistical Analysis

Analyses of phenolics and plastid pigments in the specimens pertaining to various populations collected from Ankara, Elmadağ and Imrahor walley regions were carried out in three repetitions. Analysis results were calculated as mean values for each species. The differences between the three serpentinovag species were evaluated with the Kruskal-Wallis test, and the differences between the different life strategies depending on the soil type were evaluated with the t-test.

3. RESULTS and DISCUSSION

The phenolic compounds were analyzed in three serpentinovag plant species grown in serpentine and non-serpentine soil. Higher amounts of phenolic compounds were measured in serpentine soil-grown forms of all three species. This difference is particularly high in *C. urvillei* and *S. absconditiflora* species. Although there are high amounts of phenolic substances in both forms of *S. absconditiflora* species, it was determined that especially the forms grown in serpentine soil are rich in coumaric acid (484.3 µg g^{-1}) and chlorogenic acid (186.6 µg g^{-1}) compounds. While in the serpentine soil-grown *S. absconditiflora* forms hesperidin (22.2 µg g^{-1}) was found, in non-serpentine soil-grown forms, a low amount of quersetin (9.9 µg g^{-1}) and cinnamic acid (2.4 µg g^{-1}) were determined. *C. urvillei* is the richest species in terms of phenolic substance diversity. Six different phenolic compounds were identified in the serpentine forms of *C. urvillei* and five in the non-serpentine forms. Of these compounds, except for cinnamic acid, others were measured in higher amounts in serpentine forms. Four different compounds were identified in the serpentine forms of the *A. sibiricum* species and three different compounds in the non-serpentine forms. Total phenolic content was higher in serpentine forms (Table 2).

Table 2. Phenolic substance compositions of three serpentinovag plant species. (serpentinovag on serpentine (S) and serpentinovag non serpentine (NS).

Phenol Compounds	As (S)	As (NS)	Sa (S)	Sa (NS)	Cu (S)	Cu (NS)
Benzoic acid	-	-	38.7±8.8	40.6±8.1	-	-
Chlorogenic acid	29.3±20.1	11.4±0.8	186.6±11.7	11.1±0.1	21.7±8.8	10.9±0.1
Cinnamic acid	12.5±4.4	27.9±7.1	-	2.4±0.2	0.5±0.1	7.9±1.2
Coumeric acid	2.9±0.2	8.0±0.1	484.3±97.2	98.7±1.6	18.1±0.1	9.2±0.2
Epicatechin	-	-	-	-	15.7±5.4	2.4±1.3
Hesperidin	-	-	22.2±0.2	-	64.0±0.3	19.3±0.2
Quercetin	5.5±3.7	-	-	9.9±1.8	1.0±0.1	-
Total	50.2	47.3	731.8	162.7	121.0	49.7

Unit µg g^{-1} DW.

Plants developed on serpentine are exposed to secondary water stress due to being exposed to high concentrations of heavy metals such as Ni, Co, and Cr. High concentrations of heavy metals may damage roots leading to blocking water intake from soils. High concentrations of osmoprotectants such as soluble carbohydrates and proline have been determined in plants grown naturally in serpentine soil (Özbey et al., 2017). Studies on the effects of heavy metals such as Ni, Co, Cr, which are abundant in serpentine soils, on plants have also reported similar findings (Öncel et al., 2000; Sharmila & Pardha Saradhi, 2002). Stress conditions such as heavy metal (Petukhov et al., 2019), drought (Keleş & Öncel, 2002) and nutrient deficiency (especially nitrogen and phosphorus) (Chishaki & Horiguchi, 1997) can cause the accumulation of phenolic compounds.

The researchers show that phenolic substance quantity is a suitable stress marker for many species, and they can increase under high temperature and drought conditions, particularly during summer times (Agati et al., 2013). Findings of the present study indicate that phenolic compounds are substantially high in quantity in all of the species examined (Table 2). A large number of plants naturally grown in high mountains and steppes were compared in terms of their phenolic content. However, no significant difference was found between the total soluble phenolic amounts of plants grown in these two different habitats. However, the highest phenolic substance values among the plants examined were determined in steppe plants such as *Salvia sclarea* L. and *Xanthemum annuum* L. (Oncel et al., 2004).

There is a strong correlation between the total amount of phenolic compounds and antioxidant capacity. The fact that the plants grown in serpentine soil has higher phenolic content may indicate that the serpentine stress has an oxidative property. There are studies showing that phenolic compounds and total antioxidant capacity are high in the members of the Lamiaceae family. In a study comparing Lamiacea and Poaceae species, it was determined that Lamiaceae species such as *Salvia*, *Sideritis* and *Lamium* were rich in phenolic compounds and antioxidant capacity (Sağır et al., 2018). Total phenolic substance content and antioxidant capacity of 112 anticancer medicinal plant species were analyzed utilizing methanolic and aqueous extracts. The findings proved that medicinal plants with higher content of total phenolic substances have substantially higher total antioxidant capacity. Based on these findings, it was suggested that phenolic compounds have substantial impact on antioxidant capacity of medicinal plants (Cai et al., 2004). Wojdylo et al. (2007) analyzed the relation between levels of phenolic compounds and antioxidant capacities in 32 herbaceous plants of various families, and they mentioned that there is a positive and significant correlation between phenolic compounds and antioxidant capacities. Gülcemal et al. (2010) reported that some phenolic glycosides contained in *C. urvillei* species can be used against cancer by their anti-proteosomal activities.

Chlorophyll and carotenoid compounds of three serpentinovag species subject to the study were analyzed. It was determined that the amount of chlorophyll a was higher in the serpentine soil grown forms of all three species while the amount of chlorophyll b was variable. This situation, caused chlorophyll a / b ratios in the forms grown in serpentine soils to be higher than those grown in non-serpentine soil. While the average chlorophyll a / b ratio in serpentine forms was 2.21, it was calculated as 1.56 in non-serpentine forms. Among the three investigated species, the highest chlorophyll a value were determined as *S. absconditiflora* species. The species with the highest total carotenoid values is *S. absconditiflora,* 488 µg g^{-1} in serpentine forms and 457 µg g^{-1} in non-serpentine forms. It was determined that the amount of *β*-carotene of the species subject to the study is close to each other. In plants grown in serpentine soil, *β*-carotene values are higher than non-serpentine forms. Total xanthophyll values are also parallel with *β*-carotene findings, but lower. While the amount of carotenoid per chlorophyll was lower in the serpentine forms of the *A. sibiricum* species, it did not show a significant difference in the *C. urvillei* and *S. absconditiflora* species (Table 3).

One of the fastest observed stress indicators in plants is chlorophyll loss. There are many studies showing that it can cause chlorophyll loss in drought (Batra, Sharma & Kumari, 2014), heavy metal stress (Öncel et al., 2000) and mineral nutrient deficiencies (Ruamrungsri et al., 1996). In this study, while chlorophyll a amount was found higher in serpentinovag species, in the forms grown in serpentine soils, a significant change in chlorophyll b amount could not be determined. This may indicate the success of the three species studied in the adaptation of serpentine soils, as well as the forms grown in non-serpentine soils under the influence of various environmental stresses. The high rate of chlorophyll a / b in the forms grown in serpentine soils indicates that there is a difference in terms of regulation of photosystems. It is

difficult to interpret the effect of serpentine on the amount of chlorophyll since there is not enough study on the physiological and biochemical adaptation of plants to serpentine habitats.

Carotenoids have critical importance in terms of protecting chloroplasts under stress conditions with their antioxidant properties. In the samples grown in serpentine soils of the three serpentinovag species subject to this study, both the amounts of *β*-carotene and xanthophylls were found to be significantly high. This indicates that the role of carotenoids should be studied in more detail in terms of tolerance to serpentine stress. Carotenoids protect chloroplasts against photoinhibition by dissipating excess energy under heat and light stress conditions (Choudhury & Behera, 2001). It can be suggested that chloroplasts and photosynthetic structures of on-serpentine forms could be better protected against oxidative stress effects than non-serpentine forms. Çekiç et al. (2018), determined higher car/chl ratios in gypsophile plants adapted to gypsum soil. However, in gypsovag plants, a significant change from the soil type could not be identified.

Although there are some studies on the distribution, properties, ecology and plant endemism of serpentine soils (Brady et al., 2005; Kurt et al., 2013; Anacker, 2014) studies on the physiology and biochemistry of serpentine tolerance in plants are insufficient (Özbey et al., 2017). Determining the effects of serpentine stress on secondary metabolism in wild plants may contribute to the understanding of serpentine tolerance. In the three serpentinovag plant species that are the subject of this study, it has been determined that not only the amount but also the variety of the phenolic compounds varies depending on the soil properties. Similarly, changes in the amount and rates of isoprenoids (chlorophyll, *β*-carotene and xanthophylls) have been found.

Table 3. Pigment compositions of three serpentinovag species. (serpentinovag on serpentine (S) and serpentinovag non serpentine (NS).

Species (soil type)	Chl-a (μg g^{-1} DW)	Chl-b (μg g^{-1} DW)	Chl a/b	β-Carotene (μg g^{-1} DW)	Xanthophyll (μg g^{-1} DW)	Car / Chl
A. sibiricum (S)	686 ± 94	346 ± 24	1.98	265±37	123±31	0.38
A. sibiricum (NS)	389 ± 34	269 ± 26	1,44	228±32	106±23	0.51
S. absconditiflora (S)	873 ± 36	334 ± 18	2.61	277±29	211±27	0.40
S. absconditiflora (NS)	709 ± 53	368 ± 25	1,92	253±41	204±34	0.42
C. urvillei (S)	561 ± 23	274 ± 22	2.04	246±38	111±15	0.43
C. urvillei (NS)	473 ± 37	355 ± 23	1.33	235±46	106±25	0.41
Statistics: Species (KW)	10.38 (**)	5.46 (ns)	4.85 (ns)	7.30 (ns)	10.38 (**)	3.62 (ns)
Soil type (t)	0.00 (**)	0.60 (ns)	0.00 (**)	0.00 (**)	0.00 (**)	0.09 (ns)

Kruskal-Wallis (KW) and t-test (t) results are shown at the bottom of each column (ns not significant, * $p \leq 0.05$, ** $p \leq 0.01$).

4. CONCLUSION

The findings in the present study show that antioxidant compounds such as phenolics and carotenoids may play a role in stress adaptation of plants growing in serpentin soils. In addition, it was concluded that serpentinovag plant species do not have common properties in terms of phenolic substance compositions, and show unique differences for each species. In serpentine-grown forms, the increase of antioxidants such as *β*-carotene and xanthophyll in addition to phenolic compounds can be attributed to the need for protection against oxidative stress. The findings of this research have shown that serpentine soils cause an increase in the phenolic substance content. However, it is not clear enough whether the variety of phenolic compounds synthesized in plants varies depending on the soil properties. For example, benzoic acid, chlorogenic acid and coumaric acid compounds were determined in both soil types, while the

presence of cinnamic acid, quersetin and hesperidin compounds varied depending on the soil type. This subject is important for understanding phenolic metabolism under stress conditions and needs to be studied with more species.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s).

Authorship contribution statement

Fazilet Ozlem Albayrak: HPLC analysis of phenolic compounds. **Ebru Ozdeniz:** Collection of plant material from the field and preparation for analysis. **Latif Kurt:** Planning the study, identification of plant material and writing of the article. **Yuksel Keles:** Planning the study, analysis of isoprenoid compounds, writing of the article.

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International Journal of Secondary Metabolite
2021, Vol. 8, No. 1, 11–19

https://doi.org/10.21448/ijsm.739771

Published at http://dergipark.gov.tr/en/pub/ijsm Research Article

Effect of Gibberellin on Some Fatty Acid Profiles Under Nitrogen Starvation in Green Algae *Chlorella vulgaris*

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Abstract: Plant growth substances could be stimulating algal growth rate and alter lipid compositions. In the present study, we tested hypothesis that exogenous gibberellin (GA) has any effect on growth rate and some fatty acid profiles in green algae *Chlorella vulgaris*. In Bold Basal Medium with 100 µM GA_3, cell density increased to 68.57% on third day as compared to the control cells. These results indicated that GA_3 enhanced microalgal growth and cell size. The lipid profile was also altered compared to control using Gas Chromatography-Mass Spectrometry (GC-MS). GA_3 promotes the production of C16:0, C18:0, C18:1 and C18:3 on day-3 and-5. Under nitrogen starvation condition, application of GA_3 provide enhanced algae growth and stimulated C16:0 and C18:1 production. In conclusion, this study demonstrated that gibberellin could be a good candidate as a hormone for increasing lipid production in microalgae culture system.

ARTICLE HISTORY

Received: May 19, 2020
Revised: November 02, 2020
Accepted: February 22, 2021

KEYWORDS

Chlorella vulgaris,
Gibberellin,
GC-MS,
Fatty acid,
Nitrogen starvation

1. INTRODUCTION

Microalgae are photosynthetic microorganisms with a high growth rate and the ability to convert carbon dioxide into biomass. Microalgae can synthesize high levels of metabolites that play an important role in biodiesel production, such as docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), carotenoids (β-carotene, lutein and astaxanthin), and phycocyanin (Mata et al., 2016). In this respect, it is widely accepted today as a potential sustainable biomass raw material source for biofuel production (Borowitzka & Moheimani, 2013). Although microalgae are a rich source of potential molecules (such as lipids, carbohydrates and proteins) that can be converted into fuel substitutes that are renewable, non-toxic, biodegradable and carbon-neutral; therefore, they are regarded as an environmentally friendly fuel source (Dillschneider et al., 2013), microalgal biofuels are still not seen as an alternative to fossil fuels. Because the main obstacle to successfully implementing microalgal biofuels as a replacement for fossil fuels is their high cost to produce. Today, many researchers focused on obtaining cheaper and high efficiency microalgal biofuels.

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ISSN-e: 2148-6905 /© IJSM 2021

The manipulation of culture conditions or genetic engineering approaches are widely used to increase targeted compounds such as lipids, pigments, proteins and PUFAs from microalgae (Sreekumar et al., 2018). One of the most widely used approaches is enhanced the cultivation of microalgae or biomass production. This approach is the most expensive and technically difficult step in the implementation of algae biofuel production (Leite et al., 2013). Increasing the biomass productivity and/or lipid and carbohydrate production of microalgae can increase algae cultivation's economic feasibility (Abdelaziz et al., 2013). The cellular accumulation of lipids in microalgae can also be induced by different environmental factors such as high light and salinity (Solovchenko et al., 2008; Rodolfi et al., 2009; Ren et al., 2014; Benvenuti et al., 2015). Previous studies demonstrated that application of metal stress such as copper, magnesium, iron and cadmium enhanced total lipid content (Liu et al., 2008; Li et al., 2013; Ren et al., 2014). It has also known that N-starvation causes alternation on carbon flux through the pathway of protein synthesis to lipid and/or carbohydrate metabolisms (James et al., 2013; Li et al., 2013; Jerez et al., 2016). Thus, high lipid accumulation in microalgae can be occurring under N starvation (Rodolfi et al., 2009; Benvenuti et al., 2015). Previous studies demonstrated that lipid content enhanced a 2 to 4-fold under N-starvation in microalgae such as *Chlorella, Chlamydomonas*, *Dunaliella* and *Nannochloropsis* species (Rodolfi et al., 2009; Cakmak et al., 2012; Illman et al., 2000). However, algae growth, development and metabolism effected negatively under nitrogen starvation.

Exogenously applying of plant growth regulators could be one of the alternative strategies to stimulate the synthesis fatty acids (Park et al., 2013; Lu & Xu, 2015). Some research groups reported that auxin and jasmonic acid altered to fatty acid composition in *Chlorella* species (Jusoh et al., 2015[a]; 2015[b]). Gibberellins (GAs) are diterpenoid acids that affect many areas of plant growth, such as leaf growth and flower and seed development. They promote stem elongation, fruit generation and seed germination in higher plant (Nakajima et al., 2006). It has also known that gibberellins found in macro-and microalgae (Lu & Xu, 2015). Previous studies demonstrated that active GAs, GA_1 and GA_3 in brown algae *Fucus vesiculosus* and *F. spiralis* (Radley, 1961; Jennings, 1968). Additionally, many studies have focused on gibberellin's effect on the growth of microalgae and their bioproducts. In addition, previous studies have reported that GA increases biomass accumulation and triacylglycerol content in *microalgae* (Mekhalfi et al., 2014; Du et al., 2015). Although increased growth in response to GAs has been documented in some algae (Jennings, 1968; Joseph & Chennubhotla, 1999), little evidence for GAs activity on growth and developmental processes has been observed in green algae (Lu & Xu, 2015). Moreover, the effects of GA_3 under normal conditions and N-starvation on fatty acid production have not demonstrated yet. In the present work, we tested two hypotheses; (i) exogenously GA_3 altered fatty acid composition under normal conditions, (ii) exogenous GA_3 changes the fatty acids composition) under N-starvation.

2. MATERIAL and METHODS

2.1. Culture Conditions

Chlorella vulgaris was obtained from the EGEMAC culture collection, Ege University, Izmir, Turkey. Five of the 250 mL Erlenmeyer flasks of *C. vulgaris* were used for the experiment. All experiments were carried out using cells in the exponential phase. It set up four different experimental group. The first group was the culture that was grown in a Bold Basal media (BBM, as a control). The second group was 250 mL Erlenmeyer flask of *C. vulgaris* grown in a BBM containing 100 µM GA_3. Third group was that *C. vulgaris* culture was collected with centrifuge and grown in BBM without any nitrogen sources. Finally, fourth group was that *C. vulgaris* culture was grown in BBM without nitrogen and with GA_3 in a growth chamber under continuous illumination at 80 µmol $m^{-2}s^{-1}$ light intensity and 24 °C for 1, 3, 5 and 7 days.

2.2. Growth Rate and Cell Size

The absorbance of cell growth was measured on UV-spectrophotometer at 663 nm, and cell number was counted with Neubauer hemocytometer. Each experiment repeated three times.

2.3. Methyl Esters of Fatty Acids (FAMEs)

FAMEs were carried out according to the modified procedure of Bligh and Dyer (1959) and Kattner and Fricke (Kattner & Fricke, 1986). Briefly, the extraction mixture with the dissolved lipids was evaporated to dryness and trans-esterified with 2 mL of 3% H_2SO_4 in methanol (Kattner & Fricke, 1986) four hours at 70°C. After cooling to room temperature, 2 mL of hexane was added for extraction of FAMEs. The solvent was evaporated, and 50 µL of hexane was added. Each experiment repeated three times.

2.4. GC-MS Analysis

The methyl esters of fatty acids were quantified by a gas chromatograph (Shimadzu QP2010 ultra model) equipped with a flame ionization detector (FID). The GC-MS column (TRB-5MS model) was fused 30 mm x 0.25 mm x 0.25 µm. Injector and FID inlet temperature were 270°C and 250°C, respectively. Column temperature was programmed to hold at 40°C for 4 min, then rise at 8°C min^{-1} increase to 280°C and was held at this temperature for 20 min. The column head pressure of carrier gas (helium) was flow rate 0.8 mL min^{-1}. Each experiment repeated three times.

2.5. Statistical Analysis

Statistical analysis was performed with one-way analysis of variance (ANOVA) or Student's t-test followed by *post-hoc* Tukey test as appropriate (SPSS for Windows, version 11.0).

