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Determination of Antioxidant Activities of *Echinacea purpurea* (L.) Moench. Plant Extracts Harvested at Different Times

Rabia Vildan ŞAHİN [1*], Belgin COŞGE ŞENKAL [2]
[1] Black Sea Agricultural Research Institute, Samsun
[2] Yozgat Bozok University, Faculty of Agriculture, Department of Field Crops, Yozgat
[*]Corresponding author: rabiavildan.sahin@tarimorman.gov.tr

Abstract
This research was carried out to determine the antioxidant activities of extracts obtained from plant parts of *Echinacea purpurea* L. (Moench) species harvested at different times. In the preparation of plantation, seeds for seedling production were planted in peat-containing pots on 24 April 2012. Rooted seedlings were planted in the experimental area in May. After planting, necessary cultural practices (irrigation, hoeing, etc.) of the plants were performed in 2013, 2014 and 2015. In this study, cone, leaf, flower and root parts of Echinacea plant harvested at different times between September 2014 and August 2015 were used. This study was conducted using the cone, leaf, flower, and root parts of the echinacea plant. While the cone, leaf and flower parts were harvested on 3 different dates, namely 24 July, 6 August and 19 August, the root part was harvested only once on 10 October. In this study, it was stated that the antioxidant activities of *Echinacea purpurea* species vary depending on the harvest period and the organs of the plant. The values of the highest total phenolic content and total flavonoid compound content were calculated. These values were determined as 59.107 mg GAE g^{-1} extract and 1807.286 mg QE g^{-1} extract, respectively. The lowest DPPH value was found to be 1.157 mg mL^{-1}. These datas were found for the flower parts of the plant from the first harvest time, the leaf parts from the second harvest period and the flower parts from the second harvest date, respectively.

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1. Introduction

In today's societies, people work not only to eat, but also to eat healthy and improve their quality of life. In recent years, fields such as organic nutrition, herbal medicine, and alternative medicine are rapidly gaining ground and becoming widespread. Echinacea plant is also increasingly gaining ground as an important medicinal and aromatic plant. Studies have reported that approximately 25.000 plant species in the world are used for medicinal purposes (Kan, 2011). According to another study over than 50.000 species are utilized for medicinal purposes (Jamshidi-Kia et al., 2018). Asteraceae is one of the richest families of flowering plants and is represented by approximately 1000 genera and 20000 species on earth. In Türkiye, 134 genera and 1156 species belonging to this family have been identified (Davis et al., 1988). Echinacea (*Echinaceae purpurea* L.), a member of the Asteraceae family, is a medicinal plant. (Öner et al., 2023) One of the members of this family is the Echinacea species, which is very important economically.

Echinacea is a plant originating from North America. It is known by names such as "Cone Flower, Black Sampson, Red Sunflower" in English. In Türkiye, it is known by many different names such as "echinacea, purple hedgehog, hedgehog, thin-leaved lavender cocoon flower, samson root" (Mat, 2002). The word *Echinacea* is a Greek word derived from the word "echinos" meaning sea urchin or hedgehog. It is stated that the thorn-like flower structures on the flower base give the plant its name *Echinacea* (Mistrikova and Vaverkova, 2007).

Echinacea species are perennial herbaceous plants that reach 10-60 cm in height. The stem of the plant, which has taproot or fringe roots, is in a vertical position. The plant has a simple or branched stem structure. The plant is very resistant to drought and can regenerate itself (Mistrikova and Vaverkova, 2007). The leaves are oval-lance-shaped and have 3-5 veins. The center of the cone-headed flower is surrounded by radial flowers. The colors of radial flowers vary from pink, white, purple, and red (Çalışkan and Odabaş, 2011).

