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Araştırma Makalesi (Research Article)

Antioxidant Properties of *Cichorium intybus* L. (Chicory) Extracts and Their Cytotoxic Effects on HepG2 Cells

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Abstract: Chicory (*Cichorium intybus* L.) is a biennial plant belonging to the Asteraceae family. The aim of the study is to reveal the antioxidant capacities and phytochemical profile of the different extracts and to determine the cytotoxic effects of the extracts on liver cancer cell line. *In vitro* antioxidant activity was determined by using DPPH radical scavenging activity assay and total phenolic (TPC) and flavanoid (TFC) contents were measured spectrophotometrically. The cytotoxic effect of the plant on HepG2 cell line was examined by XTT colorimetric assay. The highest extraction yield was obtained from the flower. The highest total phenol content was obtained from the flower methanol extracts and calculated as 186.3±3.281 µg GAE/mg. In both quercetin and catechin standards, total flavonoid contents of the stem and leaf methanol extracts were found to be significantly higher. The IC50 values of DPPH radical scavenging activities of water and methanolic extracts of the flowers were calculated as 7.5±0.247 mg ml^{-1} and 3.593±0.1849 mg ml^{-1}, respectively. The IC50 values of the stem extracts on HepG2 cells were calculated as 0.64 mg ml^{-1} for methanol and 2.44 mg ml^{-1} for water. The IC50 values of the leaf extracts were calculated as 2.58 mg ml^{-1} for water and 0.69 mg ml^{-1} for methanol. As a result, the cytotoxic effects of the methanolic extracts on cell viability were significantly higher than the water extracts of *Chicory intybus* L. It has been demonstrated that, unlike the root of the plant, which is commonly consumed in the public, the stem, leaves and flowers of the plant should be further examined in terms of biological activities.

Cichorium intybus L. (Hindiba) Ekstraktlarının Antioksidan Özellikleri ve HepG2 Hücreleri Üzerindeki Sitotoksik Etkileri

Makale Bilgileri

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Anahtar kelimeler

Chicory intybus L.,
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HepG2.

Öz: Hindiba (*Cichorium intybus* L.) Asteraceae familyasına ait iki yıllık bir bitkidir. Çalışmadaki amaç, *Cichorium intybus* L. bitkisinin farklı ekstrelerinin sahip olduğu antioksidan kapasiteleri ve fitokimyasal profili belirlemek ve ekstrelerin kanserli hüre hattı üzerinde sitotoksik etkilerini ortaya koymaktır. *İn vitro* antioksidan aktivite DPPH radikal süpürme metodu kullanılarak belirlenmiştir. Total fenol ve flavonoid içerik spektrofotometrik olarak ölçülmüştür. Bitki ekstrelerinin hücre hatları üzerindeki sitotoksik etkileri XTT kolorimetrik analiz ile belirlenmiştir. En yüksek ekstraksiyon verimi, çiçekten elde edilmiştir. En yüksek total fenol içeriği çiçek metanol ekstrelerinden elde edilmiştir ve 186.3±3.281 µg GAE/mg olarak hesaplanmıştır. Hem kuersetin hem de kateşin standartlarında, gövde ve yaprak metanol ekstrelerinin toplam flavonoid içeriği önemli derecede yüksek bulunmuştur. Çiçeğin su ve metanol

ekstraktlarının radikal süpürme aktivitelerine ait IC50 değerleri sırasıyla 7.5±0.247 mg/ml ve 3.593±0.1849 mg/ml olarak hesaplanmıştır. HepG2 hücreleri üzerindeki kök ekstraktlarının IC50 değerleri, metanol için 0.64 mg/ml ve su için 2.44 mg/ml olarak hesaplanmıştır. Yaprak ekstrelerinin IC50 değerleri su için 2.58 mg/ml ve metanol için 0.69 mg/ml olarak hesaplanmıştır. Sonuç olarak, metanol ekstrelerinin hücre canlılığı üzerindeki sitotoksik etkileri su ekstraktlarından önemli ölçüde daha yüksek olmuştur. Halkta yaygın olarak tüketilen bitkinin kök kısmının aksine, bitkinin gövde, yaprak ve çiçeklerinin biyolojik aktiviteleri yönünden incelenmesi gerektiği gösterilmiştir.

