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Anticholinergic Evaluation, Antioxidant Effects, and DNA Protection Potential of *Chenopodium spp* Depending on Its Phenolic Content

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Abstract: Plants are important food sources and natural therapeutics, and they are preferred as an alternative instead of synthetic medicines with harmful side effects in the treatment of routine diseases. Their unique effects are mostly attributed to specific herbal metabolites based on soil, climatic, and biogeography. *Chenopodium* species growing on barren and alkaline soils with nitrogen content are important candidates for unique biological effects. Due to their acceptance as food and wild, searching the biological activities and knowing the metabolite content are important. A series of *in vitro* biological activity tests were performed to determine the effects of *Chenopodium spp* (*Cspp*). First, leaf and flower samples were prepared using a Soxhlet device. Antioxidant tests including radical scavenging and heavy metal reduction were performed. Their phenolic contents were determined by LC-MS/MS to better interpreting the antioxidant results. Their inhibitory effects on AChE and BChE were tested and were shown to have quite significant total inhibition effect compared to Galantamine used as standard. Finally, their DNA protective effects were evaluated. In conclusion, it has been understood that phenolic content and the other biological effects are mostly parallel, and the samples have antioxidant effects at acceptable levels depending on dose.

Fenolik İçeriğine Bağlı Olarak *Chenopodium spp*'nin DNA Koruma Potansiyeli, Antioksidan Etkileri ve Antikolinerjik Değerlendirilmesi

Anahtar Kelimeler
Antioksidan, DNA koruma, İnhibisyon, Fenolikler, LC-MS/MS

Öz: Bitkiler önemli besin kaynakları ve doğal terapötiklerdir. Bu nedenle onlar özellikle rutin hastalıkların tedavisinde yan etkilere sahip olan sentetik ilaçların yerine alternatif olarak tercih edilirler. Bitkilerin özgün etkileri Çoğunlukla büyüdükleri biyocoğrafya, toprak türü ve iklim şartlarına bağlı olarak değişebilen bitkisel metabolitlerine atfedilir. Kurak ve nitrojen içeriği bol olan topraklarda büyüyen *Chenopodium* türleri özgün etkiler için önemli adaylardır. Yiyecek veya vahşi bitki olarak kabul edilmelerinden dolayı kullanmadan önce onların biyolojik yönden araştırılması ve bu etkilerin arkasındaki moleküllerin bilinmesi önem arz eder. Bu çalışmada bu türün kendine has biyolojik bazı etkilerini belirlemek amacıyla bir dizi *in vitro* biyolojik aktivite testi gerçekleştirilmiştir. İlk olarak Soxhlet ekstraksiyon aparatı kullanılarak bitkiden yaprak ve çiçek örnekleri hazırlanmıştır. Radikal süpürme ve ağır metal indirgeme testlerini içeren antioksidan testler gerçekleştirilmiş, ardından elde edinen verileri daha anlamlı hale getirmek amacıyla LC-MS/MS ile bitkinin fenolik içeriği belirlenmiştir. Daha sonra örneklerin AChE ve BChE enzimleri üzerindeki inhibitör etkileri test edilmiş ve numunelerin standart olarak kullanılan galantamine kıyasla önemli inhibisyon etkisi göstermiş olduğu anlaşılmıştır. Son olarak DNA koruyucu özellikleri test edilmiştir. Sonuçta, fenolik içerik ile diğer biyolojik etkilerin çoğunlukla paralel olduğu ve örneklerin doza bağlı olarak kabul edilebilir düzeyde antioksidan etkilere sahiptir

1. INTRODUCTION

The plants grown in wild nature have been our primary food for thousands of years and have been used as medicine because of pharmaceutical benefits [1, 2]. Although access to synthetic medicines with specific effects has become easier in today's pharmacology, public awareness about the adverse effects of modern