Echinacea was the first introduced to the medical world by Meyer, a German doctor, in the 1870s (Hobbs, 1994). It is stated that the doctor, who uses the blood purifying medicine prepared from *E. agustifolia* roots in the treatment of many diseases such as rheumatism, migraine, pain, snakebite, wounds, indigestion, plant poisoning, poisonous snake bites, learned how to use the plant from the American Indians. This prepared medicine attracted the attention of Dr. John King and Pharmacist John Uri Lloyd, and subsequently the first scientific studies on echinacea were initiated by these two scientists. It is also noted that while initially only *E. angustifolia* roots were used, later *E. pallida* roots were also used (Mat, 2002). Extracts and preparations obtained from echinacea species have gained an important place in the herbal medicine market in many countries of the world, especially European countries and the United States (Upton and Graff, 2007). In 2019, three commonly used species, including *E. angustifolia*, *E. pallida* and *E. purpurea* had a market value of $120 million in the US market, up 4.9 % compared to the previous year. In addition, it was stated that echinacea sales increased significantly by 90.9 % in the first half of 2020 in connection with the Covid-19 epidemic (Smith et al., 2020).

There are many studies have been indicated that the *Echinacea purpurea* plant has antioxidant properties. The antioxidant system is basically defined as a powerful mechanism that prevents the development of free radicals and peroxide reactions in the organism (Kobylinska and Tymochko, 2000). It has been reported that *Echinacea purpurea* can provide extra protection by maintaining normal redox status, especially when the body is exposed to infections (Chen et al., 2010; Merali et al., 2003). *Echinacea purpurea* contains important phenolic compounds. Kafaric and cichoric acids are two of these phenolic compounds. These phenolics are found in whole parts of the plant for instance,

flowers, leaves, roots, and stems. It is stated that the Asteraceae family in general and the echinacea plant in particular have antioxidant, anti-inflammatory, antiviral and immunostimulatory effects (Lee and Scagel, 2009). Studies have found that echinacea is an extremely powerful antioxidant in terms of rosmarinic acid, cichoric acid and caffeic acid derivatives, which are important antioxidants that suppress the negative effects of free radicals on metabolism (Jahanian et al., 2017). *Echinacea purpurea* powder was added to the diet of 320 (240 female, 80 male) Sudani ducks, 32 weeks old, under summer conditions and the results were investigated in terms of egg performance, serum lipid profile, antioxidant properties and semen quality. When the results were examined, egg number and mass, laying rate, feed consumption and feed conversion rate varied significantly according to the levels of *Echinacea purpurea* powder in the diet. With this study, it was determined that 2.5 g kg^{-1} dietary *Echinacea purpurea* powder supplementation had beneficial effects on productivity and reproductive performance, as well as lipid profile and antioxidant status, and was economically valuable in breeding ducks in summer conditions (Awad et al., 2020). According to a study, it was determined that tinctures of the aerial parts of *Echinacea purpurea* based on 70 % ethanol exhibited antioxidant and antimicrobial activity against *Candida albicans* and *Saccharomyces cerevisiae* (Stanisavljevic et al., 2009). It has been stated that *Echinacea purpurea* tinctures can be used in the treatment of infectious diseases occurring in the oral cavity (Yezerska et al., 2022).

Studies on *Echinacea* species have shown that the roots of the plant are used in the treatment of many diseases such as rheumatism, migraine, plant poisoning, and snakebite (Mat, 2002). In a study conducted on the elemental components of *E. purpurea* grown in Serbia, it was stated that the flowers of the plant are rich in minerals such as Cu, Zn and Ni, and the leaves are rich in minerals such as Mg, Ca, Fe, Li and Sr (Razic, et al., 2003). In the light of all these findings, the importance of examining different plant parts of echinacea is seen. In this study, extracts were prepared from the petals, leaves, central cone, and root parts of the *Echinaceae purpurea* L. plant harvested at different times, and the DPPH radical repellent effect of these extracts and the phenolic and flavonoid compounds were determined.

2. Materials and Methods

In the study, *E. purpurea* L. plants propagated from seeds were used as plant material.