1. Introduction

As they have been for thousands of years, plants are the basis of traditional medicine and they continue to offer new solutions to humanity. The use of various parts of the plant directly or the active substances derived from them in the treatment of diseases has been defined as the term medicinal plant in the literature. Medicinal plants are of great importance as they are a therapeutic phytochemical source that will allow the development of new drugs (Venugopal and Liu, 2012).

Chicory (*Cichorium intybus* L.) is a two-year plant of the Asteraceae family, with pile root system and relatively flat leaves. The name of the plant comes from Latin and Greek. The name Cichorium is given to the plant because it carries a flower bed and the name of intybus is given due to the hollow stem. Cichorium genus is a genus that can grow in almost any soil. Chicory roots are used in coffee making. The body and leaves of the plant are used for making salads and vegetable dishes. Chicory extracts are used in alcoholic and non-alcoholic beverages. Different parts of Chicory consume in the public as laxative, painkillers and diuretic (Bais and Ravishankar, 2001). Chicory is considered a medicinal plant and different parts of the plant such as root, stem, and flower contain antidiabetic, anticancer, and antiviral chemicals (Street et al., 2013).

Phytochemical analyses show that Chicory contains active substances such as sesquiterpene lactones (lactusin, lactucopyrrin, 8-desoxy lactusin, guaianolid glycosidase), caffeic acid, inulin, flavonoids, alkaloids, volatile compounds, oils, terpenes, coumarins, hydroxycoumarins, proteins vitamins, polynes. Along with all these substances, the plant suggested to have many pharmacological effects such as gastroprotective, cardiovascular, antioxidant, antidiabetic, hypolipidemic, anticancer, hepatoprotective, sedative, wound healing etc. (Al-Snafi, 2016).

Cancer is the uncontrolled or abnormal growth and reproduction of cells as a result of DNA damage to the cells. Cancer can affect different body parts and spread from one organ to another. These conditions are the result of the interaction between a person’s hereditary and other carcinogens. The WHO guess that in 2030, there will be 23.6 million new cancer cases each year, with a major increase in the world's less economically developed regions (Wiseman, 2018).

Evidence from many sources, including epidemiological and experimental, shows nutritional factors (diet, physical activity, and the consequent nutritional state including body composition) can reduced cancer incidence (Wiseman, 2018). Vegetables and fruits are excellent cancer-preventive sources. Chemoprevention by edible phytochemicals is now considered a cost-effective, easily applicable, acceptable, and accessible approach to cancer control. Phytochemicals in plants are safe to use, efficient and applicable for cancer prevention. Tumours progression may be inhibited by phytochemicals from fruits, foods and vegetables (Wattenberg, 1966).

Cancer, with its increasing incidence, is the second most common cause of death worldwide. Many studies on cancer both in vivo and in vitro models are needed to understand the biochemical mechanisms of cancer and to establish appropriate treatment protocols. For this purpose, considering the variety of secondary products it contains as indicated in the literature, *Cichorium intybus* L. plant was used in the current study to obtain various extracts from different organs of *Cichorium intybus* L. plant grown in natural environment, to reveal the antioxidant capacities and phytochemical profile of the extracts and to determine the cytotoxic effects of the extracts on cancer cell line.

2. Materials and Methods

2.1. Plant extraction

Cichorium intybus L. was collected from the Van Yüzüncü Yıl University campus on 04.07.2018. The plant was registered on the VANF Herbarium with the number 164208. The collected plants were washed with tap water then with distilled water, dried in a way that they would not be exposed to direct sunlight between the blotter. The dried root, shoot, leaf, flower parts of the plant were then powdered by grinding machine separately.

Figure 1. *Cichorium intybus* L. methanolic extracts.

For preparation of the methyl alcohol and water extracts, using 30 g of dried samples water and methanol extracts were obtained in 250 ml of distilled water and methanol with the aid of a magnetic stirrer for 1 night at 40^0C. Then, the samples filtered through a coarse filter. The obtained water extract was frozen at 80°C for 1 night and then dried with the Freeze Dryer instrument. Methyl alcohol extract was evaporated with a rotary evaporator. The residue was dissolved with water and the same procedure was done. The dried samples were weighed and the percentage of yield was determined.

Figure 2. Different organs of *Cichorium intybus* L.