3. RESULTS and DISCUSSION

3.1. Effect of Gibberellin on Cell Growth and Size

Exogenously plant growth regulators induced cell growth and algal biomass in microalgae (Joseph & Chennubhotla, 1999). GAs is a phytohormone and essential for plant growth and development processes (Sasaki et al., 2003; Tyler et al., 2004). In the present study, the stimulation effect of GA_3 on the growth was tested depend on time (Table 1). As shown in Table 1, the cell density of *C. vulgaris* at early stationary growth phase was 4.92 $x10^6\pm$ cells/mL. In BBM with 100 µM GA_3, cell density increased to 9.74 $x10^6\pm$ cells/mL on third day. The cell density also increased 127. 99% on the seventh day as compared to the control cells (Table 1). Previous studies demonstrated that GA_3 stimulated biomass production in *Chlamydomonas reinhardtii* (Park et al., 2013). Falkowska et al. (2011) also showed GA_3 had a stimulating influence on the cell number in *C. vulgaris.* Similarly, it was observed that GA treatment increased biomass productivity by 8.7-fold and 5.3-fold, respectively, in *C. ellipsoidea* and *Scenedesmus abundans* (González-Garcinuño et al., 2016). In this study, microscopic analysis in the present study showed that application of GA_3 significantly affected cell size in *C. vulgaris* culture (Table 1). These results indicated that GA_3 could be a very useful phytohormone for improving algal cell density. Similarly, Yu et al. (2016) reported that an increase in growth/biomass due to GA treatment might increase glucose uptake rate. Still, this consumption may occur with inhibition of glycolysis and the tricarboxylic acid cycle. However, more studies are needed to determine which gibberellins promote metabolic pathways.

3.2. Effect of Gibberellin on Fatty Acid Composition

Previously studies reported that plant growth regulators and growth stage altered oil compositions in microalgae and higher plants (Joseph & Chennubhotla, 1999; Lu & Xu, 2015). In the normal conditions, our results demonstrated that production of C18:0 increased on the

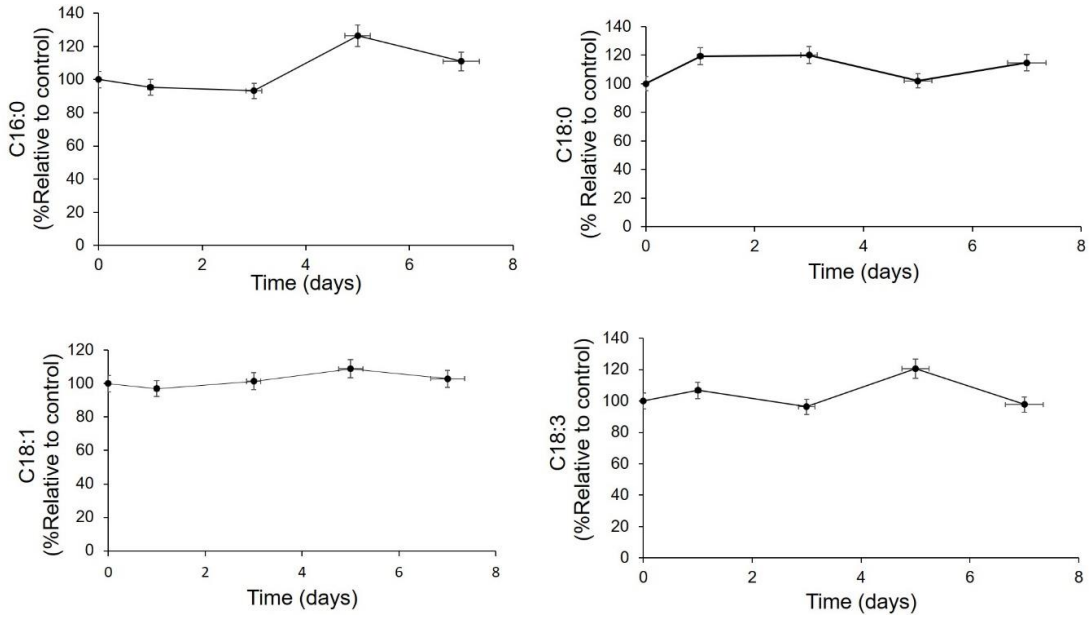
first and third days; however, interestingly, the increased amount of C16:0, C18:1, and C18:3 occurs especially on fifth day (Figure 1) under normal conditions. Grindstaff et al. (1996) demonstrated that GA_3 stimulates the degree of unsaturation of fatty acid in barley aleuronic layers in the higher plant. González-Garcinuño et al. (2016) also demonstrated the application of Gibberellins enhanced lipid productivity in *C. ellipsoidea*. In addition, treatment with GA_3 induced the amount of polyunsaturated fatty acid (especially, C18:2, and C18:3) and decreased the amount of saturated (16:0 and 18:0) fatty acid in isolated ER microsomal membranes (Grindstaff et al., 1996). In contrast, in the present study, GA_3 enhanced the production of C18:0, C18:1 and C16:0 on the first day and up to maximum production on the fifth day ($p<0.05$, Figure 2). These results indicated that application of gibberellin significantly enhanced fatty acid production in algae.

Table 1. The growth parameter of *Chlorella vulgaris* culture applied with GA_3 or control

Days after treatment	Cell density		Cell density % relative to control	Cell Size (µm)		Cell size % relative to control
	Control X±SD	GA_3 X±SD		Control X±SD	GA_3 X±SD	
1.day	4.92 x10^6± 0.14	5.33 x10^6± 0.18 ^{c}	108.33%	2.92 ± 0.43	3.17 ± 0.58	8.56%
3.day	7.61 x10^6± 0.12^a	9.74 x10^6±0.30^{bc}	127.99%	2.64 ± 0.63	3.05 ± 0.54	15.53%
5.day	14.20 x10^6± 0.21^a	18.54 x10^6±0.21^{bc}	130.56%	2.93 ± 0.62	2.95 ± 0.60	0.68%
7.day	16.07 x10^6 ± 0.28^a	22.45 x10^6±0.90^{bc}	139.7%	2.65 ± 0.43	3.01 ± 0.56	13.58%

"a" is a significant value when compared to control 1-day, "b" is a significant value when compared to GA_3 1-day, and "c" is a significant value when compared to control.

Figure 1. Fatty acid profile of *Chlorella vulgaris* grown under normal culture condition.



3.3. Effect of Gibberellin on Growth and Fatty Acid Composition Under N-Starvation

Effect of N-starvation on microalgae growth rate was demonstrated as seen in Table 2. Zhu et al. (2014) reported that N-starved cells increased twofold in number within the first two days. Similarly, our results showed that cell density of *C. vulgaris* increased 2-fold within three days under N-depletion (Table 2). It could be used for nitrogen storage during growth processes. However, cell density decreased up to approximately 1.66-fold in day-7 when compared to day-3. Application of GA_3 provides to increasing to cell growth under N-starvation (Table 2). After

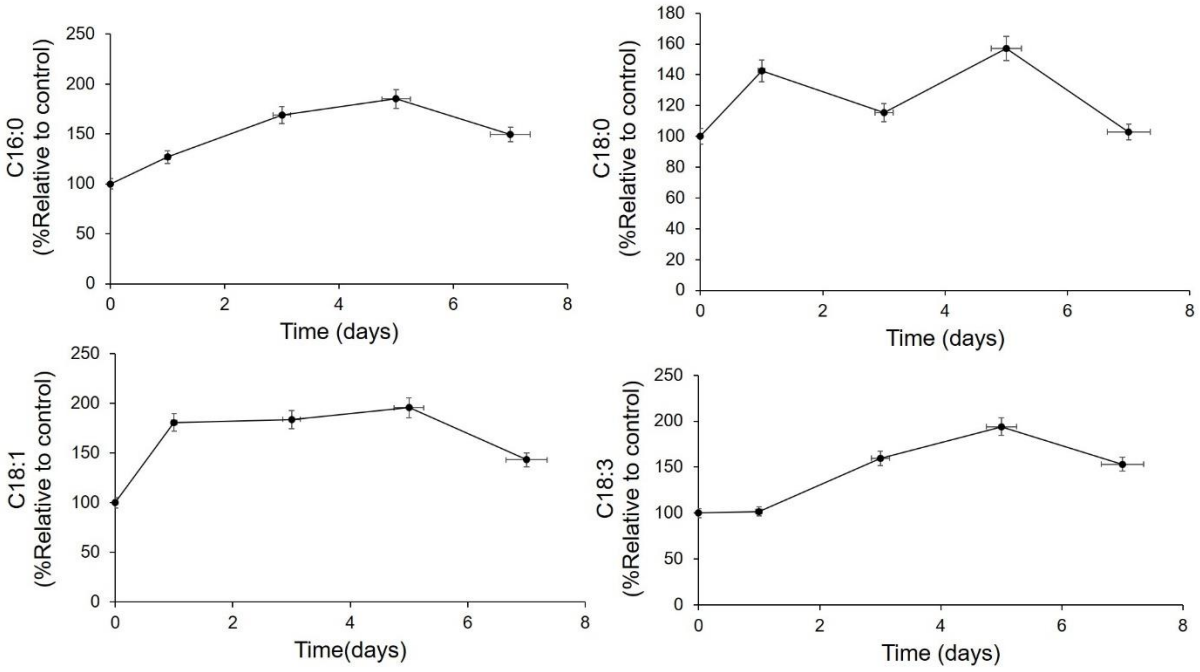
5 days, the cell growth started to decrease because of the lack of nitrogen sources. This effect could be explained that gibberellin as a phytohormone could trigger some metabolic pathways which are involved in response to nitrogen starvation. On the other hand, this hypothesis is speculation and needs to be testing in future studies.

Table 2. The growth parameter of *Chlorella vulgaris* culture applied with GA_3 or control

Days after treatment	Cell density		Cell density % relative to N-starvation
	N-starvation X±SD	N-starvation GA_3 X±SD	
1.day	4.67 $x10^6$± 0.12	4.83 $x10^6$± 0.05	103.43%
3.day	9.33 $x10^6$± 0.16^a	11.29 $x10^6$±0.12^{bc}	121.01%
5.day	7.51 $x10^6$± 0.25^a	14.02 $x10^6$±0.18^{bc}	186.68%
7.day	6.63 $x10^6$ ± 0.09^a	12.06 $x10^6$± 0.09^{bc}	181.9%

"a" is significant value when compared to control 1-day, "b" is significant value when compared to GA_3 1-day, and "c" is significant value when compared to control.

Figure 2. Fatty acid profile of *Chlorella vulgaris* grown under normal culture condition with 100 µM GA_3



N-starvation is very a general approach for changing fatty acid composition in algae. Previous studies demonstrated that N-starvation induced fatty acid production especially C16:0 and C18:1 in microalgae (Benvenuti et al., 2015; Babu, Wu, Kabra & Kim, 2017). In the present study, the fatty acid profile changed under N-starvation. The C16:0 and C18:1 increased within the first day under N-depletion (Figure 3). However, C18:3 and C18:0 profile in *C. vulgaris* significantly decreased after 3 days (Figure 3). Babu et al. (2017) demonstrated that phytohormone's application under N-limitation is a useful cultivation strategy to improve the lipid production rate of microalgae. Similarly, application of GA_3 with N-starvation significantly increased C16:0, C18:0, C18:1, and C18:3 at first and three days ($p<0.01$, Figure 4). Our results also showed that the application of GA_3 under normal conditions provided approximately 1.02, 1.18, 1.24 and 1.16 fold higher results C18:0, C18:1, C18:3 and C16:0

compared to N-starvation with GA_3 within 3 days, respectively (Figure 4). This study showed that other combinations of growth medium supplement with GA_3 100 µM and one or more published strategies such as nitrogen starvation could further increase the unsaturated fatty acid synthesis productivity of *C. vulgaris*, making its use industrially viable.

Figure 3. Fatty acid profile of *Chlorella vulgaris* grown under N-starvation condition.

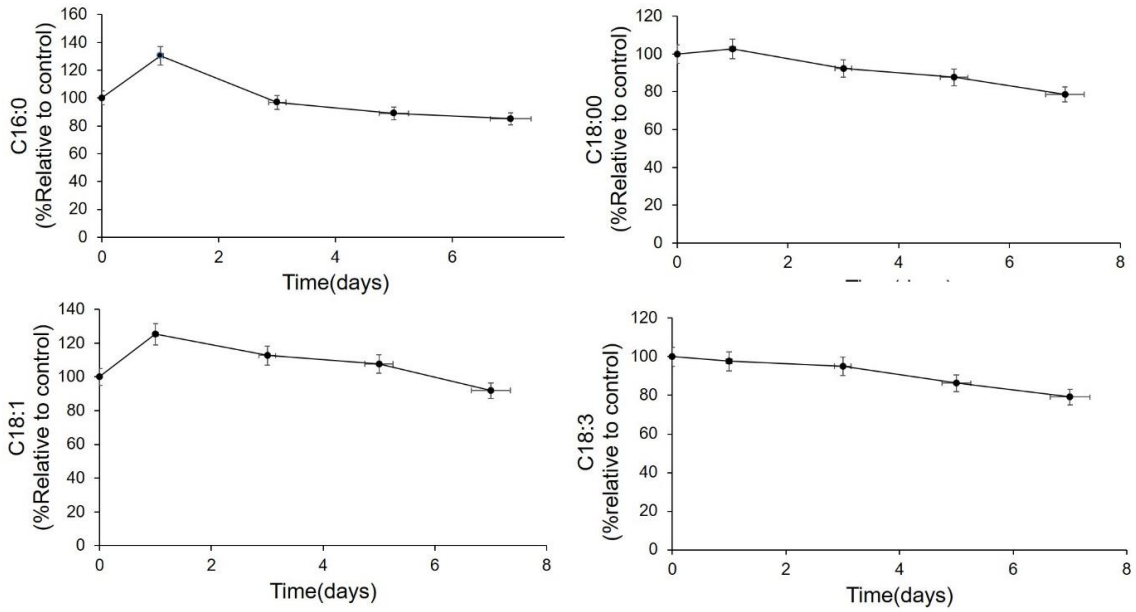
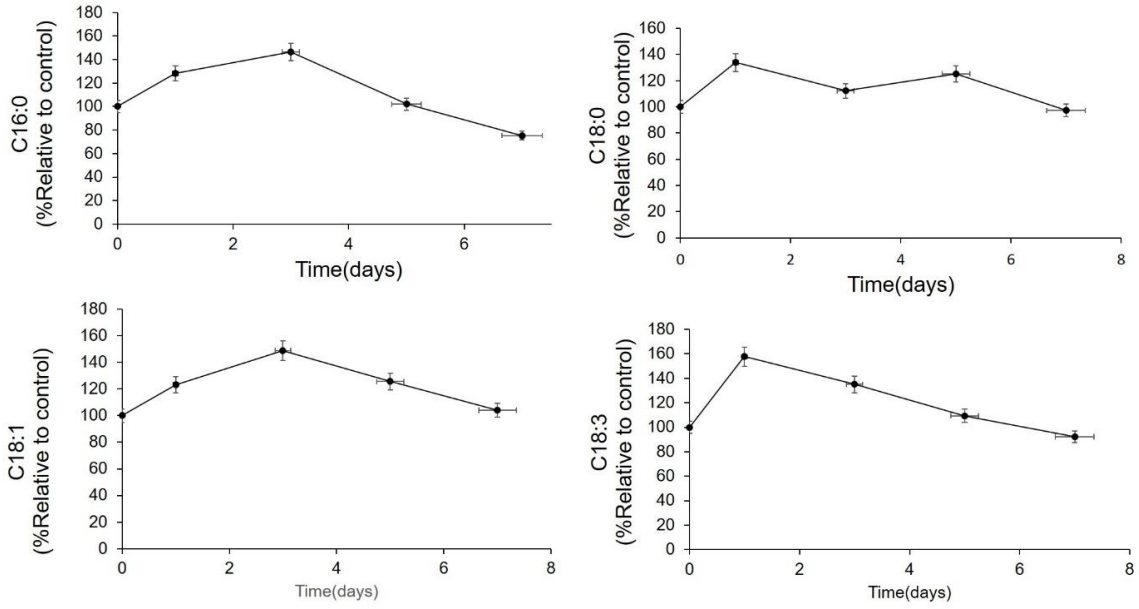


Figure 4. Fatty acid profile of *Chlorella vulgaris* grown under N-starvation condition with 100 µM GA_3



4. CONCLUSION

The combination of plant growth regulators and abiotic stress is a general approach for enhanced the accumulation of fatty acids and maintaining the microalgal biomass. In the present study, our results indicate that GA_3 supplementation increased microalgal growth rate, algal cell size and lipid production especially involved in biodiesel production under nitrogen starvation. These results showed the potential of application GA_3 in algal culture as a utilizer

for biodiesel application. This data also demonstrated that gibberellin could play a role in response to stress in algae physiology. In addition, the use of Gibberellic acid can bring us one step closer to making *C. vulgaris* suitable for biodiesel production. This study proposes using plant regulators to increase unsaturated fatty acids in combination with different stress conditions and help develop growing strategies for higher microalgal biodiesel production. Therefore, it seems necessary to study this subject in future research to create ideal culture conditions for biodiesel production.

Acknowledgements

We thanks to 2209 undergraduate student project of Scientific and Technological Research Council of Turkey (TUBITAK).

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s).

Authorship contribution statement

Uygar Kabaoglu: Investigation, Microscopic studies, Analysis of fatty acid. **Ufuk M. Aslan:** Cell growth, Fatty acid analysis. **Dilek Unal:** Experimental design, Writing-orginal draft, Statistical analysis, supervision.

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International Journal of Secondary Metabolite
2021, Vol. 8, No. 1, 20–30

https://doi.org/10.21448/ijsm.780232

Published at http://dergipark.gov.tr/en/pub/ijsm Research Article

The Antioxidant and Antimicrobial Capacities of Phenolic Profiles of Some *Salvia* L. Seeds Grown in Turkey

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Abstract: The aim of current study is to show phenolics, antioxidant capacities and antimicrobial activities of seeds of five *Salvia* L. (*S. frigida* Boiss., *S. candidissima* subsp. *candidissima* Vahl., *S. virgata* Jacq., *S. verticillata* L. var. *verticillata* and *S. russellii* Benth.) taxa grown in Turkey. The flavonoid and phenolic acid contents were measured by using HPLC whilst the antioxidant capacities were determined by using different methods. In addition, agar well diffusion method was used to determine the antimicrobial activities of *Salvia* species in this study. It was found that *S. frigida*, *S. verticillata* var. *verticillata* and *S. russellii* have the highest catechin contents and *S. frigida* and *S. verticillata* var. *verticillata* have high rosmarinic acid while *S. frigida*, *S. candidissima* subsp. *candidissima* and *S. verticillata* var. *verticillata* have high vanilic acid. Also, it was determined that *S. frigida* and *S. verticillata* var. *verticillata* have high DPPH radical scavenging activities in 150 and 250 µL while *S. frigida* and *S. verticillata* var. *verticillata* have highest ABTS radical scavenging activity in all concentrations apart from 25 µL for *S. frigida*. Furthermore, *S. frigida* and *S. verticillata* var. *verticillata* have high total phenolic contents. On the other hand, *Salvia* species have similar lipid peroxidation inhibitions. However, the metal chelating activities of *Salvia* species are different. And also, it was demonstrated that *Salvia* taxa have antimicrobial activity.

ARTICLE HISTORY

Received: August 13, 2020
Revised: October 10, 2020
Accepted: March 05, 2021

KEYWORDS

Antimicrobial activity,
Antioxidant capacity,
Flavonoids,
Phenolic acids,
Salvia L.

1. INTRODUCTION

Herbs from the Lamiaceae have been used in traditional medicine for more than 2000 years to treat different diseases such as cancer, diabetes, depression, memory enhancement and infection throughout the world (Shekarchi et al., 2012; Lopresti, 2017). Lamiaceae, contains most popular aromatic plants including marjoram, sage, basil and thyme, have strong antioxidant and antimicrobial activity due to rich in biologically effective components as caffeic

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ISSN-e: 2148-6905 /

acid, rosmarinic acid, carvacrol and thymol (Hossain et al., 2010; Khaled-Khodjaa et al., 2014; Skendi et al., 2017). And also, Turkey is accepted as a significant gene center for the Lamiaceae which is represented by 45 genera, 565 species and 735 taxa in Turkey (Dorman et al., 2004; Cetin et al., 2006).