2.1. General characteristics of the region where the trial was conducted

The plant samples used in the research were obtained from the echinacea plantation established in Gedikhasanlı Research and Application Center, which belongs to Yozgat Bozok University Faculty of Agriculture, in June 2012. In preparation for the plantation, seeds for seedling production were sown in viols containing peat on April 24, 2012. The seeds germinated in approximately 2 weeks and the seedlings were planted in the pre-planned trial area after they grew to an average height of 15 cm. Seedlings were planted in the trial area with a distance of 80 cm between rows and 50 cm on rows. The total trial area was prepared as 180 m^2.

The soil where the echinacea plantation is located is loamy in structure, slightly alkaline, salt-free, low in lime, organic matter, phosphorus and zinc, poor in nitrogen, deficient in iron content, sufficient in copper, manganese, and magnesium, and rich in potassium. Soil pH was measured as 7.6. Three different harvest times were determined for the petal, leaf, and central cone of the plant, and these were carried out on 24 July, 6 August, and 19 August 2015, respectively. The root part of the plant was harvested on a separate date, on October 10, 2015. The plant parts, collected separately according to harvest time and dried separately, were chopped into small pieces with the help of a grinder.

2.2. Preparation of extracts

Different parts of the *Echinacea purpurea* (L.) Moench plant such as leaves, flowers, cones, and roots were dried in the shade and ground with the help of a blender. 4 g of each sample obtained was weighed and 40 mL of methanol (1/10 w/v) was added. It was kept in the oven (Elektromag M 5040 P, Türkiye) at 40 ºC for 24 hours. The resulting solutions were centrifuged (Universal320 R) at 9.000 rpm. With the help of solvent evaporator (Buchi, Germany) has been removed. After determining the amounts of the extracts obtained, methanolic extracts were obtained. The extracts were stored at +4 ^{o}C until analyzed.

2.3. Determination of DPPH radical scavenger activity

1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical, a known radical, was used to determine the free radical activities of the extracts. The amount of extract that neutralized a certain amount of DPPH radicals was determined and comparisons were made between samples. To determine the DPPH radical scavenging activity, the concentration was prepared by dissolving 4 mg DPPH in 100 ml of methanol. This study was conducted using extracts dissolved in methanol. 8 mg ml^{-1} extract solution was prepared as the main stock and then 8 different concentrations were obtained by dilution. 3.2 ml of DPPH radical and 200 µl of extract solutions of different concentrations were added to each sample and incubated for 30 minutes at room temperature in the dark. After incubation, absorbance values were measured at 517 nm for each sample. Spectrophotometric measurements to determine DPPH radical scavenging activity were carried out using the PerkinElmer Lambda 25 UV/VIS spectrophotometer device. Following this process, 50 % inhibition values were calculated for each sample. Ascorbic acid was used as the standard antioxidant. For control, methanol equal to the amount of extract solution was added to the test tube. Each experiment was carried out with 3 replications and each replication was carried out twice. The following formula was used to determine the % DPPH radical scavenger (Gezer et al., 2006).

$$\%\text{DPPH radical scavenger} = [\,(A_{control} - A_{extract}) / A_{control}\,] \times 100$$

Antioxidant changes causing 50 % inhibition were obtained by linear regression from the graph drawn with the calculated % inhibition value against different concentrations. The results were expressed as mg mL^{-1}.

2.4. Determination of total phenolic content (folin method)

During this study, the Folin-Ciocalteu Reagent (FCR) method was used to determine the total phenolic content of the extracts (Singleton et al., 1999). For the study, 200 µl of the samples prepared with methanol (2 mg ml^{-1}) was taken and 9 ml of distilled water was added. After adding 200 µl of Folin-Ciocalteu reagent, it was waited for 3 minutes and added to the test tube with the chemical containing 600 µl Na_2CO_3 (20 %), making a total of 10 ml. The samples were incubated at room temperature and in the dark for 2 hours, then absorbance values were measured at 760 nm. Spectrophotometric measurements were made with the help of PerkinElmer Lambda 25 UV/VIS spectrophotometer device and total phenolic content was determined. To create the standard calibration curve, gallic acid dissolved in distilled water was used and 200 µl of methanol was added to each test tube. 0.1 mg ml^{-1} gallic acid was prepared as the main stock and 9 different concentrations were obtained by dilution. Total phenolic substance of all plant extracts was calculated as mg gallic acid equivalent GAE g^{-1} extract according to the gallic acid standard table. Each experiment was performed in 3 replicates.