2.2. Determination of antioxidant capacity

The antioxidant activity of the extracts was performed based on its ability to bind hydrogen or, in other words, its ability to capture the DPPH radical. DPPH free radical scavenging activity was measured according to the modified version of the Blois method (1958). Quercetin was used as a standard. In short, in 96 well plate, 140 µl of DPPH solution (1.6 mg ml^{-1}) in ethanol was mixed with 10 µl of plant extracts at different concentrations and incubated for 20 min. The decrease in the absorption of the reaction mixture was monitored spectrophotometrically at 517 nm following the incubation time. According to the results, RSA% were calculated versus final concentrations of the extracts (mg ml^{-1}) and IC50 (50% effective concentration) values determined.

2.3. Determination of total phenolic contents (TPC)

The total phenolic content of substances found in Chicory extracts was determined according to the modified form of the method applied by Singleton and Rossi (1965). Gallic acid (10, 50, 100, 150 and 200 µg ml^{-1}) was chosen as standard and the experiment was adapted to microplate. In the study, 20 µl of the extract and the standards were put in microplate wells, 100 µl of Folin reagent (1: 4) was added to them and mixed with pipeting. Then 80 µl of 10% (w/v) sodium carbonate was added on wells and after 30 minutes of incubation at room temperature, absorbance was read against the blank containing 20 µl of ethanol instead of the sample at 750 nm. Using the standard calibration curve created with gallic acid, total phenolic content amounts in each 1 mg extract were calculated as the gallic acid equivalents (GAE).

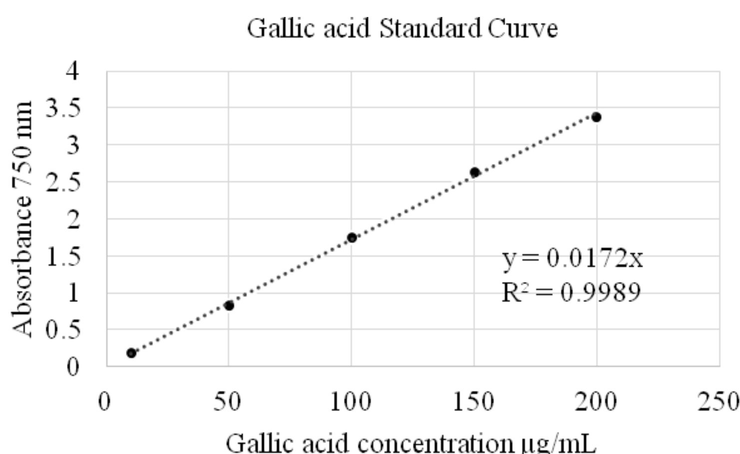

Figure 3. Gallic acid standard curve used in TPC analysis.

2.4. Determination of total flavonoid contents (TFC)

Total flavonoid contents in the extracts were determined using protocols reported by Zhishen et al. (1999) with slight modifications. For determination of TFC, Quercetin and Catechin (+) were used as standards. Different concentrations of Quercetin and Catechin (10, 50, 100, 150 and 200 µg/ml) were dissolved with 99.5% ethanol and used as a standard for TFC determination. The lyophilized extract was dissolved with distilled water different at concentrations. According to the method, 20 µl standard and sample were added to microplate wells containing 80 µl dH_2O, then 6 µl of 5% $NaNO_2$ were added. After 5 minutes, 6 µl of 10% aluminium chloride ($AlCl_3$) was added to the wells and after 6 minutes, 40 µl NaOH (1 M) was added. The total volume was made 200 µl with distilled water. The absorbance was read at 510 and 415 nm using microplate reader. Results were expressed as the quercetin equivalent (QE) and catechin equivalents (CE).