Salvia, is the from subfamily Nepetoideae of the Mentheae tribe of the Lamiaceae, which includes 1000 taxa spread out in the different regions of the world (Kahraman, Celep & Dogan, 2009). The name of *Salvia* is originated from Latin "salvare" or "salvus" and is meaning healing due to using folk medicine (Fotovvat et al., 2019). Many *Salvia* species are rich in polyphenol and terpenes and are used as digestive, antiinflammatory, antiseptic, and antioxidant agents (Dent et al., 2017; Gregorczyk-Karolak & Kiss, 2018). Phenolic compounds are in charge of antioxidant capacity in the sage and rosmarinic acid, caffeic acid, chlorogenic acid, vanillic acid, salvianolic acid, luteolin and apigenin are major phenolics in sage (Jasicka-Misiak et al., 2018; Vergine et al., 2019; Katanic-Stankovica et al., 2020).

The genus is represented by 89 species and 95 taxa in flora of Turkey and the endemism of the genus is 45% in Turkey (Kahraman et al., 2018). Generally, the studies about the determination of antioxidant and antimicrobial capacities of sage is related to plant extracts and there are lack of antioxidant capacities in sage seeds. And also the antimicrobial studies is related to plant extracts not seeds. The goal of the current study is to determine phenolic compounds antioxidant capacities and antimicrobial activities of phenolics of seeds in five *Salvia* L. (*S. frigida* Boiss., *S. candidissima* Vahl subsp. *candidissima*, *S. virgata* Jacq., *S. verticillata* L. subsp. *verticillata*., *S. russellii* Bentham) taxa grown in Turkey.

2. MATERIAL and METHODS

The plants were collected from natural habitats. The plant samples and seeds were deposited in Firat University Herbarium (FUH). The localities of studied *Salvia* L. taxa were given in Table 1.

Table 1. Localities of studied *Salvia* L. Taxa.

Taxa	Locality
Salvia frigida Boiss.	Elazig Baskil district, Hacı Mustafa Village, 1850 m
Salvia candidissima Vahl subsp. *candidissima*	Elazig Baskil district, Hacı Mustafa Village, 1750 m
Salvia virgata Jacq.	Elazig Baskil district, Bolucuk Village, 1500 m
Salvia verticillata L. subsp. *verticillata*	Elazig Baskil district, Bolucuk Village, 1490 m
Salvia russellii Bentham	Elazig Baskil district, Quercus forest around, 1400 m

2.1. Microbial Strain

In this study, fungi (*Candida albicans* FMC 17 and *Candida glabrata* ATCC 66032), dermatophyte (*Trichophyton* sp., *Epidermophyton* sp.) and bacteria [(*Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (FMC 5), *Staphylococcus aureus* (COWAN 1), *Bacillus megaterium* (DSM 32)] were used to evaluate the antimicrobial activities of studied *Salvia* taxa.

2.3. Extraction Protocol of Phenolics

PREVAIL C18 reversed-phase column (15x4.6 mm, 5 µm, USA) was used and methanol/acetonitrile/water (46/8/46, v/v/v) comprising 1.0% acetic acid is mobile phase (Zu, Li, Fu & Zhao, 2006). Morin, kaempferol, naringenin, quercetin, catechin, naringin, resveratrol, myricetin, rutin and vanillic acid, ferulic acid, rosmarinic acid, cinnamic acid, and caffeic acid were determined. 1.0 mL/min was used as flow rate and l0 µL samples were given as injection volume. Chromatographic conditions were performed at 25°C.

2.4. DPPH Radical Scavenging Activity

25, 50, 100, 150 and 250 µL of extracts were treated with 25 mg/L DPPH solved in methanol (4.0 mL). The DPPH radical protocol was performed based on Liyana-Pathiranan and Shahidi (2005)'s method in the current study. The absorbances were measured at 517 nm after the samples were stored in the dark for 30 minutes. 1 µM quercetin was used as reference. The formula (1) was used for the DPPH radical scavenging potential is following:

$$\% \text{ inhibition} = \frac{\text{Ab(control)} - \text{Ab(sample)}}{\text{Ab(control)}} x100 \quad (1)$$

The absorbance of control was represented as Ab(control) and the absorbance of sample was represented as Ab(sample).

2.5. ABTS Radical Scavenging Activity

ABTS [2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt] assay was determined according to the Ree et al. (1999) methods. 7 mM ABTS and 2.45 mM potassium persulphate were mixed to form $ABTS^{\bullet+}$ solution. The solution was stored at room temperature approximately 12–16 h. And ABTS was dissolved with water to provide an absorbance of 0.700 ± 0.020. Lastly, three mL of diluted ABTS were mixed with 25, 50, 100, 150 and 250 µL of extract and absorption was determined in the 6 min at 734 nm (Skotti et al., 2014). The formula was used for the DPPH radical scavenging potential is following (2):

$$\% \text{ inhibition} = \frac{\text{Ab(control)} - \text{Ab(sample)}}{\text{Ab(control)}} x100 \quad (2)$$

The absorbance of control was represented as Ab(control) and the absorbance of sample was represented as Ab(sample).

2.6. Determination of Total Phenolics

Folin–Ciocalteu method was used to evaluate total phenolics (Singleton et al., 1999). 100 µL extracts were mixed with 3.16 mL of H_2O and 200 µL of Folin– Ciocalteu solution. The samples were stored at room temperature about 3 min. Later, the extracts were treated with anhydrous sodium carbonate (20% w/v) and total phenolic content was observed at 765 nm after two hours in room temperature (Robya et al., 2013). The total phenolic amount was evaluated by using gallic acid equivalents (µgGAE/mg).

2.7. Chelating Effects of Ferrous Ions

The chelating activities of samples were evaluated method by Dinis et al. (1994). 50 µL of 2 mM $FeCl_2$ was injected to extracts (50, 100, 250 and 500 µg/mL). 5 mM ferrozine (0.2 mL) mixed with extracts to start the reaction. The extracts were shaken vigorously and stored at room temperature approximately 10 min. The absorbances of samples were measured at 562 nm. The inhibition (%) of ferrozine–Fe^{2+} complex was evaluated based on following formula (3):

$$\% \text{ Chelating activity} = [1 - (\frac{\text{Abs}}{\text{Abc}})] x100 \quad (3)$$

The absorbance of sample was represented as Abs and the absorbance of control was represented as Abc where 100 where Na_2EDTA was used as positive control.

2.8. Antioxidant Activity against TBARS

The antioxidant activity of samples was measured according to Shimoi et al. (1994)' method. The samples were prepared by using DMSO (dimethyl sulfoxide). The Fe^{2+} ($FeCl_2.2H_2O$) and hydrogen peroxide were used in the experiments. Also, oleic acid (3.35 mM), linoleic acid (9.01 mM) and linolenic acid (2.30 mM) were used dissolved in DMSO. Sage

extracts, control and Fenton reagent groups were formed. The control group contained 0.5 mL of fatty acid and a buffer (pH=7.4; 0.05 M Tris HCl; 0.2% Tween, 20; 0.15 M KCl) whilst the fenton group contained buffer; hydrogen peroxide (0.01 mM); 0.5 mL of fatty acid and $FeCl_2.2H_2O$ (50 µM) and the extracts comprised $FeCl_2$ (50 µM), 0.25 mL sage extract, 0.5 mL of fatty acid and hydrogen peroxide (0.01 mM). 0.1 mL of a 4% (w/v) BHT was added to all groups to protect the more oxidation and the examples were stored at the 37 °C approximately 24 h. After that, 1 mL of samples from three groups was taken and treated with 1 mL of 0.6% TBA and the samples were stored at 90 °C for 30 min. Finally, 4 mL butan-1-ol was injected to tubes, blended and centrifuged at 4250 rpm for 10 min. The absorbance of the supernatant was measured at 532 nm. MDA standard curves were formed by 1,1,3,3-tetramethoxypropane, and TBARS were written as mg MDA/kg dry matter (Keser et al., 2014).

2.9. Antimicrobial Activity

Antimicrobial activities were evaluated agar well diffusion method according to Collins and Lyne (1987)' method. Agar contained Sabouroud Dextrose Agar (Oxoid), Mueller Hinton Agar (Difco) and Malt Extract Agar (Difco) and McFarland standard. And also, bacteria (10^6 cells/mL), dermatopyhte and yeast (10^4 cells/mL), were found in 100 µL suspension. Phenolics (10 µL) were added to the well after the wells were filled with cork-borer (0.85 cm) and plates. After that, incubation for bacteria was conducted at 37±0.1°C for 24 h and for yeast and dermatophyta fungi were conducted at 25±0.1°C for 72 h. The inhibiton zone was referenced to decide the antimicrobial activity.

2.10. Statistical Analysis

All analysis were performed by using SPSS 21.0 packet program. The simple lineer regression model was used to found the correlation between antioxidant capacity (ABTS, DPPH and metal chelating) and total phenolic contents**.** Data obtained from present study represented as mean values ± standard deviation. Also, to evaluate the significance of the observed differences, the least significant difference (LSD) test was used in the antimicrobial activity. The conclusions were expressed as mean ± S.D. $p<0.0001$, $p<0.001$ and $p>0.05$ have been conceived significant when compared to the control group (ampicillin sulbactam, mycostatin). All samples were analysed in triplicate.

3. RESULTS and DISCUSSION

Present study showed that myricetin, morin, quercetin, kaempferol, naringenin and resveratrol are low or absent (Table 2). *S. verticillata* subsp. *verticillata* has the highest rutin (114.47±1.25 µg/mg), catechin (583.79±1.27 µg/mg) and naringin (128.8±1.57 µg/mg) contents. In addition, it was demonstrated that *S. frigida* (107.77±2.37 µg/mg) and *S. russellii* (306.88±1.54 µg/mg) have catechin content. And also, this study demonstrated that naringin contents of *S. candidissima* subsp. *candidissima* and *S. virgata* are low (9.27±0.81-9.17±0.57 µg/mg) (Table 2). Literature determined that *Salvia* posses ferulic acid, caffeic acid, chlorogenic acid, o-coumaric acid, p-OH-benzoic acid, protocatechinic acids, rosmarinic acid, apigenin, luteolin, kaempferol and quercetin (Kupeli Akkol et al., 2008; Hamrouni-Sellami et al., 2013; Dincer et al., 2012; Alcantaraa et al., 2019). Similarly, this study showed that studied *Salvia* seeds have vanillic acid, caffeic acid, ferulic acid and rosmarinic acid (Table 2). Whereas, it has been showed that cinnamic acid contents of studied *Salvia* seeds absent or low (Table 2). On the other hand, present study demonstrated that *S. frigida* (16.32±0.85 µg/mg) and *S. candidissima* subsp. *candidissima* (28.57±1.14 µg/mg) posses relatively high ferulic acid content compared to other studied *Salvia* taxa in this study (Table 2). And also, this study found that *Salvia verticillata* subsp. *verticillata* (152.79±1.33 µg/mg), *S. frigida* (107.38±1.51 µg/mg), *S. virgata* (88.24±0.75 µg/mg) have high rosmarinic acid amounts (Table 2). Zengin et al. (2018) found that rosmarinic acid amount of *S. verticillata* is higher than in other *Salvia*

species. Also, Yumrutas et al. (2011) showed that two varities of *S. verticillata* displayed strong antioxidant activity and they indicated that two varieties of *S. verticillata* have especially rich in rosmarinic acid and caffeic acid contents. Literature claimed that *Salvia* is characterized by the rosmarinic acid (Tepe, 2008). Further, Kan, et al. (2007) found that *S. frigida* has the highest rosmarinic acid and caffeic acid contents compare to *S. candidissima, S. virgata* and *S. verticillata*. However, the present study found that the caffeic acid content of *S. frigida* is low (19.71±1.11 µg/mg). In addition, Kan et al. (2007) indicated that *S. virgata* has low rosmarinic acid and caffeic acid contents.

Table 2. The results of flavonoid and phenolic acid contents of *Salvia* taxa (µg/mg).

		S. frigida	*S. candidissima* subsp. *candidissima*	*S. virgata*	*S. verticillata* subsp. *verticillata*	*S. russellii*
Flavonoids	Rutin	44.37±0.87	6.05±0.14	14.21±0.24	114.47±1.25	9.71±0.54
	Myricetin	-	3.67±0.21	-	0.41±0.02	0.24±0.05
	Morin	-	4.84±0.87	0.41±0.01	-	-
	Quercetin	4.87±0.25	0.21±0.02	0.22±0.04	-	-
	Kaempherol	0.83±0.79	5.47±0.68	1.41±0.32	1.63±0.2	0.39±0.03
	Catechin	107.77±2.37	-	-	583.79±1.27	306.88±1.54
	Naringin	36.78±1.14	9.27±0.81	9.17±0.57	128.8±1.57	24.4±0.64
	Naringenin	-	0.57±0.01	0.38±0.01	-	-
	Resveratrol	0.59±0.02	-	-	-	-
Phenolic acids	Vanillic acid	64.74±1.21	65.4±1.34	9.71±0.45	84.12±0.97	9.27±0.34
	Cinnamic acid	0.4±0.02	0.2±0.01	0.2±0.01	-	0.2±0.01
	Caffeic acid	19.71±1.11	29.65±0.87	31.14±0.79	72.94±1.23	7.71±0.68
	Ferulic acid	16.32±0.85	28.57±1.14	3.27±0.3	1.57±0.2	1.81±0.1
	Rosmarinic acid	107.38±1.51	28.82±0.86	88.24±0.75	152.79±1.33	17.21±0.89

On the other hand, the current study suggested that in general, *S. verticillata* subsp. *verticillata* has high DPPH and ABTS radical scavenging activities (Table 3 and Table 4). Similarly, Yumrutas et al. (2011) indicated that two varieties of *S. verticillata* have exhibited the strongest DPPH radical scavenging. The studied taxa have the highest ABTS radical scavenging activity in 150 and 250 µL whilst studied taxa except for (*S. candidissima* subsp. *candidissima*) posses highest DPPH radical scavenging activity in 250 µL (Table 3 and Table 4). On the contrary, *S. frigida* has lowest DPPH radical scavenging capacity in 25 µL and 50 µL and *S. russellii, S. candidissima, S. virgata* have the lowest DPPH scavenging activity in some concentrations (Table 3). However, Senol et al. (2010) suggested that *S. candidissima, S. virgata* and *S. russellii* have the strongest DPPH radical scavenging activity whilst Orhan et al., (2007) showed that *S. verticillata* has DPPH scavenging capacity are between 68.91±0.21% and 81.1±2.48%. Also, another study by done Orhan et al. (2013) demonstrated that *S. frigida* and *S. verticillata* have strong DPPH radical scavenging.

Table 3. The DPPH% results of extracts of *Salvia* L. taxa.

Taxa	25 µL	50 µL	100 µL	150 µL	250 µL
S. frigida	27.22±0.59	16.8±0.56	61.7±1.13	91.9±1.17	93.9±1.41
S. candidissima subsp. *candidissima*	82.14±1.25	44.97±0.84	13.12±0.58	29.4±0.87	52.9±1.18
S. virgata	80.11±1.87	93.78±1.17	43.04±0.97	27.8±0.58	84.1±1.21
S. verticillata subsp. *verticillata*	65.6±0.97	78.9±0.93	93.1±1.61	94.7±1.29	94.1±1.81
S. russellii	14.7±0.59	97.59±1.82	96.32±1.34	36.2±0.97	92.7±1.64

Table 4. The The ABTS% results of extracts of *Salvia* L. taxa.

Taxa	25 µL	50 µL	100 µL	150 µL	250 µL
S. frigida	53.44±1.12	95.68±1.64	99.67±1.45	98.71±1.12	98.14±1.24
S. candidissima subsp. *candidissima*	20.34±0.87	41.20±1.12	73.62±1.12	93.96±1.13	98.81±1.11
S. virgata	26.55±0.98	41.03±0.91	75.34±1.24	98.82±0.84	98.82±0.97
S. verticillata subsp. *verticillata*	88.44±1.29	98.65±1.29	98.87±1.14	98.57±0.51	98.65±0.79
S. russellii	28.10±0.78	41.72±0.86	90.51±1.57	98.79±0.84	98.85±0.91

Furthermore, it was found that *S. verticillata* subsp. *verticillata* (266.66±0.9 µgGAE/mg) has the highest total phenolic content whilst *S. frigida* has the lowest (76.49±1.06 µgGAE/mg) total phenolic content in the present study (Table 5). Zengin et al. (2018) showed that total phenolic content of *S. verticillata* as 53.52 ± 1.66 mg/g. Also, Tosun et al. (2009) determined the total phenolic content of *S. verticillata*, *S. virgata* and *S. candidissima* as 167.1 mg/g, 101.2 mg/g and 100.3 mg/g, respectively whilst Kupeli Akkol et al. (2008) found that *S. virgata* has 133.8 mg/g total phenolic content. Literature showed that there is a correlation between phenolics and antioxidant activities of *Salvia* species (Tosun et al., 2009). This study showed that there is a strong correlation between total phenolics and DPPH (r^2:0.752) and ABTS (r^2: 0.764) while there is moderate correlation between total phenolics and metal chelating (r^2: 0.305).

Table 5. The lipid peroxidation (mg/kg), total phenolic amounts (µgGAE/mg) and metal chelating activities (%) of *Salvia* L. taxa.

Taxa	Lipid peroxidation	Total Phenolic	Metal Chelating
S. frigida	19.95±0.82	160.87±1.72	77.84±0.95
S. candidissima subsp. *candidissima*	23.36±0.51	76.49±1.06	80.48±1.11
S. virgata	22.42±0.78	81.92±1.01	71.88±0.86
S. verticillata. subsp. *verticillata*	20.87±0.62	266.66±0.93	45.04±0.84
S. russellii	20.29±0.67	94.73±1.24	53.51±0.59

Table 6. The disc diffusion assay results of the antimicrobial susceptibility tests for growing reference microorganisms.

Reference Microorganisms	Zone of Inhibition values (mm)					Reference Antibiotics
	Sf	*Sc*	*Sv*	*Sver*	*Sr*	
E coli	11.00±0.0^{d}	-	-	-	-	11.66±0.3*
S. aureus	11.00±0.0^{d}	8.33±0.3^{c}	13.33.±0.3^{d}	13.33±0.3^{cd}	13.33±0.3^{cd}	9.66±0.3*
K. pneumoniae	8.33±0.3^{c}	11.33±0.3^{d}	13.33±0.3^{d}	11.33±0.3^{d}	8.33±0.3^{c}	11.66±0.3*
B. megaterium	14.33±0.3^{cd}	8.33±0.3^{c}	8.33±0.3^{c}	17.33±0.3^{cd}	13.33±0.3^{cd}	11.66±0.3*
C. albicans	10.66±0.33^{d}	14.33±0.3^{cd}	-	16.66±0.33^{cd}	11.33±0.3^{d}	11.66±0.3**
C. glabrata	-	11.33±0.3^{d}	-		-	8.66±0.3**
Epidermophyton sp.	-	-	-	-		8.33±0.3**
Trichopyton sp.	-	-	-	-	-	8.33±0.3**

Sf; *S. frigida*, *Sc*; *S. candidisssima*, Sv; *S. virgata*, *Sver*: *S. verticillata*, *Sr*: *S. russellii*. PS; positive control; *ampicillin sulbactam (*) and micostatin (**) 120 µL and 20µg/disc*, Interpretation of zone diameters (mm); Zone of diameter>11 mm (susceptible; p<0.0001; cd, p<0.001;d), resistant= 8-10 c: p<0.01, not susceptible (-) (a: p>0.05).