medicines supports the tendency on herbal products in the treatment of diseases. Due to their low cost, low adverse effects, and known benefits, plants are used by approximately 80% of the world's population in the treatment of diseases and as the primary nutrition source [1, 3, 4]. In parallel with this trend, it is stated that the global herbal products market size will gradually increase in recent years [5]. Most studies state that the beneficial benefits of plants depend on their secondary metabolites with low molecular weight [5]. Alkaloids, glycosides, amines, steroids, flavonoids, carotenoids, xanthophylls, vitamins, tocopherols, phenolic acids, flavonoids, and lignans are known as secondary metabolites protecting the plants against microbes, herbivores, bacteria, insects, and various adverse environmental conditions [4, 6-9]. Also, herbal molecules, including alkaloids, polyphenols, and phytoestrogens, are promising actors for natural nutrition and alternative therapy based on biological effects [10]. Depending on the pharmaceutical effects of these biomolecules, they are essential for investigations that focus on the treatment of many diseases such as cancer and Alzheimer's diseases [11, 12]. For this reason, it is stated that herbal and natural products should be added to the diet regularly, based on the positive relationship between diet and health [13].

The strong relationship between cognitive impairment depending on Alzheimer's and neurotransmitters is well known [10] and Alzheimer's disease (AD) is a cholinergic dysfunction due to impaired neuronal activity and neurotoxicity with amyloid/tau proteinopathy [14]. The cognition and recollection processes are known to greatly improve by stabilizing acetylcholine concentration via AChE enzyme inhibition [14]. Hence, the discovery of acetylcholine regulatory agents is great and promising to hold the progression of AD. Regarding this, there are some inhibitors used pharmaceutically such as tacrine, donepezil, and galantamine that are known to cause some undesirable side effects [15] and plants can be considered as anticholinergic agents. Carcinogenic molecules such as H_2O_2 can cause DNA damage. The damage in DNA has been associated with cardiovascular, many neurodegenerative diseases, and cancer [16, 17]. Plants can influence the fate of DNA through dose-dependent beneficial and lethal effects [18, 19].

The Amaranthaceae family includes 104 genera and more than 1400 species [6]. *Chenopodium* is a member of this family and is also known as white crow's feet. *Chenopodium* encompasses numerous species of perennial or annual herbaceous flowering plants that can be seen in continental climates in many parts of the world [12, 20]. *Chenopodium spp* has a widespread growth network and adaptation all over the world [12]. They are consumed as food in Northern India, Nepal, and Pakistan, as opposed to being considered wild plants in North America and Europe.

The limited data on *Chenopodium spp* (*Cspp*), its ability to survive even under stressful conditions, and the possibility of specific secondary metabolite potential raises the curiosity to investigate their biological activities and unique contents. For this purpose, *Chenopodium spp* (*Cspp*) was collected from Muş-Varto region (latitude and longitude information; 39.1929920 and 41.4457306) in Turkey, *in vitro* antioxidant activity, some herbal antioxidant contents, anticholinergic effects, and DNA protective activity were evaluated in Chenopodium spp. extracts.

2. MATERIAL AND METHOD

2.1. Plant Acquisition and Sample Preparation

Chenopodium spp (*Cspp*) samples were collected during the vegetation period and stored as herbarium material in Muş Alparslan University, Research Laboratory of Technology Research Project Coordination Unit. The flower and leaf tissues of the herbarium material were separated from each other. Leaf (*Cspp*L) and Flower (*Cspp*F) samples were prepared by extraction of tissues in ethanol (EtOH) using a Soxhlet apparatus. The solvents of stock samples were completely evaporated and stored at +4 ^{o}C until the experimental phase. The test samples with different concentrations were prepared by diluting in the same solvent.

2.2. *In Vitro* Antioxidant Studies

2.2.1. DPPH radical scavenging assay

1,1-Diphenyl 2-picrylhydrazyl (DPPH) radical scavenging assay was performed the method of Blois [21]. This method is a measurement based on the relative scavenging of DPPH radicals in alcohol by hydrogen donor molecules such as antioxidants. The absorbance values of samples were measured spectrophotometrically at 517 nm and different concentrations (15, 30, and 45mg/mL). Butylated hydroxyanisole (BHA) and ascorbic acid (ACS) were used as standard antioxidants. DPPH radical scavenging activity ($DPPH_{RSA}$) was calculated using the following equation (Equation 1) and Trolox standard curve.

$$DPPH_{RSA} = \frac{(ABS_{Control} - ABS_{Sample})}{(ABS_{Control})} \quad (1)$$