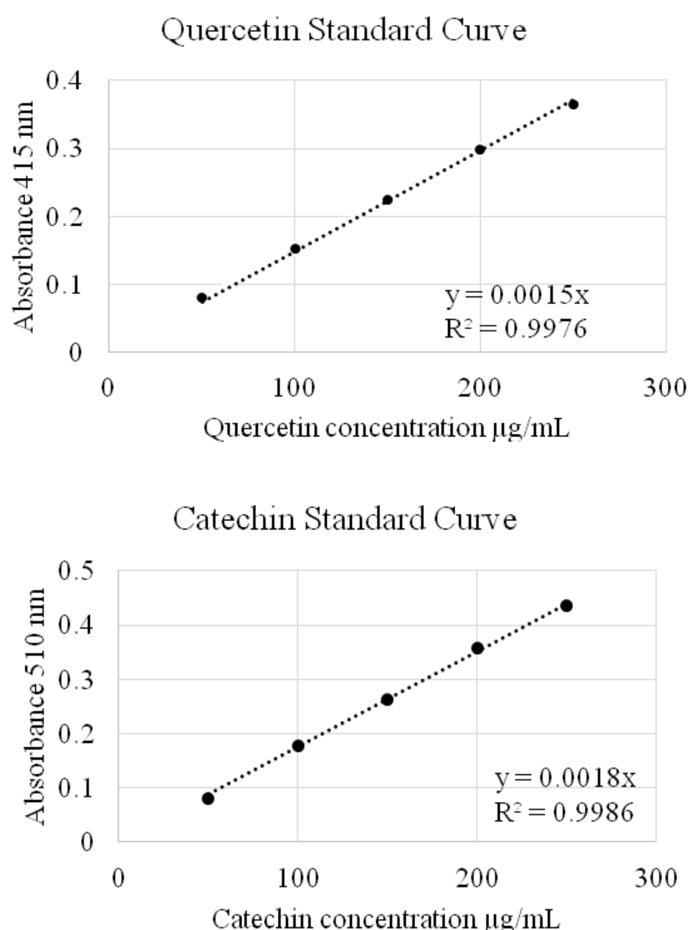

Figure 4. Quercetin and Catechin standard curve used in TFQ/C analysis.

2.5. Cytotoxicity

HepG2 (liver cancer cell line) was purchased from ATCC (American Type Culture Collection, LGC Promochem, UK). The cell line was grown in DMEM medium containing 10% FBS (fetal bovine serum) and 0.1% (100 mg ml^{-1}) Penicillin/Streptomycin (Pen-Strep), 1% L-Glutamine and 1% Na-Pruvate. The medium is changed every 2-3 days. Cells were grown at 37°C, 95% humidity and 5% CO_2.

The cytotoxic effects of Chicory (*Cichorium intybus* L.) extracts on the cell line were examined using the Cell Proliferation XTT Kit. In metabolically active cells, the XTT tetrazolium salt is converted to water-soluble, orange formazone with mitochondrial dehydrogenase activity. It is essential to measure the amount of formazan to be formed in this technique. After 24 hours of incubation in the CO_2 incubator, the wells were washed with 50 PBS and the medium was washed twice.

50 µl of fresh medium was added to the wells. 50 µl growth medium was added again for the first row and 50 µl 0.2% DMSO medium was added as control for the second row. With the serial dilution technique, the extracts were diluted in total medium and applied to the cells over 50 µl. It was applied to XTT chemical cells after 48 hours of standing. Six hours after treatment with XTT, the absorbance levels at 415 nm were read. % cell viability was calculated according to the formula below:

Total Cells/mL= (Total Cell Counted x Dilution Factor x 104) x number of Squares

2.5. Statistical analysis

Statistical analyses were carried out using Graphpad Prism 6. All results are expressed as mean with standard deviations (SD). In the analysis, multiple comparisons were made to create graphics and One-way ANOVA was used. Pairwise comparisons of the plants were analysed using t-test at the 0.05 significance level.

3. Results

3.1 Extraction

Extraction of different organs of *Cichorium intybus* L. was carried out with methanol and water. The highest extraction yield was observed in the flower organ. The flower was more efficient than the other organs in terms of the water and methanol extract's extraction yield. The yield of the root water and methanol extracts was approximately the same (Table 1).

Table 1. Extraction efficiency of different organs of *Cichorium intybus* L.

Extraction		% Yield
	Root	10
Water Extracts	Shoot	7.95
	Leaf	8.2
	Flower	22.06
	Root	9.69
Methanol Extracts	Shoot	6.56
	Leaf	6.64
	Flower	17.53

3.2 Determination of Total Phenolic Content

Gallic acid standard curve was generated for total phenolic analysis. The highest total phenolic content was obtained from the flower methanol extracts. The leaf and stem methanol and water extracts were also found to have high total phenolic content. Both the water and methanol extracts of the root have been shown to contain lower phenolic content than the other organs (Table 2).