Besides, the current study showed that *S. candidissima* subsp. *candidissima* has the highest metal chelating capacity (80.48±1.11%) and *S. verticillata* subsp. *verticillata* has lowest metal chelating capacity (45.04±0.84%) (Table 5). Senol et al., (2010) found that the methanol extracts of *Salvia* species including *S. candidissima, S. virgata* and *S. russellii* have displayed neligible metal chelating action. However, Seker Karatoprak et al. (2016) suggested that *S. virgata* may be able to protect against complexing free iron (II) ions. Moreover, the lipid peroxidation of studied taxa changed from 19.95±0.82 mg/kg (*S. frigida*) to 23.36±0.51 mg/kg (*S. candidissima* subsp. *candidissima*) in this study (Table 5). Tepe, et al. (2007) indicated that inhibition activity of the linoleic acid of *S. verticillata* subsp. *verticillata* is 74.4±1.29%. Also, Jeshvaghani et al. (2015) found that oxidation of lipid peroxidation was blocked by *Salvia* species including *S. virgata*. Besides, it was indicated that *Salvia* species mostly great protective role against lipid peroxidation study done by Asadi et al. (2010).

Moreover, the present study demonstrated that phenolic contents of *Salvia* L. taxa represented different antimicrobial activities (Table 6). It was showed that *S. verticillata* subsp. *verticillata* represented higher antimicrobial activity against *B. megaterium, C. albicans* and *S. aureus* than other studied *Salvia* taxa. And also, it was found that only *S. frigida* exhibited antimicrobial activity against *E. coli* while only *S. candidissima* subsp. *candidissima* exhibited antimicrobial activity against *C. glabrata*. On the other hand, it was determined that studied *Salvia* taxa don't show antimicrobial activity against *Epidermophyton* sp. and *Trichopyton* sp. (Table 6). It was reported that *Salvia* taxa have potent antimicrobial activity study by done Bayar and Genc (2016). They showed that the methanolic extracts of *S. candidisssima* have significant antifungal capacity (Bayar & Genc, 2018). In another study by done Akin et al. (2010). *S. russellii* is effective against micororganisms. And also, Kunduhoglu et al. (2011) suggested that *S. verticillata* exhibited antimicrobial activity.

4. CONCLUSION

The present study demonstrated that the catechin amounts of *S. frigida*, *S. verticillata* subsp. *verticillata* and *S. russellii* are high whilst the the rutin and naringin content of *S. verticillata* subsp. *verticillata* are high. Also, the current study showed that *S. frigida* and *S. verticillata* subsp. *verticillata* have high rosmarinic acid and *S. frigida* (64.74±1.21 µg/mg), *S. candidissima* (65.4±1.34 µg/mg) and *S. verticillata* subsp. *verticillata* (84.12±0.97 µg/mg) have high vanilic acid content. On the other hand, it was found that *Salvia* taxa have high ABTS (in 100, 150 and 250 µL) and DPPH (in 250 µL) except for *S. candidissima* subsp. *candidissima*) radical scavenging activities. Moreover, it was demostrated that *S. frigida* and *Salvia*

verticillata subsp. *verticillata* have high total phenolic content. And also, *Salvia* taxa represented antimicrobial activity.

Acknowledgements

Firat University Scientific Research Unit provided a financial support to this study by project number FUBAP 2041. We greatly acknowledge Firat University Scientific Research Unit. And also, some data of the study is presented and the study is placed as National Botanical Science Congress Abstract Book (25-28 October 2014, pp. 87-88).

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s).

Authorship contribution statement

İrfan Emre: The methodology (except for antimicrobial activity), the statistical analysis of results (except for antimicrobial results), the writing of original draft. **Murat Kursat:** The collection of plant materials, the nomenclature of plants, the methodology (except for antimicrobial activity). **Sevda Kirbag:** The methodology, the writing of the antimicrobial results. **Pinar Erecevit:** The methodology (antimicrobial activity), the writing of the antimicrobial results. **Mustafa Yunus Emre:** The methodology (except for antimicrobial activity). **Okkes Yilmaz:** The methodology (except for antimicrobial activity; Gas Chromatography and HPLC analysis). **Semsettin Civelek:** The nomenclature of plants.

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International Journal of Secondary Metabolite
2021, Vol. 8, No. 1, 31–39

https://doi.org/10.21448/ijsm.793336

Published at http://dergipark.gov.tr/en/pub/ijsm Research Article

Alleviation Effects of Diosmetin on H_2O_2-Induced Oxidative Damage in Human Erythrocytes

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Abstract: Free radicals (FRs) are formed in the high amounts result of the metabolic imbalance in cells and tissue. These radicals-induced oxidative damages constitute the basis of many diseases. Organisms have antioxidant defence systems (ADS) to eliminate the destructive effects of the oxidative damage. In addition to these antioxidant systems, dietary flavonoids have the antioxidant effect and the protective role against oxidative damage. In the present study, it was investigated whether a flavonoid derived diosmetin (10, 50, and 100 µM) have the elimination potential on hydrogen peroxide (H_2O_2)-induced oxidative damage in erythrocyte culture by using biomarkers such as lipid peroxidation (LP) level, catalase (CAT), total superoxide dismutase (SOD) activity and changes of SOD isozymes containing the manganese SOD (Mn SOD) and the cupper-zinc SOD (CuZn SOD). CAT, total SOD, Mn SOD and CuZn SOD activities showed a serious decline with H_2O_2 treatment, but diosmetin addition significantly increased their activities. While the H_2O_2 application critically increased LP products in erythrocytes, diosmetin considerably reduced these oxidative damage products. In conclusion, it has been determined that diosmetin can moderate oxidative damage in human erythrocytes by activating or protecting the ADS.

ARTICLE HISTORY

Received: September 10, 2020
Revised: February 02, 2021
Accepted: February 18, 2021

KEYWORDS

Diosmetin,
Flavonoid,
Oxidative damage,
Human erythrocyte

1. INTRODUCTION

Erythrocytes carry oxygen to tissues throughout their lives, therefore the survival of other cells in the body depends on their health and number in blood. They do not contain nuclei and mitochondria and have a very limited life span. These cells are in constant contact with many oxidants factors which cause oxidative stress. Therefore, their metabolism and structural components could easily be disrupted by oxidative damage, thus their life spans could reduce further. Even most oxidant drugs facilitate the conversion of oxyhemoglobin in erythrocytes to hydrogen peroxide which induces LP and protein denaturation (Smith, 1987). As a result of the reduction in the number of erythrocytes occur anemia and oxygen deficiency, and all tissues are negatively affected by this situation (Libregts et al., 2011).

Under normal conditions, antioxidant systems in the cells ensure a balance between the FRs formation and their elimination. In this situation, these systems containing antioxidant substances and antioxidant enzymes have enough strength for scavenging the reactive oxygen

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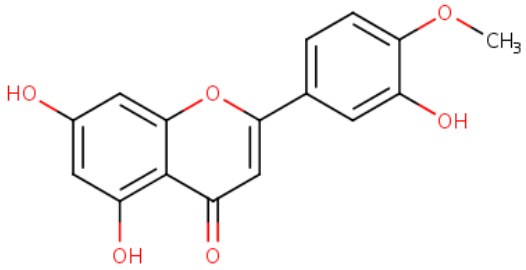
ISSN-e: 2148-6905 /© IJSM 2021

species (ROS). However, due to various reasons, an excessive increase in ROS might render insufficient the antioxidant systems and causes pathological phenomena called oxidative stress or oxidative damage (Memişoğulları, 2005; Atmaca & Aksoy, 2009). Many studies have shown that the ADS in cells could insufficient alone in protection from oxidative damage. It has been suggested that dietary antioxidant ingredients such as vitamin E, ascorbic acid and flavonoids could support antioxidant systems against oxidative damage. Therefore, these antioxidants could be effective in protecting against many diseases and delaying the ageing period (Özşahin et al., 2011). Flavonoids, natural antioxidant compounds found in abundance in plants, are known to reduce damages result from oxidative stress in cell and tissues. The antioxidant substances are lipophilic such as vitamin E and ascorbic acid. They have the ability to suppress LP by eliminating FRs in subcellular fractions such as liposomes, mitochondria, microsomes and erythrocyte membrane (Yang et al., 2001; Sánchez-Gallego et al., 2010). Recently, diosmetin (Figure 1), a flavonoid derivative, stands out with reducing oxidative damage in many tissues. It shows a cardio protective effect by reducing oxidative damage and mitochondrial apoptosis (Mo et al., 2020). Also, diosmetin attenuates ischemia and reperfusion-induced kidney damage (Yang et al., 2017). Moreover, it has been suggested that it can be used in the treatment of asthma (Ge et al., 2015). However, the role of diosmetin in protecting oxidative damage in erythrocytes is still not completely understood. In the present study, it was investigated whether the diosmetin effect on the alleviation of oxidative damage in human erythrocyte cells.

Figure 1. Chemical structure of diosmetin.



2. MATERIAL and METHODS

2.1. Preparation of Erythrocyte Samples

Erythrocytes used in the present study were obtained from Agri State Hospital Blood Center. Care was taken to ensure that erythrocyte samples are vital unexpired and their human sources were people who did a healthful, non-smoker, non-alcoholic between ages 20-30. The blood samples were centrifuged at 4500 rpm for 10 minutes, and the separated plasma was discarded. Afterwards, erythrocytes were washed with physiological saline. These processes were repeated 3 times without haemolysis of erythrocytes. The cells were transferred to Roswell Park Memorial Institute (RPMI)1640 medium to keep to them live during the experiment. This medium was purchased from Sigma Aldrich. It has been developed at RPMI, and is used as a growth medium in many different cell cultures, including mammalian cells (Moore, Gerner & Franklin, 1967). This research was conducted with the approval of the Scientific Research Ethics Committee of Agri Ibrahim Cecen University, dated 03.04.2020 and numbered 67.

2.2. Preparation of Oxidative Damage Model for Erythrocytes

All materials and devices used in the experiment were sterilized. To determine the concentration of H_2O_2 used as an oxidant agent were conducted preliminary studies with 25, 50, 100 and 200 µM H_2O_2. Considering the LP findings obtained from these studies and literature data, the H_2O_2 concentration was decided to be 100 µM.

2.3. Diosmetin Treatment Model to Erythrocytes

Diosmetin and other chemicals were purchased from Sigma Aldrich. Diosmetin was dissolved in the least amount of dimethyl sulfoxide (DMSO) as proposed by the vendor. DMSO was added to the H_2O_2 group as much as the amount of DMSO used in the diosmetin groups. This experiment was designed as five experimental groups, including control, H_2O_2, diosmetin (10, 50, and 100 µM), respectively. Diosmetin concentrations were decided based on previous study (Wang et al., 2020). The changes in oxidative damage parameters were determined after the erythrocytes were incubated at 37°C for 24 hours after the treatments.

2.4. Determination of Total SOD Activity and The Changes in SOD Isoenzymes

The total SOD activity was measured by the method of the Beauchamp and Fridovich (1971). This method is fundamentally based on the measurement of amount of formazan, which a blue-coloured complex. This blue complex is formed by nitro blue tetrazolium chloride reacting with superoxide anions produced on illumination of riboflavin in the presence of methionine as an electron donor. The total SOD amount is directly proportional with the decrease in formation of formazan, and one-unit total SOD is accepted as the amount of SOD that reduces the formazan formation 50% ratio.

Erythrocyte samples were subjected to polyacrylamide gel electrophoresis (Native-PAGE) not contain sodium dodecyl sulphate according to Laemmli method (1970). Electrophoretic separation of SOD isoenzymes was performed in 10% polyacrylamide gel (PAGE) at 120 V by Beuchamp and Fridovich methods (1971). Band densities of Mn SOD and CuZn SOD were calculated using the Gel Analyser Program.

2.5. Determination of Catalase Activity

Catalase activity was measured with methods described by Aebi (1984). The erythrocyte lysates were suspended in 50 mM potassium phosphate buffer (pH 7.0) for 30 min at 25°C. Then, 30 mM hydrogen peroxide was added to the lysates. The decomposition of hydrogen peroxide was recorded at 240 nm for 3 min. Catalase activity was calculated using an extinction coefficient of 0.0436 $mmol^{-1}\ cm^{-1}$.

2.6. Determination of Lipid Peroxidation in Erythrocyte Cultures

LP levels were determined with the method described by Chang et al. (2013). At the end of 24-hour incubation, erythrocytes were lysed and then centrifuged for 5 minutes at 4°C and 3500 *g*. Samples were taken from the supernatant part, and HCl solution containing thiobarbituric acid (TBA) and trichloroacetic acid (TCA) was added. The samples were left to incubate for 30 minutes in boiling water, and then the reaction was stopped by standing in an ice bath. After centrifugation for 5 minutes at 4°C at 5000 *g*, absorbance at 532 nm were recorded. Thiobarbituric acid reactive substances (TBARS) values were calculated and expressed as $\mu M.g^{-1}$Hb according to the haemoglobin amount of the blood samples.

2.7. Statistical Analysis

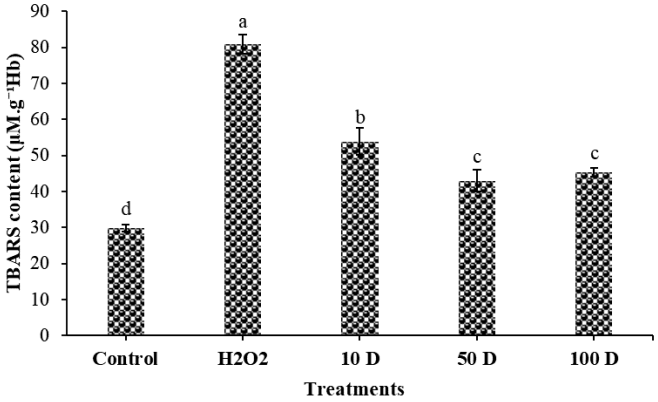
The study results are presented by taking the average of the values obtained after 3 repetitions of each sample. The results were compared with one-way analysis of variance (ANOVA) using SPSS 20 package program and Duncan's Multiple Comparison Test at $p<0.05$ significance level.

3. RESULTS and DISCUSSION

FRs are formed in organisms as a result of various metabolic events and disorders. These radicals with low molecular weight and unstable structure are highly reactive substances due to their suitability for electron exchange (Alugoju et al., 2015). Erythrocytes are highly susceptible to ROS-induced oxidative stress due to the high content of unsaturated fat in the membrane

membranes and the excess of iron groups in haemoglobin. The decrease in the number of erythrocytes as a result of oxidative damage could cause anaemia and thus oxygen deficiency in tissues (Libregts et al., 2011). Although hydrogen peroxide is a non-radical, it can be the precursor of FRs such as hydroxyl radical, therefore it could be used to create an experimental of oxidative stress in vitro studies. In the study carried out by Becker (2003), it was shown that H_2O_2 caused oxidative stress by forming hydroxyl radicals through Fenton type reactions that formation of radicals by the H_2O_2 breakdown catalysed by metals such as iron and copper. Also Morabito et al. (2016) reported that H_2O_2 causes oxidative stress by increasing the LP level in erythrocyte cultures. Consistent with these studies, the results of present study also revealed that the LP level showed a serious increase with H_2O_2 treatment to the erythrocytes (Figure 2). The probable reason of this increase might be triggered oxidative damage by forming ROS through Fenton-type reactions of H_2O_2. Meanwhile, the high iron content in erythrocytes makes more likely to acceleration of the Fenton reactions (Yee & Liu, 1997). However, the H_2O_2 concentration used in this experiment is much lower than the H_2O_2 concentration used to cause oxidative damage in erythrocytes in previous studies (An et al., 2016). On the other hand, in previous studies, the exposure of erythrocytes to H_2O_2 has been kept considerably short (about 90 mins). In the present study, the oxidative damage model could be created with a lower concentration of H_2O_2 due to their longer exposure to oxidative stress (24 hours).

Figure 2. Effects of alone H_2O_2 and H_2O_2 plus diosmetin (10 (10 D), 50 (50 D), and 100 μM (100 D)) treatments on LP level. Different letters in the graph mean statistically significant differences ($p < 0.05$).



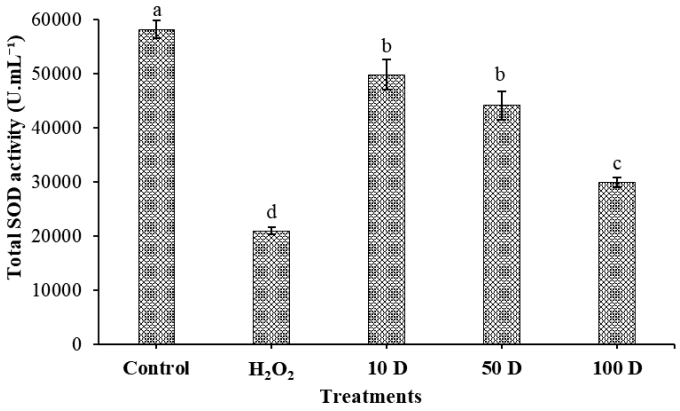
FRs naturally formed in cells or exogenously originated are attempted to be scavenged by ADS containing antioxidant enzymes and substances. However, when the radicals reach high amounts for whatever reason, the strength of these systems insufficient to scavenge the molecules. In this instance, the ROS cause irreversible oxidative damages in the cell structural components such as proteins, lipids and nucleic acids (Schieber & Chandel, 2014). Also, this damage might be ended the life of cells and is among the main reasons for diseases such as cancer, diabetes and autoimmune disorders (Ratnam et al., 2006; Cemeli et al., 2009; Pellegrini et al., 2009).

The balance between oxidants and antioxidants in cells is essential for health, but sometimes this balance might be broken in favour of oxidants. Recent studies showed that the dietary antioxidant supplement is extremely important to reduce oxidative damage. Also, it has been argued that vitamin E constitutes one of important defence factor that protects from the harmful effects of FRs (Brigelius-Flohe & Traber, 1999). Dietary antioxidants might play a role in protecting the body against radicals, and preventing many diseases and even cancer (Noroozi & Angerson, 1998). At this point, it is of great importance to take antioxidant substances from outside before being exposed to oxidant substances. It is well known that flavonoids, plant secondary metabolites in polyphenolic structure, have strong antioxidant effects, and are among

the important dietary antioxidants with their non-toxic properties for organisms (Yang et al., 2001). In the present study, it was also revealed that diosmetin, a flavonoid derivate, considerably mitigated the H_2O_2-induced oxidative damage by decreasing the LP level. However, there was no significant difference between the values of the 50 and 100 µM diosmetin groups (Figure 2).

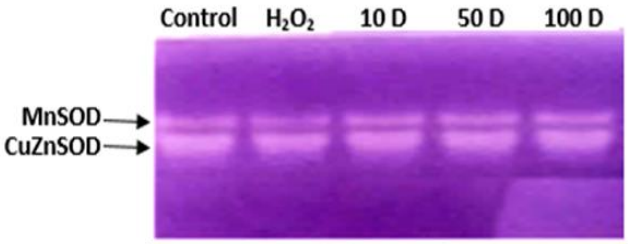
The SOD converting superoxide radicals into hydrogen peroxide and molecular oxygen constitutes the first step among cellular antioxidant enzymes that have an important role in scavenging reactive oxygen species. Besides this effect of SOD, catalase (CAT) converts hydrogen peroxide into water and oxygen. As a conclusion, two toxic oxygen species, hydrogen peroxide and superoxide radical, are eliminated by CAT and SOD. (Weydert & Cullen, 2010). The total SOD enzyme in the eukaryotic cells presence three isomers containing Mn SOD in 88 kDa, Cu SOD in 36 kDa, and Zn SOD in 36 kDa (Wong et al., 1989). These isoenzymes can be determined as two bands Mn SOD and CuZn SOD in natural electrophoresis (Beauchamp & Fridovich, 1971). Although it is considered to be present in mitochondria, Mn SOD has been reported to be present in erythrocytes that do not contain mitochondria (Adžić et al., 2004). Cu SOD and Mn SOD show antioxidant effect in the cytoplasm, intercellular areas, and another cell component. In the present study, in addition to determining the total SOD and CAT activities with spectrophotometrically methods, changes in the isomers of the SOD enzyme were determined by electrophoretic methods. The total SOD activity of erythrocytes treated with only H_2O_2 decreased seriously compared to the control group. However, especially 10 and 50 µM diosmetin applications significantly increased the SOD activity compared to the H_2O_2 group (Figure 3).