2.2.2. ABTS radical scavenging assay

ABTS radical scavenging activities were determined following the method of Wu [22]. According to this, the ABTS radical scavenging of a molecule is determined by measuring the colorless ABTS solution after the addition of the antioxidant molecule to the dark blue ABTS radical solution. The samples were prepared at 15, 30, and 45mg/mL concentrations and were incubated for 2 hours. Butylated hydroxyanisole (BHA) and ascorbic acid (ACS) were used as standard antioxidants. The absorbance values were recorded at 734nm. ABTS radical scavenging activity ($ABTS_{RSA}$) was calculated using the following Equation 2 (Equation 2) and the Trolox standard curve.

$$ABTS_{RSA} = \frac{(ABS_{Control} - ABS_{Sample})}{(ABS_{Control})} \quad (2)$$

2.2.3. CUPRAC assay

Cupric ion reducing power (CUPRAC) assay was applied by following the teachings of the method proposed by [23]. This test is based on the spectrophotometric measurement of cuprous ion (Cu^{1+}) with blue color formed by the reduction of cupric ion (Cu^{2+}) after the treatment with reductive molecules such as antioxidants. The absorbance values of different concentrations of the samples were recorded at 450 nm. Butylated hydroxyanisole (BHA) and ascorbic acid (ACS) were used as standard antioxidants. The Cu^{2+} reducing activity was calculated using the following Equation 3 (Equation 3) and the curve of Trolox as a reference. The elements in the equation are as follows; ABS_{sample}; The absorbance value of sample, Ɛ; Molar absorption coefficient of Trolox molecule (16700 L $mol^{-1}.cm^{-1}$), V_T; Total volume, V_S; Sample volume, DF; dilution factor, V_{MS}; main sample volume, and m; The amount of dry material in the main sample.

$$CUPRAC = \frac{ABS_{Sample}}{Ɛ} x \frac{V_T}{V_S} x\ DF\ x \frac{V_{MS}}{m} \quad (3)$$

2.2.4. FRAP assay

The ferric ion (Fe^{3+}) reducing antioxidant power assay (FRAP) was performed using the method of Oyaizu [24]. FRAP assay is based on reducing capacity of an antioxidant to reduce a ferric salt (Fe^{3+}) to ferrous salt (Fe^{2+}) with blue color by electron transfer reaction. The absorbance values of the samples at 15, 30, and 45mg/mL concentrations were recorded at 700 nm. Butylated hydroxyanisole (BHA) and ascorbic acid (ACS) were used as standard antioxidants. FRAP was calculated by accepting the Trolox as a reference and using following Equation 4 (Equation 4). The elements in the equation are as follows; c; sample concentration obtained from Trolox standard curve equation, V; Sample volume, DF; dilution factor, and m; Dry material weight [25].

$$FRAP = c\ x\ Vx \frac{t}{m} \quad (4)$$

2.2.5. Iron chelating assay

The iron chelating activity was performed according to the basic teachings of the method proposed by Decker, Welch [26]. The essence of this method is a measure of the binding affinity between a metal ion such as Fe^{2+} and a reagent. Butylated hydroxyanisole (BHA) and ascorbic acid (ACS) were used as standard antioxidants. The absorbances of the samples at 15, 30, and 45mg/mL concentrations were recorded at 562nm and converted to concentrations with the help of trolox standard curve, and Fe^{2+} chelating activity was calculated by following Equation 5 (Equation 5).

$$Fe^{2+}\text{chelating activity} = \frac{(ABS_{Control} - ABS_{Sample})}{(ABS_{Control})} \quad (5)$$

2.3. Chromatographic Assay

Quantitative phenolic compounds were determined chromatographically in LC-MS/MS device (Agilent 6460 Triple Quadrupole LC-MS/MS (Liquid Chromatography-Tandem Mass/Mass Spectrometer, Agilent Technologies) equipped with a Zorbax SB-C18 (4.6x100mm; 3.5 Micron) column). The analyzes were performed in Atatürk University, Eastern Anatolia Advanced Technologies Research and Application Center (DAYTAM) laboratories. The analysis mode is a multiple reaction monitoring mode (MRM). The mobile phase was filtered with a 0.45 µm Millipore membrane filter before loading to LC-MS/MS. Samples were then maintained at a concentration level of 5 mg/mL. The identification of the samples was done by comparison with the standard samples. İnjection volume was studied as 5µl and working time was determined as 20 min. Solvent A contains 0.1% (v/v) formic acid in water and solvent B contains 0.1% (v/v) formic acid in acetonitrile.