2.5. Determination of total flavonoid content

Flavonoids protect cells from free radicals as potent antioxidants (Şenkal, 2020). Total

amounts of flavonoid compounds in the extracts were calculated by the method of Arvouet-Grand et al (Arvouet-Grand et al., 1994). 2 mg ml^{-1} was prepared from the extract solution dissolved in methanol as the main stock. During the preparation of the experiment, 100 µl of 10 % aluminum nitrate and 100 µl of 1M potassium acetate were taken and the extract was added to make the final volume of the plant extract 100 µg ml^{-1}. The final concentration of the experiment was completed with 99 % ethanol to 5 ml. The absorbance values at 417 nm were measured for the samples kept in the dark and at room temperature for 40 minutes. For the quercetin standard, 0.5 mg ml^{-1} was prepared as the head stock and 8 separate concentrations were acquired by dilution. Total flavonoid substance content is expressed as mg quercetin equivalent QE g^{-1} extract. Spectrophotometric measurements to determine total flavonoid content were carried out using the PerkinElmer Lambda 25 UV/VIS spectrophotometer device. Each experiment was carried out in three replicates.

2.6. Statistical analyses

The data discussed in the study were evaluated in the TARIST program according to the Random Blocks Trial Design and variance analysis was performed. Differences in variance analysis were checked with the Least Significant Difference (LSD) test (Açıkgöz and Gökçol, 2004).

3. Results

3.1. Extraction efficiency of samples

The extraction efficiencies of the petal, leaf, central cone and root parts of the Echinacea plant at different harvest times were compared. The difference between the samples was found to be statistically insignificant. Extraction yields were calculated based on 4 mg dry matter amount. The amount and percentage of extract obtained from the petal parts of echinacea at the end of the first and second harvest periods were found to be the same, but a decrease was observed in the values obtained in the third harvest period. In the leaf, higher values were observed in the third harvest period compared to the first and second harvest periods. Although the highest value from the central cone was obtained in the third harvest period, the lowest value was observed in the second harvest period. The root part of the plant was harvested once at the end of the vegetation time, and 0.344 mg of extract was obtained from the root harvest.

Table 1. Extraction yields of leaves, petals, central cone and root parts of *Echinacea purpurea* L. (Moench) plant

Samples[1]	Amount of Extract Obtained (g)	Percentage of Extract Obtained (%)
F1	0.452	11.3
F2	0.452	11.3
F3	0.401	10.0
L1	0.290	7.3
L2	0.290	7.2
L3	0.332	8.3
C1	0.524	13.1
C2	0.507	12.7
C3	0.533	13.3
R	0.344	8.6

[1] F: Flower, L: Leaf, C: Cone, R: Root 1,2,3: Harvest Time, Amount of Dry Matter: 4 g

3.2. Total flavonoid, total phenolic and dpph radical scavenging activity

Total flavonoid, total phenolic and DPPH values of plant parts of the Echinacea plant harvested at different times were calculated. Values were found by taking the average of 3 repetitions. The differences between the mean values of DPPH radical scavenging activity and phenolic content of the samples were found to be statistically significant at the 1 % level, and the total phenolic content was found

to be significant at the 5 % level. The highest value in total phenolic substance content was reached with 59.10 mg GAE g^{-1} extract from the flower parts of the first harvest period, while the lowest value was observed with 21.94 mg GAE g^{-1} extract from the flower parts of the third harvest period. The highest value in total flavonoid content was obtained from the leaf parts with 1807.28 mg QE g^{-1} extract in the second harvest period, while the lowest value was recorded as 68.23 mg QE g^{-1} extract in the root harvest period. The highest DPPH content was 8.09 mg ml^{-1} in the root period, and the lowest was 1.15 mg ml^{-1} in the flowers in the second harvest period. It was determined that antioxidant activity decreased as the DPPH value increased. The value of ascorbic acid was measured as 0.07 mg. All values of the findings obtained are presented in Table 2.