Figure 3. Effects of alone H_2O_2 and H_2O_2 plus diosmetin (10 (10 D), 50 (50 D), and 100 µM (100 D)) treatments on superoxide dismutase activities in human erythrocytes. Different letters in the graph mean statistically significant differences ($p< 0.05$).



The change in SOD isoenzymes can be clearly seen in native gel electrophoresis. Moreover, band densities of Mn SOD and CuZn SOD were calculated using the Gel Analyser program and shown in Figure 4. Images and calculations obtained from the gel were shown that the application of H_2O_2 caused a critical decrease in the amount of Mn SOD and CuZn SOD protein. On the other hand, diosmetin applications compared to H_2O_2 application significantly increased the band density in SOD isoenzymes. As in total SOD activity, highest density SOD isoenzyme bands in erythrocytes exposed to oxidative stress were observed in 10 and 50 µM diosmetin applications.

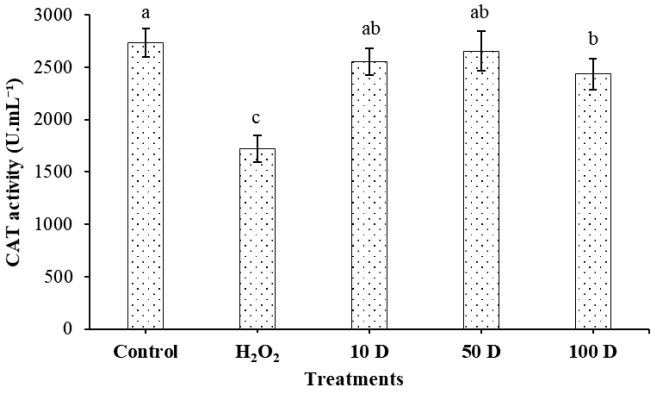
Figure 4. Effects of alone H_2O_2 and H_2O_2 plus diosmetin (10 (10 D), 50 (50 D), and 100 µM (100 D)) treatments on relative band intensity of different types of superoxide dismutase isoenzymes in human erythrocytes. (*) The band intensity of control was accepted as one hundred percent and the other groups were compared to the control.



	Control	H_2O_2	10 D	50 D	100 D
Mn SOD (%)	100*	74	86	86	80
CuZn SOD (%)	100	73	78	80	76

Similar trends to the changes in SOD activity were also detected in CAT activity. While CAT activity is declined by H_2O_2, diosmetin treatments considerably increased as compared to H_2O_2 group. In all of diosmetin treatments, CAT showed higher activity than H_2O_2 groups but there were no statistical differences among the diosmetin groups (Figure 5) ($p<0.05$).

Figure 5. Effects of alone H_2O_2 and H_2O_2 plus diosmetin (10 (10 D), 50 (50 D), and 100 µM (100 D)) treatments on catalase activities in human erythrocytes. Different letters in the graph mean statistically significant differences ($p< 0.05$).



The high ADS power resulted from diosmetin can be explained by the fact that it directly removes reactive oxygen species or indirectly contributes to antioxidant enzyme activities. The diosmetin-induced upward trend in total SOD activity was compatible with the increases of band densities in SOD isoenzymes. It is well known that erythrocytes do not contain organelles such as nuclei and ribosomes, therefore the diosmetin-induced increases in these isoenzyme bands cannot be explained with newly synthesized SOD proteins. The possible reason for these increases might be a protective effect of diosmetin on the activity of existing SOD isoenzymes. Moreover, the lowest LP levels were detected in the diosmetin applications where total SOD, CAT activities and SOD isoenzyme bands were highest except for control groups. In a recent study, it has been reported to be low oxidative damage in erythrocytes with high SOD activities as compatible with our results (Schieber & Chandel, 2014).

4. CONCLUSION

In this study, the mitigating effects of diosmetin on human erythrocytes exposed to oxidative damage were revealed with changes in antioxidant enzyme activities and lipid

peroxidation. Also, for the first time, the changes in SOD isoenzymes of damaged erythrocytes were detected. These results indicated that diosmetin, a flavonoid derivate, has a considerable effect on mitigating oxidative damage by reducing lipid peroxidation and, by increasing antioxidant enzyme activities in erythrocyte cells. Further studies of diosmetin is recommended being done and, investigated its possible drug active substance potential.

Acknowledgements

The authors thank the Agri Ibrahim Cecen University Central Research and Application Laboratory for providing their laboratory facilities.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s).

Authorship contribution statement

Mucip Genisel: Resources, Investigation, Formal Analysis, Methodology, Writing-original draft, Supervision, Validation. **Fatma Kubra Yildizoglu:** Investigation, Methodology, Validation.

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International Journal of Secondary Metabolite
2021, Vol. 8, No. 1, 40–48

https://doi.org/10.21448/ijsm.793715

Published at http://dergipark.gov.tr/en/pub/ijsm Research Article

Cytotoxic Activities of Methanol Extract and Compounds of *Porodaedalea pini* Against Colorectal Cancer

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Abstract: *Porodaedalea pini* is a medicinally important mushroom with antioxidant, cytotoxic, immunostimulating, antitumor, antiviral and immunomodulating activities. Therefore, in this study, *P. pini* methanol extract and isolated compounds from the methanol extract were tested for cytotoxic activities against DLD-1 (colorectal cancer) and CCD-18Co (human colon fibroblast cell line) by using Alamar Blue assay. Cytotoxic activity on DLD-1 was decreased in the order of *P. pini* methanol extract> 4-(3,4-dihydroxyphenyl)but-3-en-2-one (**3**)> pinoresinol (**2**)> ergosta-7,24(28)-dien-3*β*-ol (**1**). *P. pini* methanol extract was determined to have the best cytotoxic activity with the lowest IC_{50} value on DLD-1 (IC_{50}: 25.33±0.29 µg/mL) and the highest IC_{50} value on CCD-18Co (434.30±1.45 µg/mL). Within the scope of the findings, it is thought that *P. pini* mushroom can be used as a new and natural agent in the treatment of colorectal cancer.

ARTICLE HISTORY

Received: September 11, 2020
Revised: October 11, 2020
Accepted: February 18, 2021

KEYWORDS

Porodaedalea pini,
Colorectal cancer,
Cytotoxic activity,
Extract,
Isolation

1. INTRODUCTION

Cancer ranks second in deadly diseases after cardiovascular diseases worldwide. Approximately 10% of diagnosed cancers or cancer-related deaths each year consist of colorectal cancer (Dekker et al., 2019). Colorectal cancer is the second most common type of cancer diagnosed in women, while it is third cancer in men. The incidence and mortality in women are about 25% lower than in men. The worldwide incidence of colorectal cancer is estimated to increase to 2.5 million new cases by 2035 (Arnold et al., 2017; Dekker et al., 2019). Among the causes of colorectal cancer, various risk factors including diets are rich in animal fat, in vegetables and fruits, smoking, diabetes and obesity have been identified (Kelly et al., 2012; Perdue et al., 2014). Also, studies have reported that high consumption of red meat increases the risk of colorectal cancer, while adequate physical activity, especially outdoor activities, and the level of vitamin D in the bloodstream reduces the risk of colorectal cancer (Oba et al., 2006; Takachi et al., 2011; Surya et al., 2016). Worldwide, nutrition and diet are the most remarkable factors causing colorectal cancer. The literature studies revealed that many

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ISSN-e: 2148-6905 /© IJSM 2021

natural diet products can be beneficial in the prevention of cancer (Tao et al., 2018). Also, plants, fruits, vegetables, herbal teas, and mushrooms have been reported to have cytotoxic effects on colorectal cancer cells and due to the presence of dietary fibers and phytochemical compounds (Turati et al., 2015; Seidel et al., 2017).

In Chinese medicine, *Phellinus* genus has been reported to be used in the treatment of many diseases such as arthritis of the knee, stomachaches, tumors, inflammation, lymphatic and gastroenteric disorders and to promote longevity in Chinese medicine (Ganeshpurkar et al., 2010; Seephonkai et al., 2011). Bioactive compounds such as sesquiterpenes, triterpenes, steroids, pigments, and polysaccharides, responsible for estrogenic, antiviral, antioxidant, antitumor and anti-estrogenic activity have been purified from *Phellinus* species (Wu et al., 2010; Song et al., 2014; Wang et al., 2014; Pei et al., 2015). *Porodaedalea pini*, a member of *Phellinus* genus and Hymenochaetaceae family, is usually grown under the pine trees. It is known to lower blood sugar, increase immunity, and have anti-cancer properties (Ayer et al., 1996). Antioxidant, antifungal, anticholinesterase, anti-tyrosinase, cytotoxic activities, and inhibition effects on NO production in murine macrophages-RAW 264.7 cells by the compounds isolated from *P. pini* have been revealed by previous studies (Ayer et al., 1996; Wangun & Hertweck, 2007; Jang &Yang, 2011; Deveci et al., 2019[a]). In recent years, the discovery of bioactive compounds from natural sources, and obtaining compounds and possible drug candidates with therapeutic properties have attracted more attention. In this context, mushrooms are considered as sources of natural bioactive compounds and are defined as promising therapeutic agents. The study was aimed to evaluate cytotoxic activities against DLD-1 (colorectal cancer) and CCD-18Co (human colon fibroblast cell line) of both *P. pini* methanol extract and ergosta-7,24(28)-dien-3*β*-ol, pinoresinol, and 4-(3,4-dihydroxyphenyl)but-3-en-2-one compounds isolated from the methanol extract in our previous study (Deveci et al., 2019[a]).

2. MATERIAL and METHODS

2.1. Mushroom Material

Porodaedalea pini (Brot.) Murrill. was collected from the villages of Mentese district of Mugla in November-December 2014 and January 2015 from the area of Muğla, Turkey. The voucher specimen has been stored at the Research and Application Center for Mushrooms, Mugla Sitki Kocman University (Fungarium No AT-2446).

2.2. Spectral Measurements and Chemicals Used

For purification of the compounds, silica gel (Kieselgel 60, 70-230 mesh, Merck) was used for column chromatography; RP-18 $F_{254}S$ and silica gel 60 F_{254} plates for thin-layer chromatography (TLC). Separation and isolation of the compounds were performed by using C_{18} and GS-320 column-linked recycling HPLC (Japan Analytical Industry Co. Ltd.). UV-254-nm light was used for detection of TLC spots and $CeSO_4$ solution for visualization. Chemical structures of the isolated compounds were elucidated by using FT-IR, 1D-NMR, 2D-NMR spectroscopy techniques. Thermo Scientific one Nicolet IS10 FT-IR spectrometer was used to record the IR spectrum. The NMR spectrum was on Agilent-600-MHz instruments coupled with cooled cryoprobes probe for ^{1}H- and ^{13}C-NMR including HSQC, HMBC, and COSY. A 96-well microplate reader (MultiskanGo, Thermo Scientific Co., MA, USA) was used to analyze cytotoxic activity studies. Cytotoxic activity results were measured and calculated by using GraphPad Prism (GraphPad Software v5.0, USA).

2.3. Extraction and Isolation

For extraction of the aerial parts of *P. pini* (1900 g), powdered mushroom samples were macerated separately and respectively in solvents with increasing polarity: *n*-hexane,

chloroform, acetone, and methanol for 24 h; and four times at room temperature. Solvents were vaporized by using a rotary evaporator. The methanol extract obtained was 49.70 g. The methanol extract was stored at +4 °C for further analysis. As a result of the chromatographic isolation of *P. pini* methanol extract, ergosta-7,24(28)-dien-3*β*-ol, pinoresinol, and 4-(3,4-dihydroxyphenyl)but-3-en-2-one were obtained. Details about the isolation and characterization of the compounds can be seen in our previously published research (Deveci et al., 2019[a]).

2.4. Cell Viability

CCD-18Co (human colon fibroblast cell line) and DLD-1 (colorectal cancer) were cultivated in EMEM and RPMI-1640 growth mediums (ATCC, Virginia, USA), respectively and incubated with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 2 mM L-glutamine (Sigma, St. Louis, Missouri, USA) in 5% CO_2 at 37 °C and 90-95% humidity.

2.5. Cell Viability Assay

$1x10^4$ cells were put into 96-well plate with a growth medium and incubated in 5% CO_2 at 37 °C for 24 h until they attached to the bottom. Different concentrations (between 1 µg/mL and 1000 µg/mL) of the methanol extract and isolated compounds of *P. pini* were added to each well. Viability and proliferation of the cells were tested according to the previously described Alamar Blue assay (Karakurt & Adali, 2016). The results were measured at 570 nm and 610 nm by using a 96-well microplate reader. Doxorubicin was used as a positive control. The sigmoidal plot of the inhibition rate (%) versus the log concentration was used to calculate the IC_{50} values of *P. pini* methanol extract and the isolated compounds.

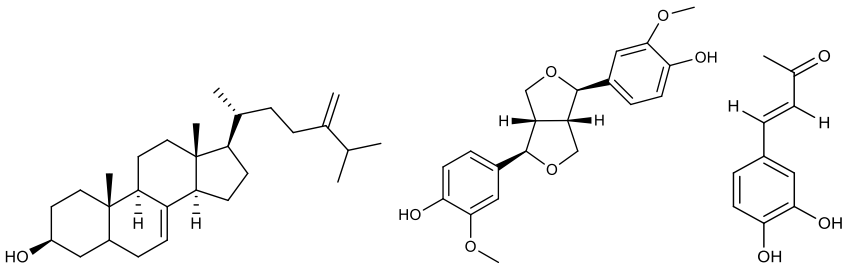
2.6. Statistical Analysis

All data on cytotoxic activity tests were the average of three parallel sample measurements. Data were recorded as mean ± S.E.M. Significant differences between means were determined by *t*-test, $p < 0.05$ were regarded as significant.

3. RESULTS and DISCUSSION

Ergosta-7,24(28)-dien-3*β*-ol (**1**), pinoresinol (**2**), and 4-(3,4-dihydroxyphenyl)but-3-en-2-one (**3**) were obtained as a result of the isolation of *P. pini* methanol extract. Spectroscopic data of the isolated compounds can be seen in our published previous research (Deveci et al., 2019[a]). Figure 1 shows the chemical structures of the compounds isolated from *P. pini* methanol extract.

Figure 1. Chemical structures of the compounds isolated from *P. pini* methanol extract.



1 **2** **3**

Cytotoxic activities against DLD-1 (colorectal cancer) and CCD-18Co (human colon fibroblast cell line) of *P. pini* methanol extract and ergosta-7,24(28)-dien-3*β*-ol (**1**), pinoresinol (**2**) and 4-(3,4-dihydroxyphenyl)but-3-en-2-one (**3**) compounds isolated from the methanol extract were tested by Alamar Blue assay. DLD-1 and CCD-18Co cells were treated with different concentrations of the methanol extract and isolated compounds. Figure 2 represents

the cytotoxic effects of *P. pini* methanol extract and isolated compounds on DLD-1 and CCD-18Co.

Figure 2. Cytotoxic effects of *P. pini* methanol extract and isolated compounds on DLD-1 and CCD-18Co (**a**) IC_{50} values on DLD-1 (**b**) IC_{50} values on CCD-18Co (**c**) Heat Map analyses of dose-dependent inhibition against DLD-1 cells. Cell viability decreased from red to pink color (**d**) Heat Map analyses of dose-dependent inhibition against CCD-18Co. Cell viability decreased from green to pink color.

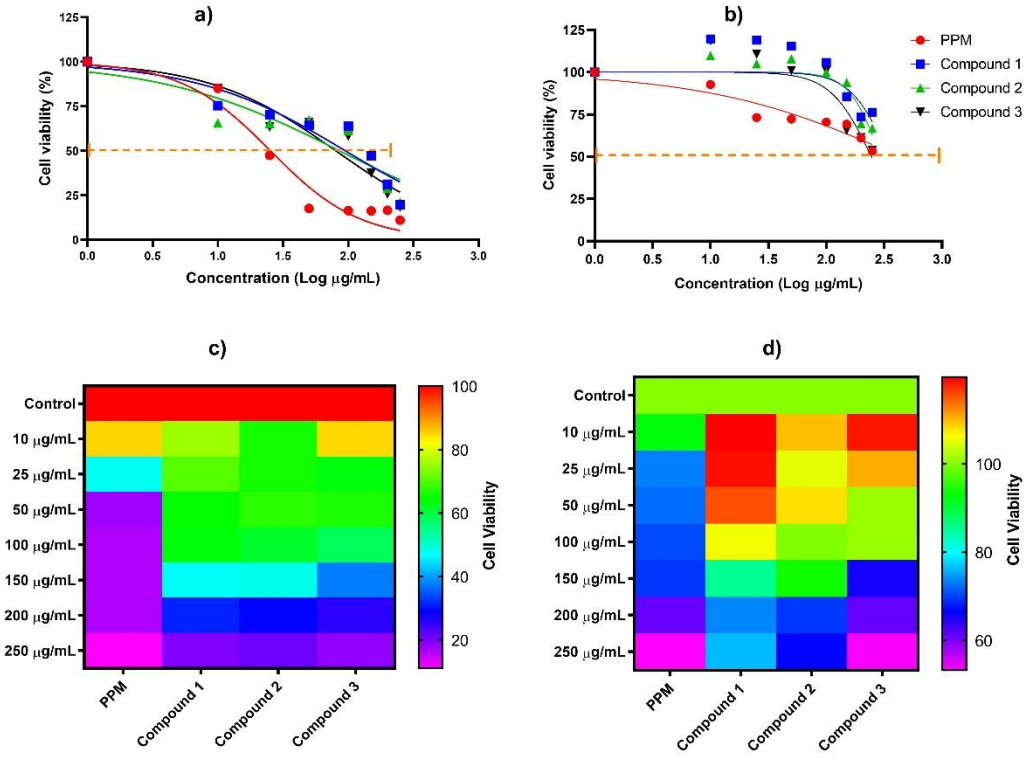


Table 1. Calculated IC_{50} values of the methanol extract and isolated compounds of *P. pini*[a]

	DLD-1 IC_{50} (µg/mL)	CCD-18Co IC_{50} (µg/mL)
Methanol extract (PPM)	25.33±0.29	434.30±1.45
Ergosta-7,24(28)-dien-3*β*-ol (**1**)	95.05±1.25	347.20±0.78
Pinoresinol (**2**)	85.69±0.87	293.90±0.46
4-(3,4-dihydroxyphenyl)but-3-en-2-one (**3**)	80.32±0.98	245.20±0.34
Doxorubicin[b]	6.10±0.55	NT[c]

[a]: IC_{50} values represent the means ± SEM of three parallel measurements ($p<0.05$). [b]: Positive control. [c]: NT: Not tested.