Table 2. Total phenolic content (TPC) of *Cichorium Intybus* L. extracts

Different Plant Extracts TPC (µg GAE/mg)				
	Root	Shoot	Leaf	Flower
Water	34.14±0.4754	148.2±0.7953****	143.9±0.559****	123.8±0,8727****
Methanol	96.67±1.711	144.4±0.8804****	168.3±0.7635****	186.3±3.281****

*The results are the mean of the triple measurements obtained from three different experiments (n = 3). Comparisons were made between the root and different organs in the same solvent. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

3.3 Determination of Total Flavonoid Content

Quercetin and catechin were used as the standards for the determination of total flavonoid content. In both quercetin and catechin standards, total flavonoid contents of methanol extracts were higher than water extracts. Total flavonoid contents of flower, stem and leaf methanol extracts were found to be higher than the root, especially in the flowers at the highest rate.

Table 3. Quercetin equivalent total flavonoid content of *Cichorium Intybus* L. extracts

Different Plant Extracts TFQ (µg QE/mg)				
Quercetin	Root	Shoot	Leaf	Flower
Water	211.1±2.222	897.8±34.92****	888.9±2.222****	730±5.092****
Methanol	516.7±1.92	1200±42.56****	1171±2.222****	1248±2.94****

*The results are the mean of the triple measurements obtained from three different experiments (n = 3). Comparisons were made between the root and different organs in the same solvent. * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001

Table 4. Catechin equivalent total flavonoid content of *Cichorium Intybus* L. extracts

Different Plant Extracts TFC (µg CE/mg)				
Catechin	Root	Shoot	Leaf	Flower
Water	100±3.208	654.4±10.6****	578.5±4.271****	388.3±5.357****
Methanol	575.6±9.686	680.7±10.45**	891.1±2.222****	550±14.53^{ns}

*The results are the mean of the triple measurements obtained from three different experiments (n = 3). Comparisons were made between the root and different organs in the same solvent. * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001

3.3 Determination of Antioxidant Activity

Antioxidant capacity of the plant was determined by DPPH method. The highest antioxidant scavenging activity was observed in the methanol extract of the flower. The flower methanol extract showed two times more antioxidant effect than the water extract. The methanol extracts of the stem, leaf and root are also extracts with high antioxidant scavenging capacity. The root water extract was the least antioxidant organ and solvent. In general, methanol extracts showed higher antioxidant effect than water extracts.

Table 5. IC50 values of *Cichorium Intybus* L. extracts

Different Plant Extracts Antioxidant Activity (mg ml^{-1})				
	Root	Shoot	Leaf	Flower
Water	25.62 ± 0.1009	7.267 ± 0.04509****	15.18 ± 0.7059***	7.5 ± 0.247****
Methanol	6.014 ± 0.4352	4.653 ± 0.061*	6.143 ± 0.5345^{ns}	3.593 ± 0.1849**

*The results are the mean of the triple measurements obtained from three different experiments (n = 3). Comparisons were made between the root and different organs in the same solvent. * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001

3.4 Determination of Cytotoxicity

In contrast to the flower organ, leaf and shoot extracts had little difference in phytochemical results in water and methanol extracts that made leaf and shoot preferred for in vitro cytotoxicity studies. Extract concentrations required to kill 50% of cancer cells were determined. The shoot and leaf methanol extracts showed high cytotoxicity in HepG2, hepatocellular carcinoma cell line. The methanol extracts of the mentioned plant parts showed about 4 times more antiproliferative effect than the water extracts. The success of alcoholic extracts with IC50 values below zero was demonstrated in each analysis.

Table 6. Cytotoxic effects of *Cichorium Intybus* L. extracts

Different Plant Extracts Cytotoxic Effects (mg ml^{-1})		
	Shoot	Leaf
Water	2.44	2.58
Methanol	0.64	0.69

4. Discussion and Conclusion

The high phenolic and flavonoid content in medicinal plants is associated with their antioxidant activity, and they are particularly involved in the prevention of age-related diseases caused

by oxidative stress. The world market for plant-based chemicals (medicines, perfumes, flavours and colorants) alone exceeds several billion dollars per year (Singh, 2015). The beneficial phytochemicals of medicinal plants, as well as the demand for natural products in the pharmaceutical and cosmetic industries, make research on medicinal plants as essential and important as research on traditional medicines (Azwanida, 2015).