Table 2. Total phenolic, total flavonoid and DPPH values of extracts obtained from plant parts of *Echinacea purpurea* L. (Moench) species

Samples	Total phenolic (mg GAE g^{-1} extract)	Total flavonoids (mg QE g^{-1} extract)	DPPH (mg ml^{-1})
F1	59.107^{A1}	305.381^{D}	3.807^{D}
F2	38.358^{B}	168.873^{E}	1.157^{G}
F3	21.947^{F}	104.428^{G}	3.597^{D}
L1	41.250^{B}	1426.333^{G}	2.257^{F}
L2	31.470^{CD}	1807.286^{F}	2.487^{EF}
L3	23.222^{F}	1284.429^{G}	2.630^{E}
C1	26.028^{EF}	112.365^{G}	2.250^{E}
C2	30.875^{CD}	142.523^{F}	7.057^{B}
C3	27.559^{DE}	120.619^{G}	4.097^{C}
R	32.406^{C}	68.238^{H}	8.090^{A}
AA^{2}			0.070^{H}
LSD (0.05)	4.114	19.913	0.276

[1]The difference between means marked with the same letter is statistically insignificant at the 5% level. [2] Ascorbic Acid

According to the analysis of variance, the differences observed between the DPPH radical scavenging activities of leaves, petals, central cone, and root parts obtained from three different harvest times were found to be statistically significant at the 0.01 % level. Flower parts were in the same group at the 5 % importance level in the first and third harvest periods. In addition, the difference between the cone parts in the first harvest period and the leaf parts in the third harvest period was found to be statistically insignificant (Table 2).

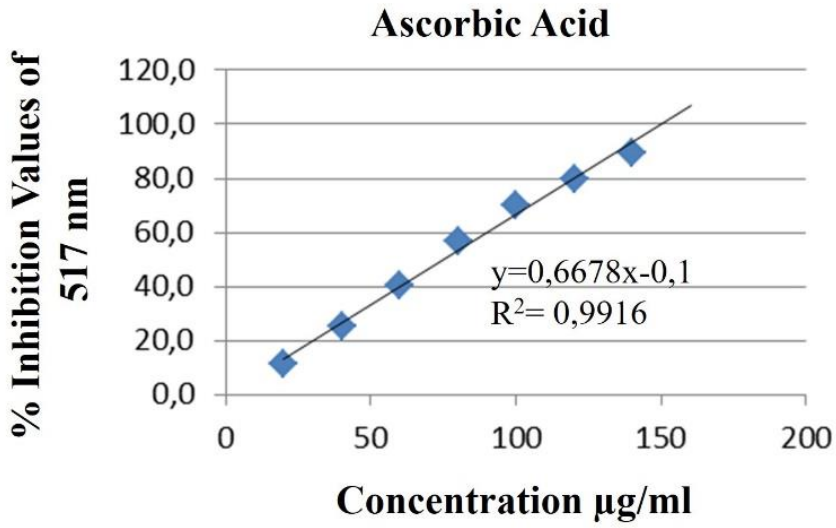

Figure 1. Percentage inhibition of dpph radical by standard ascorbic acid according to concentration

Total phenolic content of plant extracts of *Echinacea purpurea* L. (Moench) species harvested in different periods were calculated as mg GAE g^{-1} extract. According to the analysis of variance, the differences recorded between the total phenolic content values of leaves, petals, cone, and root parts obtained from three different harvest times were found to be statistically significant at the 0.01 % level. The grouping values of total phenolic content values of plant parts obtained from three different harvest periods of the *Echinacea purpurea* L. (Moench) species are shown in Table 2. As a result of statistical analysis, cones, and leaves in the second harvest period were in the same group. In addition, the flowers in the second harvest period and the leaves in the first harvest period were in the same group. As a result of the evaluations, the highest value in terms of total phenolic content was acquired from the flower parts in the first harvest period with 59.10 mg GAE g^{-1}, and the lowest value was obtained from the flower parts in the third harvest period with 21.94 mg GAE g^{-1}. As a result of the statistical analysis, no divergence was observed between the cone and leaf parts of the plant in the second harvest period. The absorbance value R^2 of the gallic acid standard curve of *Echinacea purpurea* L. (Moench) species at 760 nm was calculated as 0.9989. The specified values are shown in Figure 2.