Table 1 shows the calculated IC_{50} values of *P. pini* methanol extract and isolated compounds. As seen in Figure 2, *P. pini* methanol extract and isolated compounds inhibited the viability of DLD-1 and CCD-18Co in a dose-dependent manner. Cytotoxic activity on DLD-1 was decreased in the order of *P. pini* methanol extract (IC_{50}: 25.33±0.29 µg/mL)> 4-(3,4-dihydroxyphenyl)but-3-en-2-one (**3**) (IC_{50}: 80.32±0.98 µg/mL)> pinoresinol (**2**) (IC_{50}: 85.69±0.87 µg/mL)> ergosta-7,24(28)-dien-3*β*-ol (**1**) (IC_{50}: 95.05±1.25 µg/mL). Toxicity on CCD-18Co was decreased in the order of *P. pini* methanol extract (IC_{50}: 434.30±1.45 µg/mL)>

ergosta-7,24(28)-dien-3*β*-ol (**1**) (IC_{50}: 347.20±0.78 µg/mL)> pinoresinol (**2**) (IC_{50}: 293.90±0.46 µg/mL)> 4-(3,4-dihydroxyphenyl)but-3-en-2-one (**3**) (IC_{50}: 245.20±0.34 µg/mL) (Table 1).

From ancient times, people have described mushrooms as culinary wonders and valuable in folk medicine. In recent years, mushrooms have witnessed the intense interest of scientists due to their pharmaceutical potentials (Dimitrijevic et al., 2017). Until this time, the medicinal uses of mushrooms have been associated with their nephroprotective, antioxidant, antitumor, hypocholesterolemic, antidiabetic, immunomodulatory, anti-cancer, antiallergic, and antimicrobial properties. Literature studies have shown that *Phellinus, Agaricus, Pleurotus, Clitocybe, Ganoderma, Antrodia, Cordyceps, Trametes, Calvatia, Xerocomus, Flammulina, Schizophyllum, Suillus, Inonotus, Funlia, Inocybe, Lactarius, Russula*, *Albatrellus*, and *Fomes* mushroom species are effective against cancer. These mushroom species are defined as new generation biotherapeutics (Patel & Goyal, 2012). It is prominent that not only active isolated compounds but also extracts containing active compounds can be used as alternative agents with fewer side effects in cancer treatment and hence it is important to examine the cytotoxic effects of the mushroom species. According to the results obtained, it was determined that *P. pini* methanol extract has the best cytotoxic activity. In earlier studies, purification and bioactive properties of steroids, phenolics, terpenoids, and polysaccharides from *P. pini* have been reported (Deveci et al., 2019[a]; Deveci et al., 2019[b]; Hong et al., 2013; Jang &Yang, 2011). Therefore, the higher cytotoxic activity of the methanol extract compared to other isolated compounds can be explained by the synergistic effect of various bioactive compounds contained in it. Among the isolated compounds, phenolic compounds indicated higher cytotoxic properties. It is known that phenolic compounds have anticancer activity associated with their high antioxidant properties or have direct cytotoxic effects on cancer cells (Ivanova et al., 2014). Also, the cytotoxic mechanism of action of phenolic compounds has been elucidated in previous studies as modulating carcinogen metabolism, altering gene expression levels, arresting the cell cycle, inducing apoptosis, and inhibiting various cell proliferation signaling pathways (Huang et al., 2010).

Previously, cytotoxic effects of the extracts, fractions, and pure compounds obtained from different *Phellinus* species on colorectal cancer were determined with a limited number of studies. In the study of Reis et al. (2014), the methanol (GI_{50}: 70±3 µg/mL) and ethanol (GI_{50}: 61±1 µg/mL) extracts as also polysaccharides (GI_{50}: 87±4 µg/mL), glucans (GI_{50}: 202±4 µg/mL), and triterpenoids (GI_{50}: 65±1 µg/mL) fractions of *P. linteus* were tested for cytotoxic effects on HCT-15 (colorectal cancer). The hispidine isolated from *P. linteus* has been reported to be a promising new anticancer agent due to inducing both intrinsic and extrinsic apoptotic pathways mediated by ROS in CMT-93 (mouse colorectal cancer) and HCT-116 (human colorectal cancer) cells (Lim, Lee, Park, Kim, & Lim, 2014). He et al. (2015) purified two illudin type sesquiterpenoids, sulphureuine A and phellinuin J from *P. tuberculosus*, tested these compounds for their inhibitory effects on SW480 (colorectal cancer) and no significant inhibitory activity was reported (IC_{50}: >40 mM). Inhibition rates of 24-ethylcholesta-5,22-dien-3*β*-ol, ergosterol, 3,4-dihydroxy benzaldehyde, ergosta-7,22-dien-3*β*-yl pentadecanoate, baicalein and inoscavin A purified from *P. baumii* on SW620 (colorectal cancer) were reported as ~40, ~30, ~50, ~40, ~90 and ~90%, respectively at 100 µg/mL concentration. Also, inoscavin A and baicalein were found to have higher cytotoxic activity than 5-fluorouracil that served as a positive control (Zhang et al., 2017). Ethyl acetate extract (IC_{50}: 149.9 µg/mL), *n*-hexane (IC_{50}: 69.8 µg/mL), *n*-butanol (IC_{50}: >100µg/mL) and ethyl acetate (IC_{50}: 77.8 µg/mL) fractions and atractylenolide I (~20% cell viability at 100 µg/mL concentration) from *P. linteus* were investigated for their cytotoxic effects on HT-29 (colorectal cancer) by Jeon et al. (2013). There are studies on the cytotoxic effects of different mushroom species on DLD-1 (colorectal cancer) in the literature. IC_{50} values of the *n*-hexane, chloroform, and ethyl acetate subfractions of *Tremella fuciformis* were found as 350, 400, and 450 ppm, respectively against DLD-1

(colorectal cancer) (Kim, Chang, Choi, Yoon, & Lee, 2006). In a different study, at 0.2 and 0.5 mg/mL concentrations, *Inonotus obliquus* water extract inhibited 48% and 62% proliferation of HCT-l16 and 40% and 60% proliferation of DLD-l colorectal cancer cells and it was reported that the water extract downregulated the *β*-catenin and NF-kB signaling, which exerted anti-inflammatory and antiproliferative activities in colorectal cancer cells (Mishra et al., 2013). Ergosterol peroxide isolated from *Inonotus obliquus* was screened for cytotoxic activity against HCT-116, HT-29, SW620, and DLD-1 colorectal cancer cells at 0, 5, 10, 20 µg/mL concentrations. When the cell growth of HCT-116, HT-29, SW620 were found nearly ~20% at 20 µg/mL concentration, the cell growth of DLD-1 was found nearly ~80% at 20 µg/mL concentration (Kang et al., 2015). Our results are in agreement with the literature studies. This is the first investigation on cytotoxic activities of *P. pini* methanol extract and isolated compounds against DLD-1 and CCD-18Co cells.

4. CONCLUSION

Chemotherapy, cytotoxic drugs, radiotherapy, and surgery are the main methods used in the treatment of colorectal cancer. Among these methods, the most effective method is surgery, and treatment is continued with chemotherapy or radiotherapy since there is a high possibility of recurrence of colorectal cancer after surgery. The negative side effects of chemotherapy compounded by the tendency of colorectal cancer reoccurrence, has made it imperative to search for new, natural, and effective agents with fewer side effects in its treatment. Cytotoxicity of *P. pini* methanol extract and ergosta-7,24(28)-dien-3*β*-ol (**1**), pinoresinol (**2**), and 4-(3,4-dihydroxyphenyl)but-3-en-2-one (**3**) isolated compounds were investigated on DLD-1 and CCD-18Co cells for the first time. The methanol extract of *P. pini* displayed significant cytotoxicity on DLD-1. As a result, this study recommends that *P. pini* mushroom, especially the methanol extract, can be used for further research that could lead to the development of new natural remedies in the treatment of colorectal cancer.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s).

Authorship contribution statement

Ebru Deveci: Investigation, Visualization, Software, Formal Analysis, Methodology, Writing-original draft, Supervision. **Gülsen Tel-Cayan:** Investigation, Visualization, Software, Formal Analysis, Methodology, Writing-original draft, Supervision. **Serdar Karakurt:** Resources, Methodology, Validation. **Mehmet Emin Duru:** Resources, Methodology, Formal Analysis, Validation.

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International Journal of Secondary Metabolite
2021, Vol. 8, No. 1, 49–58

https://doi.org/10.21448/ijsm.725512

Published at http://dergipark.gov.tr/en/pub/ijsm Research Article

Formononetin Production by Large-Scale Cell Suspension Cultures of *Medicago sativa* L.

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Abstract: In this study, calli of *Medicago sativa* L. cv. Elçi (alfalfa Elçi) were inoculated in cell suspension culture and analyzed for aggregate assay, cell viability test, total phenolic content assay, DPPH free radical scavenging activity and formononetin assay by means of High-Performance Liquid Chromatography (HPLC). Hypocotyl, cotyledon and apical meristem explants were taken from 15-day-old aseptic seedlings and germinated in MS medium. 10 g calli were grown for each explant and then transferred into cell suspension culture. The highest cell viability rate, which was 75%, and the highest DPPH free radical scavenging activity with 51.36% was measured in 1000 mL cell suspension culture, while the highest total phenolic content, i.e. 40.2 mg/g, was quantified in 250 mL cell suspension culture. In accordance with the findings of the study, the production of formononetin was higher in the calli derived from cell suspension cultures than in herb samples of *M. sativa*. Moreover, in 1000 mL cell suspension culture, 4.99 mg/g of formononetin concentration was quantified, which scored the highest. In large-scale cell suspension cultures of *M. sativa*, it was possible to increase the production of formononetin production. Hence, due to its medicinal significance, a method has been tested to obtain higher amounts of this compound.

ARTICLE HISTORY

Received: April 23, 2020
Revised: September 21, 2020
Accepted: January 19, 2021

KEYWORDS

Cell viability test,
DPPH,
Formononetin,
Medicago sativa,
Total phenols

1. INTRODUCTION

Medicago sativa L. (alfalfa) is an important feed plant in all over the world with its ability to adapt to different climates and its high feed efficiency and quality. *M. sativa* spreads in world's temperate regions (such as the USA, Southern Canada, Europe, China, South America and South Africa). In general, it shows spreading characteristics up to 2400 m. It is more resistant to drought compared to other feed types.

The plant has been used as a herbal supplement for increasing strength and energy, detoxifying blood, fighting against infections and treating anemia. *Medicago* is also known as a model plant because of its ability to regenerate *in vitro* in plant tissue culture (Erişen, 2006). *M. sativa* is also reported to reduce the menopausal symptoms in women by increasing the levels of estrogen (Gülen, 2013; Çölgeçen et al., 2014; Franciscis et al., 2019). *M. sativa* is rich in flavonoids, too. The term "flavonoids" is derived from the Latin word "flavus," meaning yellow. The basic flavonoid structure consists of 15 carbon atoms (C_6-C_3-C_6 system) and 2-phenyl benzopyrone (diphenylpropane). Additionally, flavonoids are polyphenolic compounds

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ISSN-e: 2148-6905 /© IJSM 2021

(Kahraman et al., 2002). They are found in all organs of the plants such as flower, leaf, root, stem, seed and fruit (Işık, 2005). Although the flavonoids have been studied for many years, their biological activities and cellular mechanisms have not yet been fully elucidated. However, studies have shown that they have free radical-scavenging (antioxidant) effect, cardiovascular and liver protective role, antiviral, and as recently suggested, anticancer properties (Birman, 2012).

Analyzed in this study, formononetin is an isoflavonoid. Clinical trials have shown that isoflavonoids reduce menopausal symptoms by increasing estrogen levels. It is also known that Asian women are less likely to have estrogen-dependent cancers due to high soybean-based nutrient intake with isoflavonoid content. Formononetin inhibits tumor growth and is anti-allergic (Evcimen & Aslan, 2015; Franciscis et al., 2019, Tay et al., 2019). From this research, it was aimed to inoculate *M. sativa* calli in cell suspension culture. Also, it was analyzed for aggregate assay, cell viability test, total phenolic content assay, DPPH free radical scavenging activity and formononetin assay by means of HPLC.

2. MATERIAL and METHODS

2.1. Plant Material

M. sativa was grown in the trial garden of Department of Biology of Bülent Ecevit University. Two different herb samples were collected from the trial garden in May and September. The samples with an average size of 40-50 cm were dried in a lyophilizer and stored at -80 ° C.

2.2. Aseptic Seedling Plant

The seeds were sterilized in 96% ethanol for one minute and then transferred to 10% commercial sodium hypochlorite solution for 5 minutes. Then, they were rinsed 3 times in autoclaved distilled water and germinated in hormone-free Murashige and Skoog (MS) medium (Murashige & Skoog, 1962). Hypocotyl, cotyledon and apical meristem explants were taken from 15-day-old aseptic seedlings, and later calli were grown.

2.3. *In vitro* Culture Medium

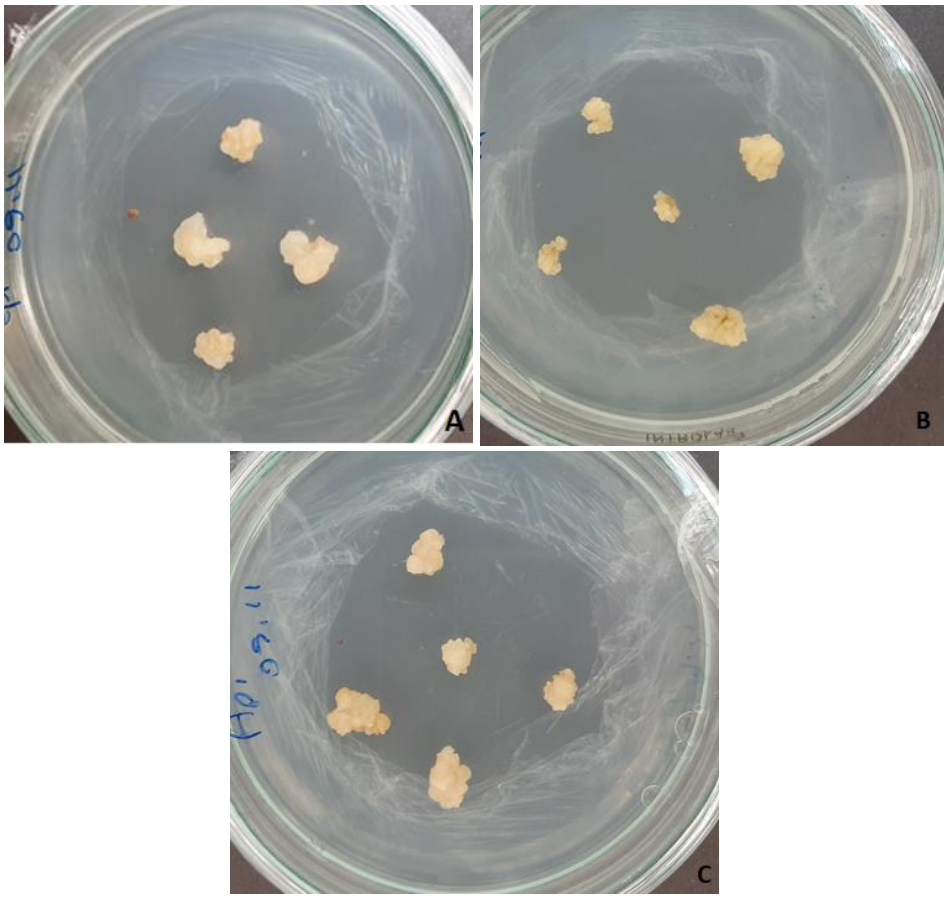
Standard MS medium was used as the callus culture medium. 1.5 mg/L Kinetin, 1.5 mg/L NAA, 0.7 mg/L 2,4-D were added to MS medium as plant growth regulators. All MS media were autoclaved for sterilization with 20 g/L sucrose and 7 g/L agar. Agar-free MS medium + 1.5 mg/L Kinetin, 1.5 mg/L NAA, 0.7 mg/L 2,4 D, 20 g/L sucrose were used for cell suspension cultures. Hypocotyl, cotyledon and apical meristem explants from the 15 days-old aseptic seedlings were germinated in MS medium as 5 explants on each petri dish (Figure 1). The calli were subcultured in every three weeks, and they were stored in the dark at 24 ± 2 ° C. Adequately matured friable calli were grown in cell suspension cultures in 4 different volumes (2.5 g/100 mL, 6.25 g/250 mL, 12.5 g/500 mL and 25 g/1000 mL). The calli that was transferred to the cell suspension cultures were shaken at 180 rpm on the shaker for 20 days. The experiment was repeated 3 times.

2.4. Cell Viability

The calli were shaken for 20 days on the shaker and then filtered with a 200 mesh Sigma-Aldrich Cell Dissociation Kit for 1 min. The filtered cells were then transferred to microcentrifuge tubes, stained with 0.5 mL of 0.2%. Trypan Blue for 20 minutes and then washed with pure distilled water. The washed cells were placed on the microscope slide and covered with cover slips. Cell count was performed by Olympus BX51 Microscope and Olympus SC100 Camera, and Digimizer Image Analysis Software was used for image

processing (Figure 2). Percent viability was calculated by this formula: % viability = (live cell count / total cell count) x 100 (Patel et al., 2009).

Figure 1. Matured calli **A)** hypocotyl, **B)** cotyledon, **C)** apical meristem.



2.5. Extraction

Lyophilized samples were pulverized for extraction. 1 g of each sample was used. They were shaken on the shaker with 100 mL of 80% MeOH at 180 rpm, then filtered. The remaining extract was shaken with 150 mL of 80% MeOH on a shaker at 180 rpm for 24 hours and filtered with filter paper. After filtration, 80% of MeOH was evaporated in a water bath at 45 ºC in the rotary evaporator. After evaporation, the remaining extract was dissolved with 10 mL of 99.9% MeOH.

2.6. Total Phenolic Content Assay

Total phenolic content was determined according to the Folin-Ciocaltaeu method (Wang & Lee, 1996; Ismail et al., 2010; Karimi et al., 2013). The samples were prepared as 1 mg/mL. Each sample (20 µL) was reacted with 100 µL of Folin-Ciocalteu reagent. Sodium carbonate (20% w/v) for 300 µL, and 1580 µL distilled water were then added. The mixture was incubated at room temperature for 30 min. Each reaction was replicated 3 times. The absorbance of each reaction was determined at 765 nm by VWR V-1200 Spectrophotometer. Gallic acid (15.62 mg/L, 31.75 mg/L, 62.5 mg/L, 125 mg/L, 250 mg/L, 500 mg/L and 1000 mg/ mL) was used as a positive control.

2.7. DPPH Free Radical Scavenging Activity Assay

DPPH free radical scavenging activity was measured using the Sanchez-Moreno method (Sanchez-Moreno et al.,1998,1999[a],1999[b]). The samples were prepared as 1 mg/ mL, 0.5

mg/mL, 0.25 mg/mL, 0.125 mg/mL. For comparative purposes, Ascorbic Acid, Butyl hydroxy toluene (BHT), Butyl hydroxy anisole (BHA) solutions were also prepared as 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL. The absorbances of the samples were read at 517 nm by V-1200 Spectrophotometer including 4 cuvettes. Percentage inhibition was calculated with this formula (1):

$$\% \text{ inhibition} = \frac{\text{Ab(control)} - \text{Ab(sample)}}{\text{Ab(control)}} \text{x}100 \quad (1)$$

2.8. HPLC-UV Analyses

Formononetin standard was prepared as 300 ppm, 150 ppm, 75 ppm, 37.5 ppm, 18.75 ppm, 9.37 ppm, and 4.68 ppm dissolved 99.9% MeOH. The formononetin of the *M. sativa* extracts was identified on a Shimadzu 1200 HPLC chromatographic system. Separation was performed using a column (C18 5 µm 250 × 4.6 mm). The samples were run in 70% MeOH with a flow rate of 0.35 mL/min and injection volume of 20 µL. The maximum absorbance of the HPLC was set at 254 nm. Formononetin was monitored in the scheduled multiple reaction monitoring mode (Rodrigues et al., 2014; Krakowska et al., 2018).