Extraction is an important process for obtaining valuable secondary products in plants. The process can be affected from solid-liquid ratio, particle size, extraction time and solvent type used in the experiment. In current study, solid/liquid extraction technique was used. The highest extraction yield was obtained from the flower methanol extracts. The lowest yield was obtained from the methanol shoot extracts. Most extraction is carried out in alcohol. Phytochemical profile of alcohol extracts was higher than water extracts in the study. A similar trend has been demonstrated in the phytochemical profile of the plant in previous studies (Jasim, 2018).

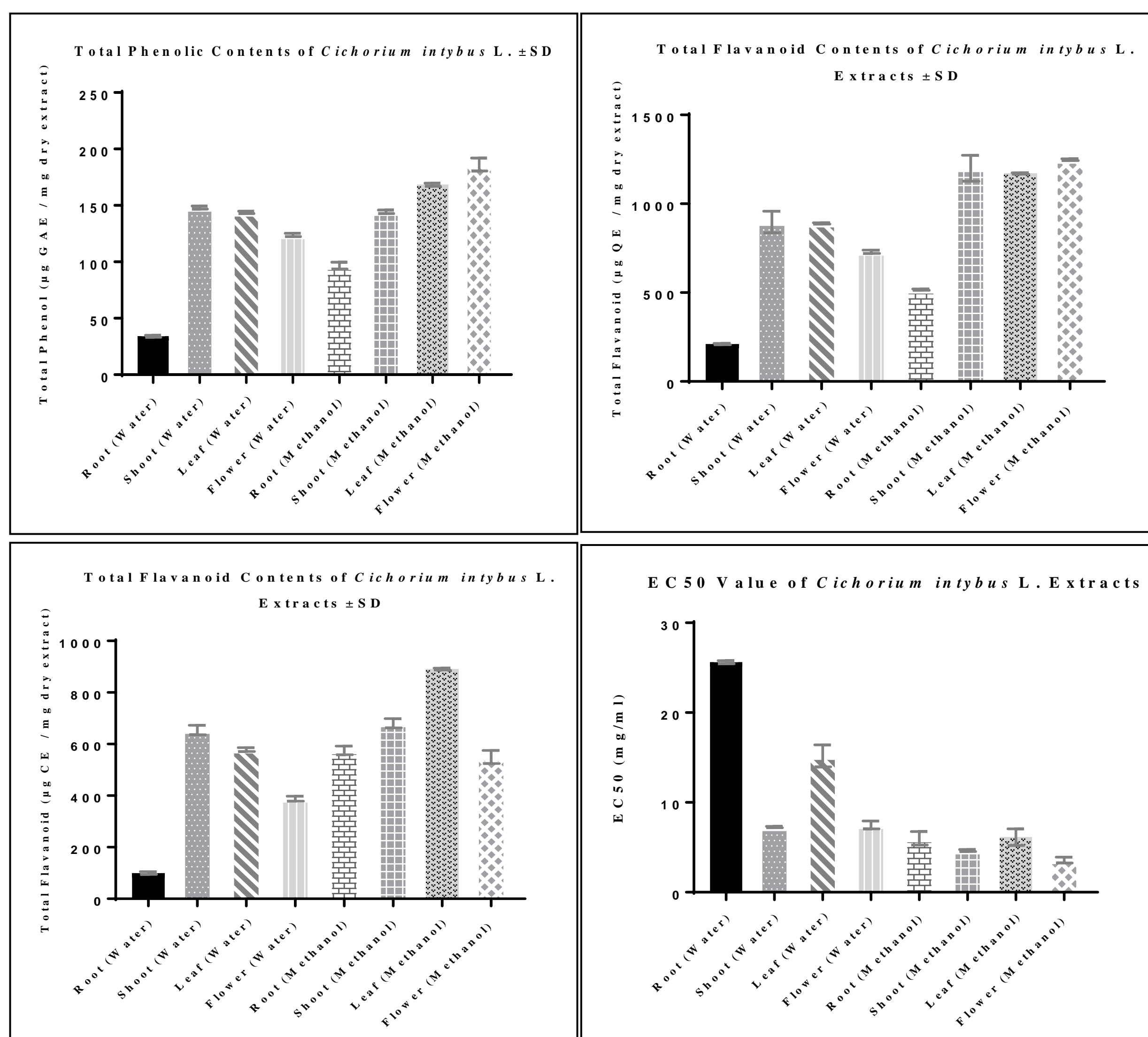

Figure 5. Phytochemical analysis graphs of different organs of *Cichorium intybus* L.