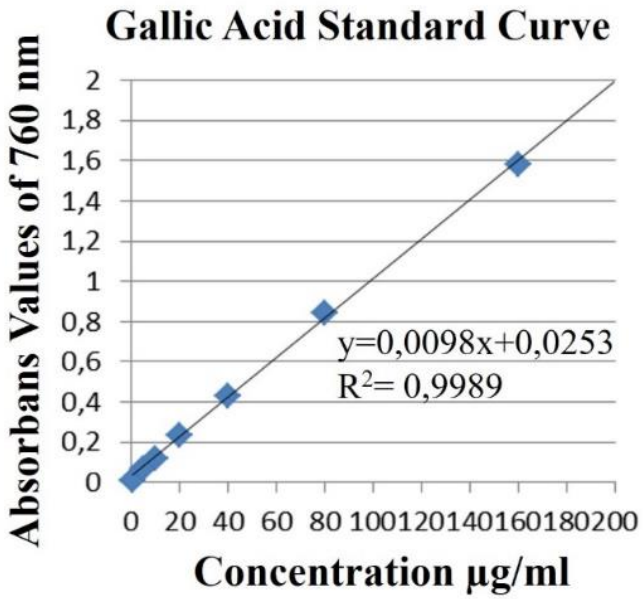

Figure 2. Standard curve for phenolic content determination in *Echinacea purpurea* L. (Moench)

Total flavonoid content values of plant extracts gained from different harvest periods of *Echinacea purpurea* L. (Moench) species were determined as mg QE g^{-1} extract. According to the analysis of variance, the differences recorded between the total flavonoid content values of leaves, petals, cones, and root parts obtained from three different harvest times were found to be statistically significant at the 0.05 % level (Table 2). As a result of the evaluations, the highest value in terms of total flavonoid content was obtained from the second period leaf harvest with 1807.28 mg QE g^{-1}, and the lowest data was obtained from the root harvest with 68.23 mg QE g^{-1}. As a result of statistical analysis, the cone and flower parts of the first harvest period and the cone and flower parts of the third harvest period were in the same group. The absorbance values of the Quercetin Standard Curve of *Echinacea purpurea* L. (Moench) species at 417 nm were found to be R^2 0.9994 and it specified in Figure3. In Figure 4, data expressing the changes in total phenolic, total flavonoid and DPPH values are presented.