2.9. Statistical Analyses

SPSS 13 (SPSS Inc., Chicago, IL, USA) (Snedecor & Cochran, 1967) and Microsoft Office 2010 Excel Software were used in viable cell count, total phenolic content assay, DPPH free radical scavenging activity assay and HPLC quantification. $p<0.05$ was considered statistically significant.

3. RESULTS and DISCUSSION

In the present study, MS3 medium was used as the most successful of previously tested media (Çölgeçen et al., 2014). Çölgeçen et al. (2014), had used hypocotyl, cotyledon, apical meristem, epicotyl and young primary leaf explants taken from seedlings. Due to the low callus growth rate observed in epicotyl and young primary leaf explants, trials were carried out with hypocotyl, cotyledon and apical meristem explants in the study. The rate of contamination in callus and suspension culture media has decreased down to 5% due to use of UV-C lamps in the laboratory. There was no problem in callus production and suspension cultures.

3.1. Aggregate Measurement Results

Friable callus was used in cell suspension cultures. Although calli were distributed in the suspension cultures, their distribution was not homogeneous. Prior to filtration in cell suspension culture media, photographs were taken and aggregate length was measured, yet no significant differences were observed. Generally, aggregates were about 5 mm. The lowest aggregation size was 3 mm and the highest was 7.4 mm (Table 1).

As callus darkening started after the 21st day, 21-day-old yellow friable calli were taken into an agar-free MS media for large-scale cell suspensions of 100 mL, 250 mL, 500 mL, 1000 mL. Callus cells were left in the shaker in the suspension media and stained with 0.2% Trypan Blue for viability assays. In their study on *M. sativa* L. cv. Chaubet, Steward et al. (1999), used 0.375% Trypan Blue for cell viability assays. They followed the viability of cells for 11 days and found that the best result of 80% was achieved on the first day. Cell viability displayed a decreasing trend and dropped to 20% by the end of the 11th day. As the viability in the cells could not be detected at high concentrations of Trypan Blue, 0.2% of it was used in this study. Cell count was made for 20-day-old suspensions and thus the cell viability was determined. There was no significant difference in cell viability among 100 mL, 250 mL, 500 mL and 1000 mL cell suspension cultures. However, the highest cell viability of 75% was obtained in the

1000 mL culture. The lowest cell viability of 62.40% was quantified in 500 mL culture, which could be attributed to the lack of cell homogeneity.

Table 1. Aggregate measurement results (mm) ($p<0.05$).

Extract	Result (mm)
100 mL	4.67±0.1
250 mL	5.18±0.08
500 mL	4.95±0.09
1000 mL	5.81±0.06

3.2. Cell Viability Measurement Results

In cell viability analyses, the best result was obtained as 75% in 1000 mL cell suspension cultures. Percentage viability was calculated as 71.40% in the 100 mL, 67.80% in 250 mL and 62.40% in 500 mL cell suspension cultures (Table 2).

Table 2. Percentage viability (%) ($p<0.05$).

Extract	Percentage viability (%)
100 mL	71.40±4.1
250 mL	67.80±2.2
500 mL	62.40±1.8
1000 mL	75.00±0.9

3.3. Total Phenolic Compound Measurement Results

Comparing May and September herb samples, the best result for total phenolic content was obtained as 44.2 mg/g in the September one. Total phenolic content was measured as 32.2 mg/g in the May sample. In cell suspension cultures, 250 mL sample gave the best result for total phenolic content with 40.2 mg/g. The 100 mL cell suspension culture had the lowest amount of total phenolic compounds (Table 3).

Table 3. Cell suspension cultures, total phenolic contents in May and September herb samples ($p<0.05$).

Extract	Total phenolic content (mg/g)
100 mL	30.2±0.4
250 mL	40.2±0.3
500 mL	32.2±0.3
1000 mL	34.2±0.2
May herb sample	32.2±0.5
September herb sample	44.2±0.1

3.4. Total Phenolic Compound Measurement Results

DPPH free radical scavenging activity of May and September herb samples was higher than that of cell suspension cultures. Although there was no significant difference among the cell suspension cultures, the best result of 51.36% was obtained in the 1000 mL one (Table 4).

Figure 2. Stained cells **A)** 100 mL, **B)** 250 mL, **C)** 500 mL, **D)** 1000 mL cell suspension cultures.

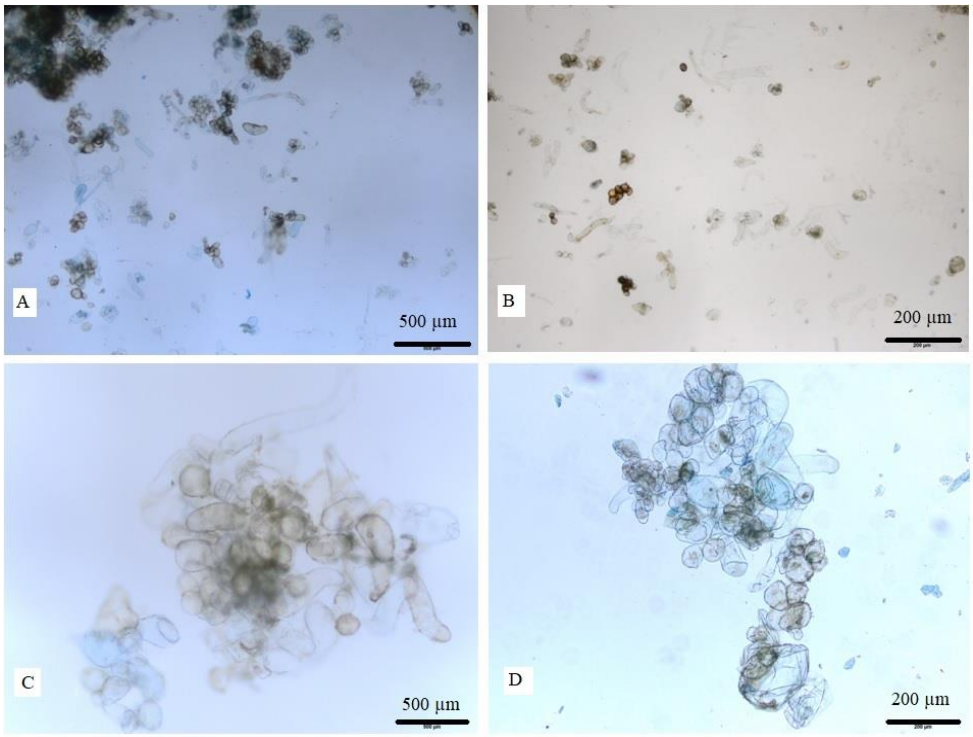


Table 4. DPPH free radical scavenging activity results (%) (p<0.05).

Extract	DPPH scavenging activity (%)
100 mL	41.63±3.1
250 mL	45.22±1.1
500 mL	47.26±1.3
1000 mL	51.36±1.2
May herb sample	80.51±2.5
September herb sample	85.69±2.1
Ascorbic acid	97.83±0.9
BHT	95.24±0.9
BHA	95.29±1.1

Antioxidants are radical scavenging compounds used in the treatment of various diseases. High antioxidant capacity in plants is an indication that the plant has medicinal importance. Bora and Sharma (2010), reported that *M. sativa* Linn. has 71.05% DPPH scavenging activity. Karimi et al. (2013), analyzed dry leaf samples of *M. sativa* (provided from a Taghavi farm in Iran) and quantified the total phenolic content as 45.2 mg/g and DPPH scavenging activity as 54%. Zinca and Vizireanu (2013), evaluated 2, 3, 4, 5, 6 and 7-day-old *M. sativa* L. seedlings (provided from a health store in Canada) for their total phenolic content and antioxidant activity. The highest total phenolic content (0.9 mg/100 g) was measured in 4-day-old seedling and the highest antioxidant activity (64%) was shown by 6-day-old seedling. Silva et al. (2013), analyzed *M. sativa* L. (provided from market) herb samples and reported 56% DPPH scavenging activity. Different from these researchers, in this study, *M. sativa* L. herb samples were collected separately in May and September. These samples were compared according to their total phenolic content and DPPH scavenging activity. The highest total phenolic content

(44.2 mg/g) and the highest DPPH scavenging activity (85.69 %) was observed in the September herb sample.

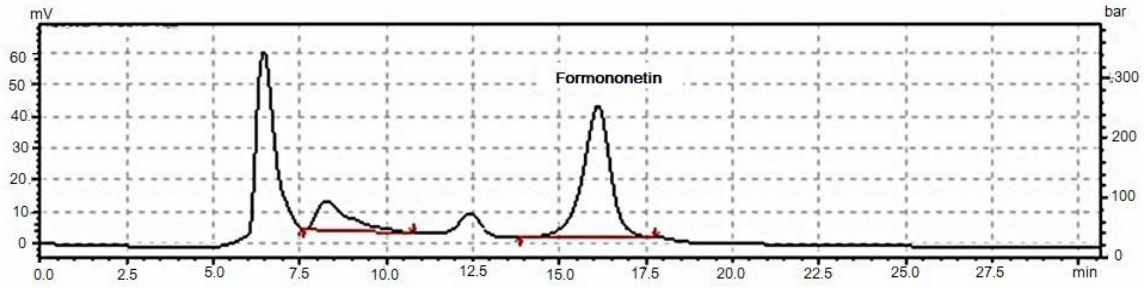
3.5. HPLC-UV Measurement Results

The calli derived from large-scale cell suspension cultures of *M. sativa* L. cv. Elçi (alfalfa Elçi) and the herb samples collected in May and September were analyzed by HPLC-UV method. The retention time for formononetin was 16 mins (Figure 3). Of the herb samples, May sample had the highest formononetin content with 2.07 mg/g, while of the cell suspension cultures, 1000 mL has shown the highest formononetin concentration of 4.99 mg/g. Additionally, formononetin content increased in cell suspension cultures compared to naturally occurring *M. sativa* L. cv. Elçi (Alfalfa Elçi) herbs (Table 5).

Table 5. HPLC-UV analysis results (Formononetin) (mg/g) (p<0.05).

Extract	Quantity (mg/g)
100 mL	3.72±0.04
250 mL	3.46±0.06
500 mL	3.36±0.08
1000 mL	4.99±0.09
May herb sample	2.07±0.05
September herb sample	1.84±0.09

Figure 3. Chromatogram of 1000 mL cell suspension culture.



M. sativa L. is a valuable medicinal plant which is rich in flavonoid compounds. The levels of these compounds increase in response to various factors. For example, elicitor trials may increase the amount of these compounds. *M. sativa* L. cv. Moapa 69 had been exposed to *Rhizobium meliloti* bacteria (Dakora et al., 1993). No formononetin was detected in the control group yet it was detected in the treatment group subjected to *Rhizobium meliloti* bacteria, but it could not be quantified. *M. sativa* cv. Nagyszenas was subjected to to different concentrations of potassium nitrate (KNO_3) and its formononetin content was determined by HPLC analysis (Coronado et al., 1995). Formononetin content was lower than 1 mg. The data suggested that low concentrations of KNO_3 increased formononetin content compared to high concentrations. Although no elicitor trials were conducted in the present study, formononetin was determined in herb sample and the quantity was 2,5 times higher.

Culture media are also important to increase flavonoid production. SH (Shenk and Hildebrandt) callus and suspension culture media was prepared for *M. sativa* cv. Apollo (He et al., 1998). The scale of cell suspension culture media was 50 mL. The researchers revealed formononetin content during HPLC analyses of calli, but the amount of formononetin was not measured. Tetrahydrofuran and distilled pure water were used as solvent for HPLC analysis. No formononetin content could be observed in *M. sativa* L. cv. Elçi (alfalfa Elçi) herb samples,

yet they revealed formononetin production in cell suspension cultures (Çölgeçen et al., 2014). Formononetin content was measured as 0.32 mg/100 mg in 100 mL suspension cultures. In this study, MS medium was used both for callus and suspension culture. Herb samples were found to contain formononetin. The highest formononetin content of 4.99 mg/g was found in 1000 mL cell suspension cultures while the lowest of 0.37 mg/g was found in 100 mL cell suspension cultures.

The use of different solvents or different chromatographic methods may result in varying flavonoid contents. A quantitative analysis had been conducted on some flavonoids in *M. sativa* cv. Lucerne (Martin et al., 2006). 260 nm wavelength was used in LC-MS analysis for this quantification of flavonoids in herb samples. Formononetin content was measured as 40 mg/kg. The herb samples of *M. sativa* cv. Azurara and 6 different *Medicago* species were evaluated for their flavonoid content (Rodrigues et al., 2014). HPLC analysis was performed with the columns C18 150 mm x 4.60 mm. *M. sativa* extracts prepared with aqueous and ethanol solutions were compared. No formononetine and daidzein could be detected in the *M. sativa* extract that was prepared with aqueous solution, while 2.40 mg/kg formononetine was quantified in the *M. sativa* extract that was prepared with ethanol extract. In this study, analyses were performed by HPLC at a 254 nm wavelength with C18 4,6 x 250 mm columns. 70% MeOH-distilled pure water was used as solvent. The highest formononetin content was measured as 4.99 mg/g. As a remarkable finding, this study indicated that herp samples of *M. sativa* L. cv. Elçi collected in May (2.07 mg/g) and those collected in September (1.84 mg/g) contain different amounts of formononetin. It was clearly revealed that seasonal changes have significant effect on formononetin content in plants.

4. CONCLUSION

Clinical trials have shown that formononetin reduces the symptoms of menopause by increasing estrogen levels. Thus, the extracts of *M. sativa* with high content of formononetin can be a starting point for the development of new pharmacotherapies. Hence, a method has been tested to increase the production of this compound with such high medicinal value. In this study, formononetin production was increased in large-scale cell suspension cultures of *M. sativa* L. cv. Elçi (Alfalfa Elçi). Future studies should focus on the development and application of modern sample preparation techniques, and better cell suspension culture methods should be developed for the production of formononetin in *M. sativa.* Meanwhile, the development of advanced methods for purifying special and biologically active compounds will enable our future understanding of their actions on organisms. Also, this study provides a basis for achieving high-efficiency production with bioreactors and can be investigated in further studies.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s).

Authorship contribution statement

Tayfun Aktaş: Investigation, Resources, Visualization and Writing -original draft. **Hatice Çölgeçen:** Methodology, Supervision, and Validation. **Havva Karahan:** Investigation, Resources, Visualization and Corresponding.

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International Journal of Secondary Metabolite
2021, Vol. 8, No. 1, 59–69

https://doi.org/10.21448/ijsm.794617

Published at http://dergipark.gov.tr/en/pub/ijsm Research Article

Determination of Photosynthesis-Related and Ascorbate Peroxidase Gene Expression in the Green Algae (*Chlorella vulgaris*) Under High-Temperature Conditions

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Abstract: Increasing water temperatures because of climate change resulted in population shifts and physiological responses in aquatic environments. In this study, short-term high-temperature condition effects on green algae *Chlorella vulgaris* were investigated at transcriptional and physiological levels. The photosystem II D1 protein (*psbA*) gene, a large unit of Rubisco (*rbcL*) gene and chloroplastic ascorbate peroxidase (*cAPX*) gene expressions were quantified using semi-quantitative real time-PCR. The *psbA* gene transcription level at 45˚C for 48 and 72 h was reduced by approx. 2.22 and 2.86-folds, respectively. The *rbcL* gene transcription level was also reduced by 1.54 relative to the control at 72 h. Our *APX* gene transcriptional level results indicated that the transcription of this gene was significantly increased at 35˚C at 24, 48, and 72 h. In contrast, the *cAPX* mRNA transcript level was reduced by approx. 2 times compared with the control. Our data demonstrated that alteration *cAPX* gene expression could play an essential role in high-temperature acclimation in *C. vulgaris*.

ARTICLE HISTORY

Received: September 14, 2020
Revised: February 22, 2021
Accepted: March 05, 2021

KEYWORDS

cAPX gene,
Microalgae,
psbA gene,
rbcL,
Heat stress

1. INTRODUCTION

Temperature stress can be counted as a critical abiotic factor due to stimulated changes in some physiological processes like membrane stability, development, photosynthesis, plant growth, and respiration (Sinsawat et al., 2004). The high temperature also inhibits Calvin cycle activity by decreasing the activation state of Rubisco enzyme (Weis, 1981; Feller et al., 1998; Law & Carfts-Brandner, 1999). Photosystem II (*PSII*) also displays susceptible responses to increasing temperatures and heat-inhibition of photosynthesis. The inhibition of electron transport in photosynthetic organisms has been attributed to the thermal accumulation ability of *PSII*, which occurs in the formation of reactive oxygen species (*ROS*) from water (Allakhverdiev et al., 2007).

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ISSN-e: 2148-6905 /

The PSII reaction center includes two main proteins, *D1*, and *D2* proteins. *D1* proteins in the thylakoid membranes are known to be susceptible to many environmental factors (Giardi et al., 1997). *D1* proteins are generally affected by oxidative stress, and they can be degraded (Prasil et al., 1992) and leads to *PSII* photodamage. Photosynthetic organisms get typically harmed by the instability of synthesis/degradation balances of *D1* protein during stress conditions. *D1* protein is encoded by the *psbA* gene, which has a role in the replacing damaged *D1*. During the repair of damaged *PSII*, firstly, the damaged *D1* protein is removed then the new *D1* protein is synthesized instead of the damaged one. As a result, a new *D1* protein was added to the *PSII* system.

It has been demonstrated by previous studies some environmental stress factors such as metal and salt stress restrain the *PSII* repairment by the inhibition of *psbA* gene transcription and translation (Nishiyama et al., 2004; Allakhverdiev et al., 2008; Qian et al., 2009). The electron transport system is the primary basis of *ROS* in chloroplasts. Besides, the location of *ROS* generation changes according to stress types (Foyer & Noctor, 2003; Mittler et al., 2004). The *ROS* production is highly detrimental for the protein and lipid metabolisms and leads to the inhibition of algal growth (Sainju et al., 2001; Tang et al., 2007). Many photosynthetic organisms have robust antioxidant systems, embracing antioxidant enzymes and antioxidants. Antioxidant enzymes have played an essential role in reducing oxidative stress. Ascorbate peroxidase (*APX*) enzyme is fundamental in the ascorbate-glutathione cycle. They are found in green plants and algae and catalyze the transformation of H_2O_2 into the water using ascorbate as an electron donor (Asada, 1999). Photosynthetic organisms with escalated tolerance to several environmental stresses, comprising temperature stress, achieve such tolerance through the excited expression of *APX* genes. *APX* gene expression was induced after potato tubers were exposed to low temperatures (Kawakami et al., 2002). In chloroplasts, the over-expression of *APX* has a vital role for detoxification of H_2O_2. Up-regulation of *APX* could alleviate photooxidative depredation during temperature stress. Researches conducted with transgenic plants demonstrated that they have higher photochemical efficiency of *PSII* compared with wild-types under cold stress (Sun et al., 2010). Du et al. (2013) demonstrated that the transcript levels of cytosolic (cyt) *APX* were significantly higher in heat-tolerant *Poa pratensis* L. under long-term heat stress. However, there is less available data on the effects of high-temperature stress on *psbA, rbcL*, and chloroplastic *APX* transcription levels, and these different genes interact. The aims of this study are (i) to understand the tolerance capacity of green algae *C. vulgaris* by analyzing growth rate, chlorophyll quantity and chlorophyll degradation rate under high-temperature stress; (ii) to determine the effects of high-temperature stress on the transcription levels of *psbA*, *rbcL*, and chloroplast *APX* genes in by semi-quantitative real time-PCR.

2. MATERIAL and METHODS

2.1. Culture Conditions

C. vulgaris culture was obtained from Ege University Microalgae Culture Collection (EGEMACC). Organisms were stored in Rudic Medium (RD) (Rudic & Dudnicenco, 2000) at 25°C in laboratory conditions until experiments. Five flasks containing 100 ml of *C. vulgaris* were used for the experiment. The culture was grown in RD at 25°C (as control), 35°C and 45°C. Aeration was provided to the culture flasks continuously by bubbling air via a blower.