Table 1. Order of samples loaded into electrophoresis wells and the component amounts of the samples.

Well No	DNA(µL)	H_2O_2(µL)	DMSO(µL)	*Cspp* extract (10µL)	Water (µL)
1	10	-	-	-	15
2	10	5	-	-	10
3	10	5	10	-	-
4	10	-	10	-	5
5	10		-	*Cspp*L (0.25 mg/ml)	5
6	10	5	-	*Cspp*L (0.25 mg/ml)	-
7	10		-	*Cspp*L (0.5 mg/ml)	5
8	10	5	-	*Cspp*L (0.5 mg/ml)	-
9	10		-	*Cspp*L (1 mg/ml)	5
10	10	5	-	*Cspp*L (1 mg/ml)	-
11	10		-	*Cspp*F (0.25 mg/ml)	5
12	10	5	-	*Cspp*F (0.25 mg/ml)	-
13	10		-	*Cspp*F (0.5 mg/ml)	5
14	10	5	-	*Cspp*F (0.5 mg/ml)	-
15	10		-	*Cspp*F (1 mg/ml)	5
16	10	5	-	*Cspp*F (1 mg/ml)	-

2.4. DNA Protection Assay

DNA protection assay was performed according to the method proposed by Siddall [27]. pUC18 plasmid DNA was used as mode DNA in the DNA protection assay and the protective effects of the samples were tested on. In this scope, stock samples were prepared as 200mg/ml by dissolving the dry extraction products in ethanol, and by diluting the stock samples, fresh samples were prepared at 0.25mg/mL, 0.5mg/mL, and 1mg/mL concentrations. Electrophoresis samples were incubated at 37 ºC and dark for 24 hours before loading. 15 µl from each sample were loaded into agarose gel electrophoresis and run at 40 Volts for 2 hours. The electrophoresis product was visualized using the BIORAD ChemiDoc XRS imaging system. The component amounts of the electrophoresis samples are shown in Table 1.

2.5. Cholinesterase İnhibition Assay

Acetylcholinesterase (AChE) isolated from Electrophorus electricus (Electric eel) and butyrylcholinesterase (BChE) isolated from equine serum were purchased from Sigma- Aldrich (St. Louise, MO). Acetylthiocholine iodide (AChI) and butyrylcholine iodide (BChI) were used as a substrate, and 5′dithiobis - 2 - nitrobenzoic acid (DTNB) were used as Ellman's reagent in the measurement of inhibition. The inhibitory effects of the samples on AChE and BChE were evaluated by following the teachings of the spectrophotometric method proposed by [28]. Absorbance values showing the inhibition effects were recorded at 412 nm at 1 min intervals for 5 min. Inhibition effect of each sample were recorded in 3 replications and calculations were made using the averages of these results. %Activity graphs were plotted to determine the inhibitory effects of samples and IC_{50} values showing inhibition effects of the samples were calculated.

2.6. Statistical Analyses

The tests for each study were performed in at least 3 replicates. All statistical data were represented as Mean ± Standard Deviation (SD), and $p < 0.05$ was considered significant. In the antioxidant studies, sample results were compared statistically with standard results using One-way ANOVA followed by Dunnett's Multiple Comparisons Test, and the statistical significance levels were represented as follows; P>0.05 (not significant, ns); *P<0.05 (significant); **P<0.01 (very significant); ***P<0.001 and ****P<0.0001 (extremely significant). Inhibition values according to the increase in concentration were normalized in the range of 0-100, and $LogIC_{50}$, IC_{50}, and R^2 values were calculated. In addition to this, the band intensities in the results of the electrophoresis study were analyzed using the ImageJ 2x software for windows. The band density values of the samples were statistically compared with the control values of the same study, and the stabilization effects of the samples on DNA were calculated as a percentage. Finally, compatibility between quantitative phenolic results and biological activity results was analyzed using Spearman's correlation test. The p values and r values showing the direction of the correlation were calculated.