Phenolics are multifunctional because they act as reducing agents, hydrogen atom donors and also singlet oxygen scavengers. In addition, some phenolic compounds have the ability to chelate against transition metals to stimulate free Fenton reactions (Karaman et al., 2010). The antioxidant effects of natural products often arise from the phenolic compounds they contain (Singh, 2015). Therefore, total phenol analysis may also reflect the antioxidant capacity of the substances. In our

study, the plant part with the highest phenolic content was the flower methanol extract. At the same time, the flower methanol extracts had the most antioxidant activity with an IC50 value of 3.593±0.1849 mg ml^{-1}. The results obtained are consistent with the literature. The phenolic content of the stem, leaf and flower were higher in both water and methanol extracts than the root. This is explained by the fact that the secondary metabolites were stored different organs in the plant (Babaoğlu et al., 2001).

Total phenolic, flavonoid, and antioxidant experiments results were inefficient in the root extracts of the plant in the current study. The solvent type and concentration used in the studies effect the extraction process and thus the results. Previous studies also confirmed the insufficiency of the root in terms of phenolic contents and antioxidant effects (Dalar and Konczak (2014). Dalar and Konczak (2014) examined the antioxidant scavenging activity of *Cichorium intybus* L. by FRAP method and showed that the antioxidant activity of the leaves and flower parts were higher than the stem and root.

The water extracts of Chicory plant have been reported to contain tannin, saponin, alkaloid, flavonoid (Nandagopal and Kumari, 2007). Anticancer activities of tannins and saponins have been demonstrated (Yıldırım and Kutlu, 2015) which makes the plant valuable in cancer studies. Cytotoxic effects of water and methanol extracts of leaves and stems were investigated *in vitro* by considering the valuable metabolites of the plant.

Cichorium intybus L. caused differentiation into monocyte-macrophage-like cells in human U-937 and HL-60 leukemia cell lines (Lee et al., 2000). Hexane extracts of the aerial parts of the plant showed cytotoxic effect against Jurkat cells (human leukemia cancer cell line) (Saleem et al., 2014). Ethanolic extracts of the plant have been shown to reduce the viability of human breast cancer cell line (MCF7) by 17% (Gospodinova and Krasteva, 2015). In our study, IC50 values for the stem and leaf methanol extracts on cell viability in HepG2 cell line were determined as 0.64 mg ml^{-1} and 0.69 mg ml^{-1}, respectively, whereas water extracts of shoot and leaf were found to be 2.44 mg ml^{-1} and 2.58 mg ml^{-1}, respectively. The smaller IC50 value, the greater antiproliferative effect of the plant on the cancer cell line. The IC50 values of water extracts was about 4 times higher than the methanol extracts, indicating the efficiency of methanol extracts on the cancer cell line.

These ratios mean that the plant's stem methanol extract has a cytotoxic effect at a concentration of 0.64 mg ml^{-1} and the leaf methanol extract at a concentration of 0.69 mg ml^{-1} in 50% of HepG2 cells, In water extracts, the IC50 value was about 4 times higher. Again, the efficiency of methanol extracts was revealed.

As a result, the different organs of the plant were evaluated phytochemically and it was observed that methanol and water extracts of leaves, stems and flowers contained higher total phenol and flavonoid and showed higher antioxidant activity than the root. Contrary to the flower organ, the difference in phytochemical results of the leaf and stem extracts in water and methanol extracts, made the leaf and stem preferred for in vitro cytotoxicity studies. In this respect, the cytotoxic effects of the leaf and stem extracts were investigated. In our study methanol extracts showed cytotoxic effect on cancer cells at a very low concentration. The results needed in vivo evaluation of the plant in cancer studies. In the present study, which organs would be more beneficial for human health have been demonstrated. It has been concluded that, due to the different chemical contents of the organs selected in the consumption of medicinal plants, it is important to choose the right organ for seeing the expected pharmaceutical effect.

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