2.2. Cell Density

The absorbance at 663 nm was determined with a UV-Vis spectrophotometer (Pharo 300, Merck) at 24, 48, and 72 h. Specific growth rate μ was calculated using the equation described by Guillard (1973) as follows (1):

$$\mu=\ln(X_t/X_0)/t \quad (1)$$

X_0 indicates the initial cell density, X_t indicates the cell density after t hours.

2.3. Determination of Chlorophyll a Degradation

Chlorophyll degradation detected according to dimethyl sulfoxide (DMSO) extraction protocol (Wellburn, 1994). 20 mg of cells was extracted with 3 ml DMSO in one hour at 65°C under unilluminated conditions. Polyvinylpyrrolidone was added to DMSO to prevent chlorophyll degradation during incubation. To determine the chlorophyll degradation, extracts were read at 665 and 649 nm in the spectrophotometer (Pharo 300, Merck). Chlorophyll a, b, and a/b were calculated via specific absorption coefficients.

2.4. RNA Isolation and Reverse Transcriptase-PCR

The material ground in liquid nitrogen and 1 mL of TRIZOL Reagent (Thermo Fisher Scientific, cat# 15596026) was added into the fine powder. For the homogenization, chloroform (Sigma-Aldrich, cat# 650498) was inserted into the mixture and centrifuged at 10000 x g for 15 min. at 4°C. After the incubation for 10 min. at 15 to 30°C, the samples were centrifuged at 10000 x g at 4°C for 10 min. After washing with 75% ethanol, the pellet was air-dried for 15 min (Poong et al., 2017). The quality and quantity of obtained RNA were measured by spectrophotometer (Pharo 300, Merck). Manufacturer's instructions of cDNA Reverse Transcription Kit (Invitrogen, cat# 4398814) were followed for Reverse Transcriptase-PCR.

2.5. Semi-Quantitative RT-PCR

The oligonucleotide primers were designed from the *C. vulgaris psbA*, *rbcL*, and *cAPX* gene sequences using the PerlPrimer open source PCR primer design programme (Marshall, 2004). PCR reactions were also performed with GAPDH primers as internal control. The following sequences were used for *psbA* forward (5´-GATGAGTGGTTATACAATGGTGG-3´) and reverse (5´-GTGAGTTGTTGAAAGAAGCGT-3´), for *rbcL* forward (5´-TAACTTACTACACTCCTGAC-3´) and reverse (5´-AAGAAGACCATTATCACGAC-3´), and for chloroplastic *APX* forward (5´-CCTTTCATCCCTCTACGGCT-3´) and reverse (5´-GTCCTCTGCATACTTCTCTCGG-3´) primers. The semi-quantitative RT-PCR was performed using 5 ng cDNA, 2.5 mM PCR buffer (10X), 10 mM dNTP mix, 10 µM primers, and 1U Taq DNA polymerase enzyme (Thermo Scientific, cat # EP0402). Each PCR cycle consists of 95°C of 60 sec. denaturation, 49°C (*psbA*), 53°C (*cAPX*), and 56°C (*rbcL*) of 75 sec. annealing, 72°C of 75 sec. elongation cycles. After 32 cycles the amplification ended with a 10 min. final elongation step at 72°C (Sen et al., 2014). Each Primer set was a number of PCR cycles optimized to ensure the linearity requirement for semi-quantitative RT-PCR analysis.

2.6. Statistical Analysis

Statistical significance was assessed using a student's t and ANOVA test (SPSS, for Windows, Version 11.0). A $p<0.05$ value was considered statistically significant. All experiments were repeated three times.

3. RESULTS and DISCUSSION

According to our results, specific growth rates gradually decreased over four days at 45˚C, whereas growth rates did not change significantly at 35˚C. Figure 1 demonstrates the high-temperature effects on growth ratio. Besides, maximum cell densities and the growth rates of *C. vulgaris* at 45˚C showed a significant reduction ($p< 0.05$) after 72 h compared to the control group and cultures at 35˚C. Bajguz (2009) demonstrated that high-temperature stress leads to inhibition of photosynthetic oxygen evolution, and decreased cell division in *C. vulgaris*. Temperature optima for many commercial microalgae changes between 20-30˚C (Sánchez-Luna et al., 2007). The previous study showed the inhibition of *C. vulgaris* growth above 30˚C

(Converti et al., 2009). Sorokin and Krauss (1962) demonstrated that at 45˚C, no constant growth was observed in *C. pyrenoidosa.*

Figure 1. The effects of different temperatures (35ºC and 45ºC) on the growth rate of *Chlorella vulgaris* culture. (*) Represents a statistically significant difference of $p<0.05$ when compared with the control, (**) represents a statistically significant difference of $p<0.01$.

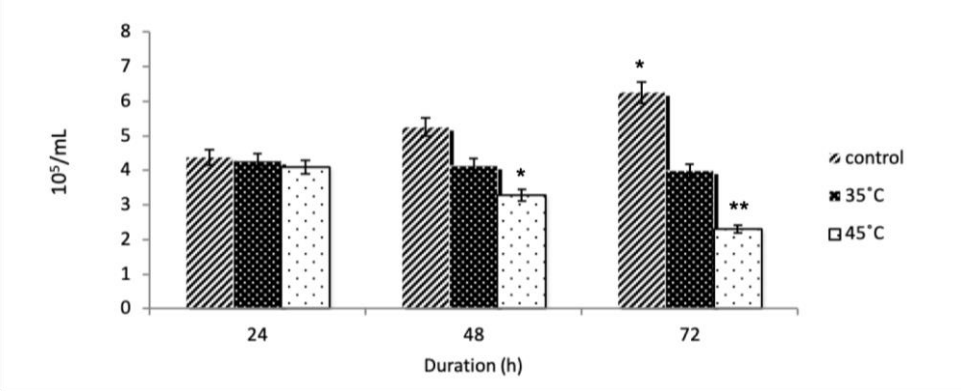


Table 1. Chlorophyll a, and b content and Chla/b rate of the algae *Chlorella vulgaris* cultivated with a growth 25 ºC (control), 35 ºC and 45ºC temperature.

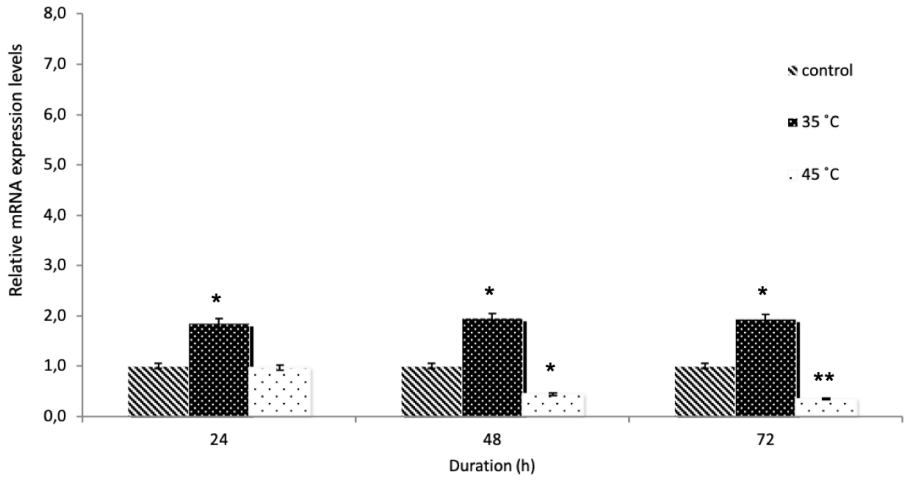
Groups	n	Chl a			Chl b			Chl a/b		
		24 h x±SD	48 h x±SD	72 h x±SD	24 h x±SD	48 h x±SD	72 h x±SD	24 h x±SD	48 h x±SD	72 h x±SD
Control	3	25.32 ±0.22	25.32 ±0.22	25.32± 0.22	7.69± 0.14	7.69± 0.14	7.69± 0.14	3.31± 0.1	3.31± 0.1	3.31± 0.1
35˚C cultures	3	24.09 ±0.2	21.81 ±0.28	19.17± 0.19	7.22± 0.19	8.75± 0.09	8.99± 0.11	3.34± 0.07	2.49± 0.06	2.09± 0.05
45˚C cultures	3	21.99 ±0.13	20.06 ±0.06	16.47± 0.06	8.58± 0.16	9.23± 0.13	11.23 ±0.08	2.54± 0.03	2.17± 0.04	1.46± 0.01

Values in bold are significantly different from control samples. Significance of differences ($p<0.05$) was checked by one-way analysis of variance (ANOVA). n=number of replicates, x=mean values, SD=standard deviations.

Chlorophyll amount is very important for photosynthesis since chlorophyll absorbs sunlight and synthesizes carbohydrates with the participation of CO_2 and water. Chlorophylls are susceptible molecules to a sense of stress-initiated oxidative stress (Puckett et al., 1973; Sandmann & Böger, 1980; Chettri et al., 1988). Under oxidative stress conditions, chlorophyll a is oxidized from the methyl group on ring II to the aldehyde groups and occurs in chl b formation (Chetri et al., 1988). For this reason, chlorophyll a/b ratio is more sensitive than chlorophyll a+b to modification. In the present study, we tested all chlorophyll parameters for understanding high-temperature effects on *C. vulgaris* culture. Based on our data, the pigment levels were not significantly different at both 35˚C and 45˚C for 24 h. Chl a, Chl b, and Chl a/b amount was significantly ($p< 0.05$) different when comparing the control group with at both 35˚C and 45˚C for 48 and 72 h (Table 1). It was observed that the chlorophyll a content and chlorophyll a/b ratio for the 72 h application period at 35˚C decreased by 20.34% and 36.56%, respectively, and at 45˚C decreased by 34.95% and 55.89%, respectively. Our present results also confirmed that a high temperature (45˚C) treatment for 72 h resulted in a significant increase in chl b and chl a consistent with the expedited conversion of one to the other (Table 1).

The high temperatures lead to *D1* protein damage and contribute to descended electron transport efficiency. Damaged *D1* protein could be immediately re-synthesis via *PSII* repair mechanisms for providing redox homeostasis in chloroplasts. Therefore, the replacement of new *D1* proteins in *PSII* needs to be the expression variations of the *D1* coding gene *psbA*. In the present study, transcription levels of two photosynthesis-related genes were analyzed by semi-quantitative RT-PCR and compared the chlorophyll degradation results under heat stress. The results revealed that the mRNA transcript level of *psbA* increased at 35˚C for 24, 48, and 72 h compared with control. The *psbA* mRNA level of *C. vulgaris* cultured at 35˚C for 24, 48, and 72 h was increased by 1.85, 1.95, and 1.94 times, respectively, as compared with the control group (Figure 2). In cultures subjected to 45˚C for 24 h, the *psbA* mRNA transcript level did not display significant differences as compared with the control group (Figure 2). However, the *psbA* mRNA transcript level was slightly decreased by 2.22 and 2.86 times, respectively, relative to control at 48 and 72 h under high temperature. Similarly, both salt stress and oxidative stress (Nishiyama et al., 2006; Allakhverdiev et al., 2008) prohibit the repair of photodamaged PSII by inhibiting the *psbA* gene transcription and translation. Qian et al. (2009) studied the effects of copper and cadmium stress on *C. vulgaris,* and the results proved that metal stress inhibits the expression of *psbA* and *rbcL* genes at the transcriptional level.

Figure 2. The effects of different temperatures (35ºC and 45ºC) on the relative expression of *psb*A of *Chlorella vulgaris* culture. (*) Represents a statistically significant difference of $p<0.05$ when compared with the control, (**) represents a statistically significant difference of $p<0.01$.



It has been shown in previous studies the maximal transcript accumulation temperature was distinctly different for several photosynthesis-related genes. Kusnetsov et al. (1993) demonstrated that the maximum transcription level for *psbA, psbE* genes, *psbB, psbC, atpA* genes, and *psbA, psbD* genes were observed 38˚C, 40˚C, and 42˚C, respectively in higher plants. Similarly, the highest *psbA* mRNA transcript levels were found with *C. vulgaris* cultures at 35˚C at 48 and 72 h (1.95 and 1.94 times higher than the control group, respectively). The rise of the transcript levels might increase the corresponding enzyme and its activity. Thus, it might protect the electron transport in *PSI* and *PSII* under moderate high-temperature stress. According to Kusnetsov et al. (1993), the rate of electron transport decreased due to the inactivation of *PSII* acceptor side at temperatures below 40-42˚C.

Vierling and Key (1985) reported that the *rbcL* transcript level was slightly varied within the temperature range of 28 to 48˚C in soybean suspension cultures. In the present study, Figure 3 shows the mRNA transcript level of *rbcL* under high temperature. The transcript level of *rbcL* was significantly changed at 35˚C for 24, 48, and 72 h compared to the control group. According to our results at 35˚C for 24, 48, and 72 h, the transcript level of *rbcL* did not

significantly change; however, the transcript level of *psbA* increased dramatically after 24 and 48 h at 35˚C. However, compared to the control, the mRNA transcript level of *rbcL* was decreased significantly (1.07, 1.3, and 1.54, respectively) after exposure to 45˚C for 24, 48, and 72 h (Figure 3). In the present study, the mRNA transcript levels of *psbA* and *rbcL* decreased significantly after 72 h at 45˚C. The decrease in transcript levels might be the result of the prevention of normal electron transport in PSI and PSII and block carbon assimilation.

Figure 3. The effects of different temperatures (35ºC and 45ºC) on the relative expression of *rbcL* of *Chlorella vulgaris* culture. (*) Represents a statistically significant difference of $p<0.05$ when compared to the control, (**) represents a statistically significant difference of $p<0.01$.

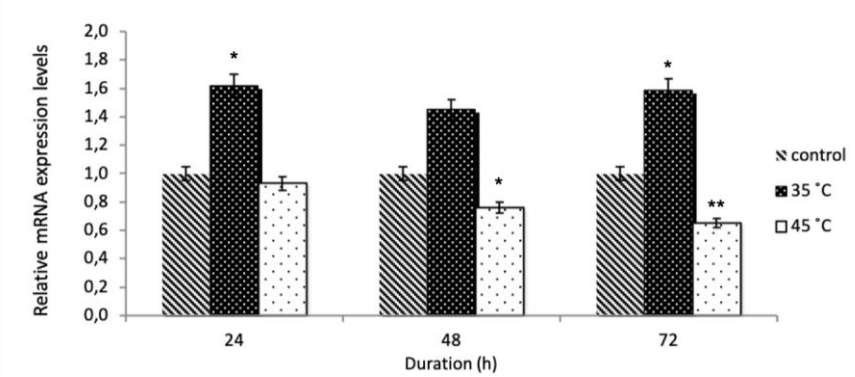
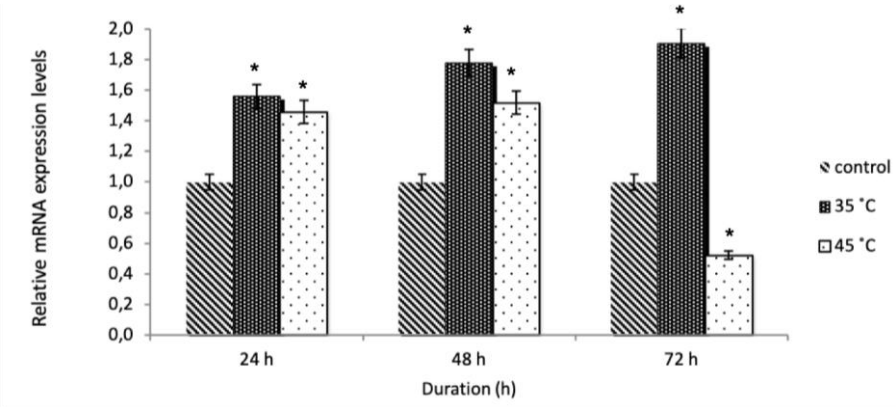


Figure 4. The effects of different temperatures (35ºC and 45ºC) on the relative expression of *Apx* of *Chlorella vulgaris* culture. (*) Represents a statistically significant difference of $p<0.05$ when compared to the control.



In living organisms, ROS accumulation occurs as a result of various stress conditions. Plants neutralize ROS by antioxidant systems, such as ascorbate peroxidase enzyme. *APX* encoding gene expressions are modulated by multiple environmental stresses, such as drought, salinity, extensive light, pathogens, and low temperature (Zhang et al., 1997; Yoshimura et al., 2000; Agrawal et al., 2003; Menezes-Benavente et al., 2004; Lin & Pu, 2010). Lin and Pu (2010) reported escalating cytosolic accumulation of *APX* transcripts in a salt-tolerant sweet potato. Goyary (2009) also demonstrated the increment of ascorbate content and *APX* gene expression in transgenic tomato plants compared to wild-type under cold temperatures. *APX* is known to have an important function against high temperatures by intercepting the oxidation of enzymes and the degradation of membranes. Previous studies reported that over gene expressions of *APX* enhanced the tolerance capacity and minimized photooxidative damage under temperature stress (Caverzan et al., 2012; Sato et al., 2011; Shi et al., 2001; Miller et al., 2007). Park et al. (2004) also emphasized the highly induced *cAPX* gene levels in sweet potato

leaves after high-temperature exposure. Moreover, Ma et al. (2008) showed the increment expression levels *APX* in apple leaves at 40˚C for 4 h exposure and decreasing afterward. In the present study, c*APX* gene was up-regulated at both 35˚C and 45˚C with different time periods, as shown in Figure 4. The c*APX* gene transcription level after exposure at both 35˚C and 45˚C for 24 h was significantly different from that of the control. According to our results, cultivation at 35˚C for 24, 48, and 72 h, the *cAPX* mRNA levels were increased by 1.56, 1.78, and 1.91 times, respectively, as compared with the control group (Figure 4). However, the c*APX* mRNA transcription level was also decreased by approx. 2 times in the *C. vulgaris* culture at 45˚C for 72 h.

4. CONCLUSION

Green alga *C. vulgaris* was used to determine the affects of the moderate and high temperature stress. The experiments were conducted with 3 different temperatures; 25ºC as control group; 35ºC as moderate temperature group and 45ºC as high temperature group. All measurements (cell density and growth rate) and analysis (*rbcL*, *psbA*, *cAPX* genes transcription levels) applied on 24th,48th and 72nd hours of the experiments. According to our results, moderate temperature does not show a significant affect on growth rate and cell density. However, at high temperature conditions growth rate decreased after 4 days. High temperature stress leads to inhibition of photosynthetic oxygen evolution, and decreased cell division in *C. vulgaris* as suggested in previous studies. Chl a content and chl a/b ratio decreased under moderate and high temperature stresses after 72 h. Besides pigment ratio changes, some differences are determined on stress genes transcription levels. For example, *psbA* gene transcription levels decreased at high temperature stress conditions after 48 hours. The *cAPX* levels of moderate and high temperature exposed groups were up-regulated after 24 hours. Our results suggest that the *cAPX* gene expression could mitigate high temperature-induced oxidative damage in *C. vulgaris*, depending on the application period, through increased *psbA* and *rbcL* transcript levels and decreased chlorophyll degradation. Future work will focus on how the *cAPX* interacts with the *psbA* and *rbcL* expression responses to high-temperature stress.

Acknowledgements

The study founded by 2209 Projects by Turkish Scientific and Technological Research Institution (TUBITAK).

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s).

Authorship contribution statement

Inci Tuney Kizilkaya: Writing, editing, validation. **Sedef Akcaalan:** Laboratuary work. **Dilek Unal:** Experiment design, supervision, statistical analysis, validation.

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