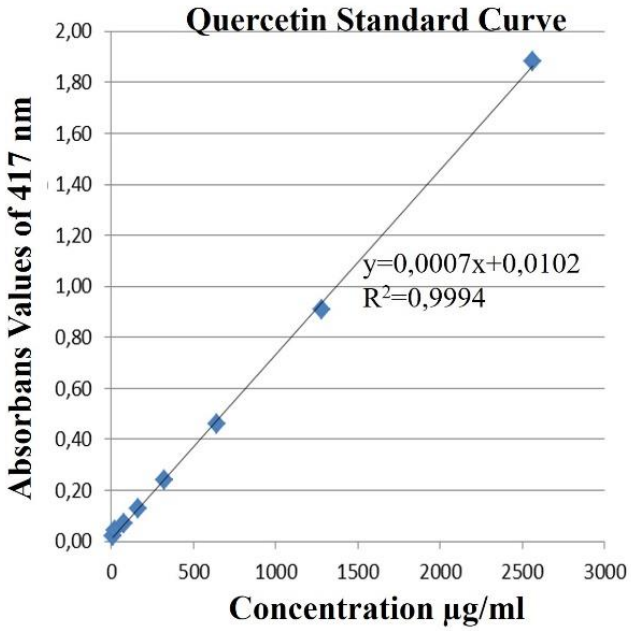

Figure 3. Flavonoid substance content quercetin standard curve graph

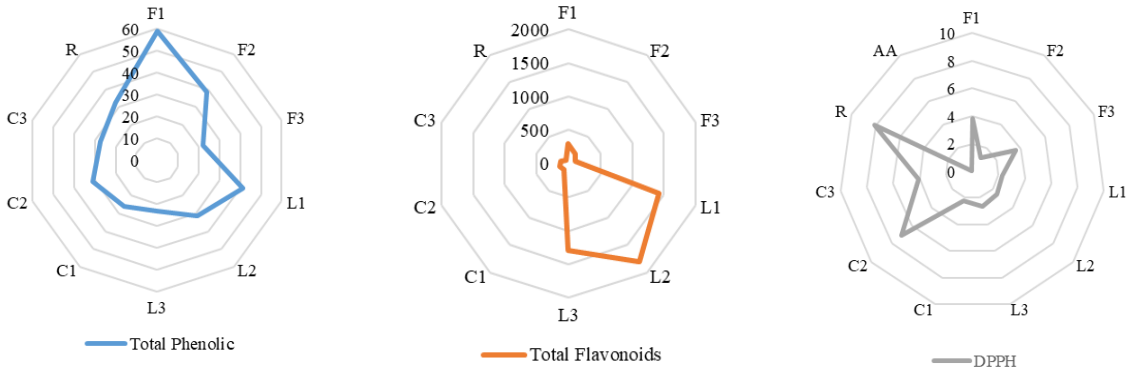

Figure 4. Changes in total phenolic, total flavonoid and DPPH values of the samples

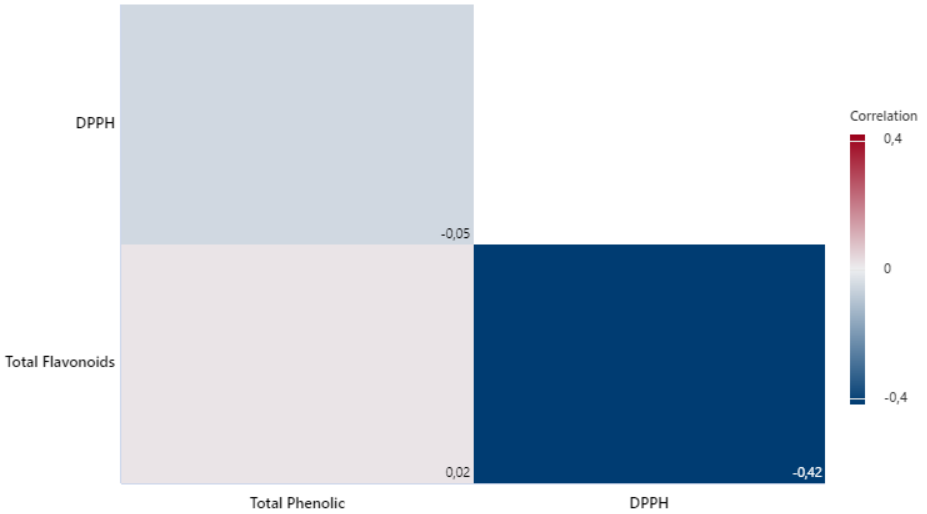

Figure 5. Correlation between total phenolics, total flavonoids and DPPH

Correlation Coefficient: 0.00-0.10 negligible correlation, 0.10-0.39 weak correlation, 0.40-0.69 moderate correlation, 0.70-0.89 strong correlation, 0.90-1.00 very strong correlation (Schober et al., 2018). Phenolic and flavonoid molecules are important antioxidant components responsible for neutralizing free radicals as they can donate hydrogen atoms to free radicals. In addition, it is stated that they have ideal structural properties for free radical scavenging (Amarowicz et al., 2004). Different studies indicate that total phenolic and flavonoid content has a linear correlation with antioxidant capacity (Shrestha and Dhillion, 2006). In our study, a moderate correlation was observed between DPPH and total flavonoids. No significant relationship was determined between total flavonoids and total phenolics and DPPH and it shown in Figure 5.