3. RESULTS

3.1. *In vitro* antioxidant results

3.1.1. Radical scavenging activities

The scavenging effect of *Cspp* samples removing the half effects of DPPH and ABTS radicals were calculated as IC_{50} values, and the results were evaluated statistically (Figure 1 A and D). Trolox Equivalent Antioxidant Capacity (TEAC) values of DPPH and ABTS were calculated (Figure 1 B and E). In addition, heavy metal reduction and radical scavenging capacities were expressed as µM TE/g (Figure 1 C and F). IC_{50} values of *Cspp* samples were detected to be higher than standards and TEAC results were determined as lower than standards.

3.1.2. Heavy metal reduction activities

Reduction activities of Fe^{3+} and Cu^{2+} ions were determined using FRAP and CUPRAC methods, respectively. The results were shown as IC_{50}, TEAC, and µM TE/g (Figure 2). In the FRAP results, the similarity of *Cspp*F IC_{50} values to BHA results was noteworthy. However, the IC_{50} value of *Cspp*L was found to be quite high compared to the standards (Figure 2A). Similarly, unlike *Cspp*L, it was observed that *Cspp*F TEAC result was close to the BHA result (Figure 2B). The CUPRAC results clearly show that the samples have a higher IC_{50} and lower TEAC results compared to the standards (Figure 2D and 2E). Better heavy metal reduction activities depend on sample concentrations were observed however, the results at the same concentrations were weaker than standards (Figure 2C and 2F). The iron-chelating activities of *Cspp* samples were also determined. When compared with the results of standards, the IC_{50} values of iron chelation as lower and the TEAC values as higher were calculated (Figure 2G and 2H). However, interestingly, the iron chelating capacities of the samples were detected to be quite close to the standards (Figure 2I).

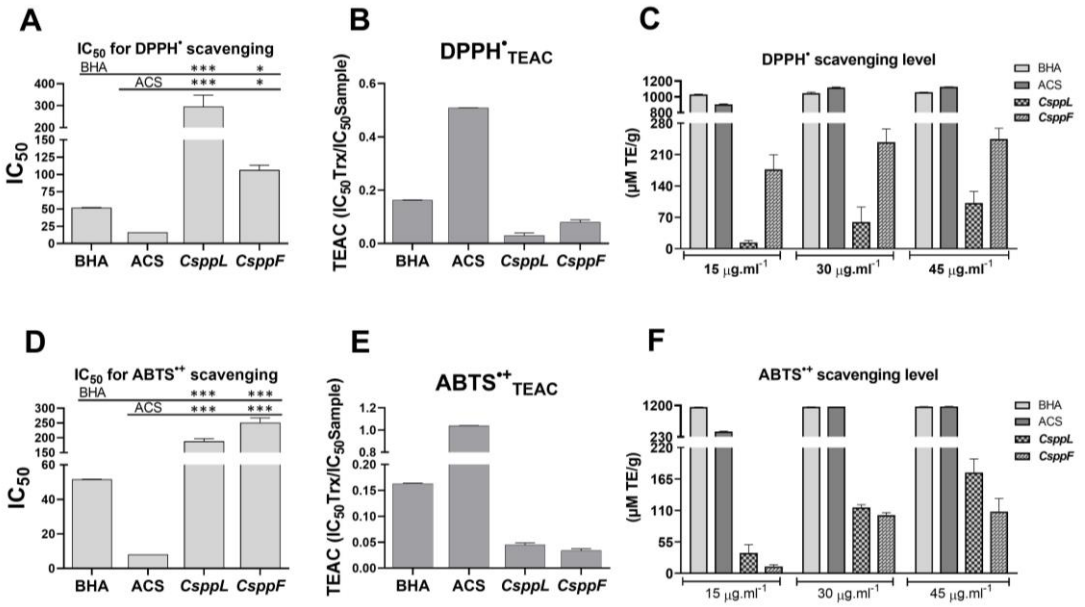

Figure 1. Radical scavenging results of the samples **A)** The IC_{50} values based on DPPH radical scavenging, **B)** The TEAC results of DPPH radical scavenging using Trolox reference ($DPPH^{\bullet}_{TEAC}$), **C)** DPPH radical scavenging levels based on sample concentration, **D)** The IC_{50} values based on ABTS radical scavenging, **E)** The TEAC results of ABTS radical scavenging using Trolox reference ($ABTS^{\bullet+}_{TEACH}$), **F)** ABTS radical scavenging levels based on sample concentration.

