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

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

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

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

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

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

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1. Introduction

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

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

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

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

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

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

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

2. Materials and Method

2.1. Plant Material

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

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

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

2.2. Total Phenolic Content

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

2.3. Total Flavonoid Content

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

2.4. Total Antioxidant Activity Measurements

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

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

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

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

3. Results

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

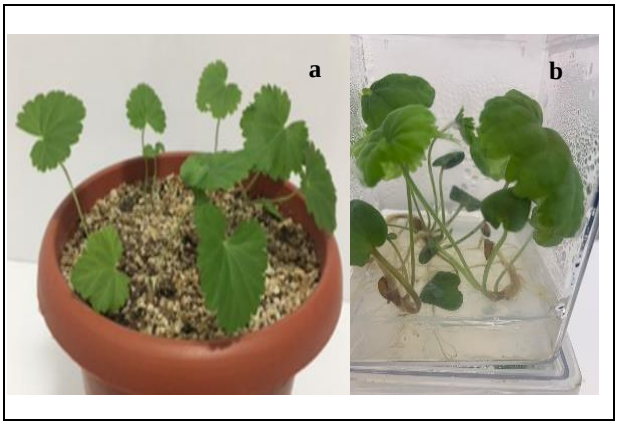

Figure 1. *In vivo* and *in vitro* development of *Pelargonium quercetorum* plants (a. *in vivo*; b. *in vitro*)

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

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

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

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

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

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

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

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

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

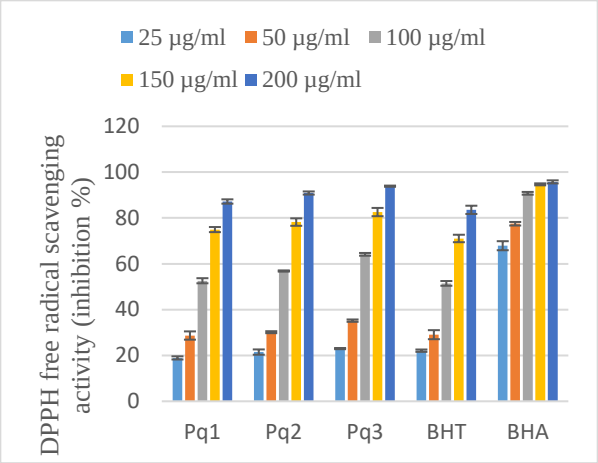

Figure 2. DPPH free radical scavenging activity of *Pelargonium quercetorum* and positive controls (Pq1: *In vivo* rhizome; Pq2: *In vivo* aerial parts; Pq3:*In vitro* shoot)

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

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

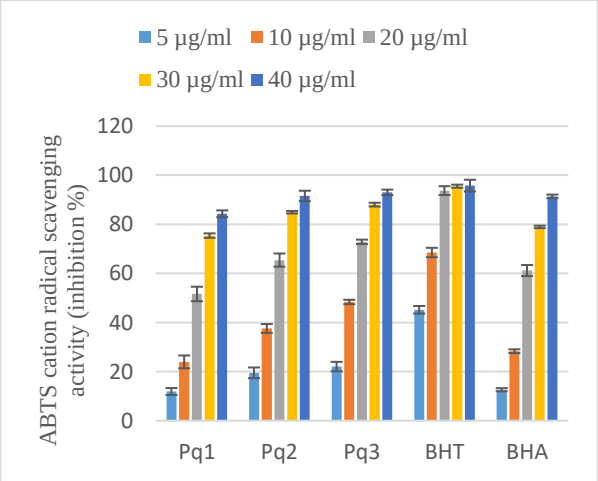

Figure 3. ABTS cation radical scavenging activity of *Pelargonium quercetorum* and positive controls (Pq1: *In vivo* rhizome; Pq2: *In vivo* aerial parts; Pq3:*In vitro* shoot)

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

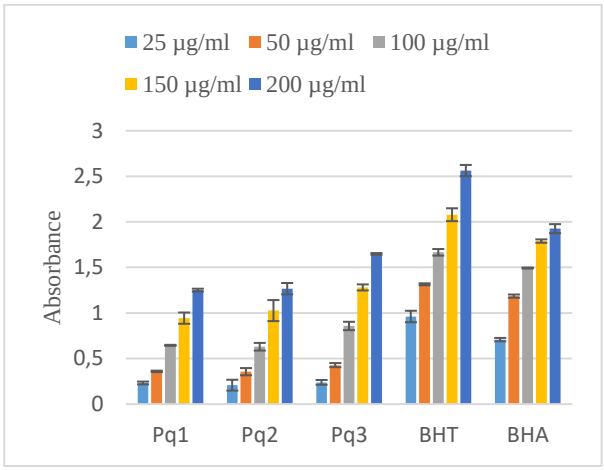

Figure 4. Cuprac reducing antioxidant capacity of *Pelargonium quercetorum* and positive controls (Pq1: *In vivo* rhizome; Pq2: *In vivo* aerial parts; Pq3:*In vitro* shoot)

4. Discussions

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

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

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

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

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

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

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

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

5. Conclusion

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

Conflict of Interest

Authors have declared no conflict of interest.

Authors' Contributions

The authors contributed equally.

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