4. Discussion and Conclusion

Echinacea purpurea plant contains important phenolic compounds. Secondary metabolites such as phenolic compounds are of great importance for the continuity of the medicinal properties of the *Echinacea purpurea* plant (Cao and Kindscher, 2016). One of the important points regarding medicinal plants is to evaluate the effect of environmental conditions on the quality of the plant. In addition, studies on medicinal plants have focused on finding the conditions under which the plant reaches the highest point in terms of secondary metabolites (Daniel, 2016). Flower, leaf, root and stem parts may contain these phenolics (Lee and Scagel, 2009). According to studies conducted on the echinacea plant, it has been stated that the highest phenolic acid content is observed in the leaves and flowers and reaches the highest point (6.6 %) in the full flowering phase. In addition, the researcher found that the chemical compound contents of the echinacea flower may differ depending on the parts of the plant and that the highest caffeic acid concentration is in the flowers and roots (Foster, 1991). Caffeic acid derivatives such as echinocoside, cichoric acid, caftaric acid, cynarin and chlorogenic acid are among the important phenolic compounds for Echinacea purpurea (Cao and Kindscher, 2016). These compounds can have positive effects on the immune system thanks to their antioxidant and antimicrobial properties. Caffeic acid concentrations may vary depending on the organ in which it is found, environmental conditions and development period. In general, the concentration of caffeic acid derivatives is higher in roots than those in leaves and stems in Echinacea purpurea (Billah et al., 2019). Hu and Kitts (2000) reported in their study that methanol extracts of freeze-dried *E. purpurea* roots exhibited antioxidant activity. In this study, the highest phenolic content value of *E. purpurea* was obtained from the petals in the first harvest period. On the other hand, different organs of the plant exhibited different antioxidant capacities at different harvest times. Researchers stated that the total phenolic substance content in ethanol extracts obtained from *E. purpurea* was 11.0±1.0 gallic acid equivalent on dry matter and that DPPH exhibited radical scavenging activity (Lee et al., 2009). In this study mentioned, the total phenolic substance of methanolic extracts obtained from E. purpurea was recorded as 21.9±59.1 gallic acid equivalent on dry matter. This value is higher than reported by researchers. As a result of a study, it was specified that the DPPH radical scavenging activity determination was 85.1 % in 0.5 mg mL^{-1} extract and the IC50 value was 0.23 mg mL^{-1}. As a result of a study published in 2015, the total phenolic compound content of Echinacea purpurea extracts was found to be 10.57 % GAE and the IC50 value was 15.67 µg/mL. It has been stated that *Echinacea purpurea* extracts show strong antioxidant activity (Facino et al., 1995; Jukić et al., 2015). In this study, the IC50 value of the samples was recorded between 1.157-8.090 mg mL^{-1}. In conclusion, Echinacea purpurea L. plant has strong antioxidant capacity. When compared in terms of DPPH properties, the flower parts of the plant showed the highest activity. Considering the DPPH analysis, it was stated that the first harvest period was suitable for the flower and leaf parts and the second harvest period was suitable for the cone. It was

observed that the first harvest period was suitable for flower and leaf parts and the second harvest period was suitable for cones in terms of phenolic compounds. In terms of flavonoid compounds, the highest value was obtained from the leaf parts in the second harvest period. In addition, the organ of the plant with the highest flavonoid content was found on the leaves. Factors such as plant organs and harvest time cause differences in the antioxidant activities of Echinacea purpurea plants. It has been stated by many researchers that the differences in antioxidant activities of Echinacea purpurea species vary depending on factors such as genotype, climate, and growing conditions.

Declarations of Author Contributions

The authors declare that they have contributed equally to the article. All authors declare that they have seen/read and approved the final version of the article ready for publication.

Declaration of Conflicting Interests

The authors declare no conflict of interest.

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