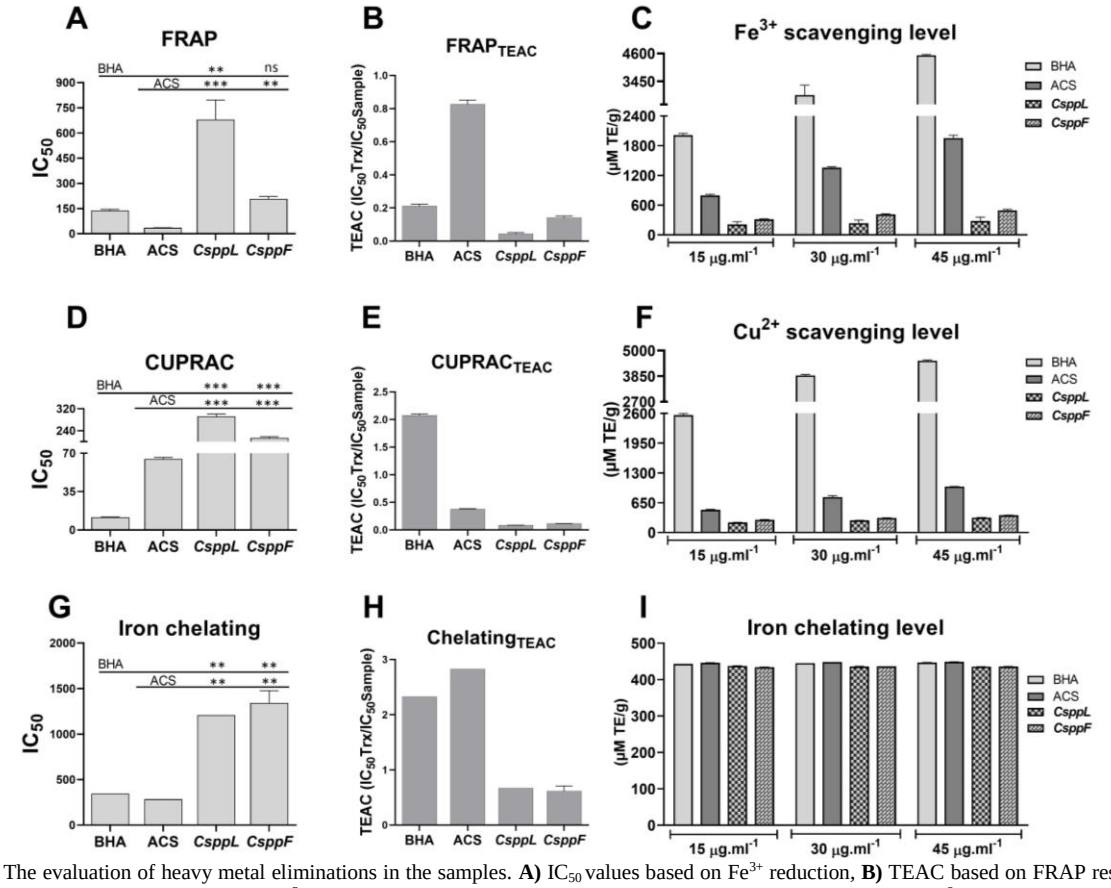

Figure 2. The evaluation of heavy metal eliminations in the samples. **A)** IC_{50} values based on Fe^{3+} reduction, **B)** TEAC based on FRAP results using Trolox reference ($FRAP_{TEAC}$ results), **C)** Fe^{3+} reduction levels based on sample concentration, **D)** IC_{50} values based Cu^{2+} reduction, **E)** TEAC based on CUPRAC results using Trolox reference ($CUPRAC_{TEAC}$ results), **F)** Cu^{2+} reduction levels based on sample concentrations, **G)** IC_{50} values based on Fe^{2+} chelating capacity, **H)** TEAC based on Fe^{2+} chelating capacity using Trolox reference ($Chelating_{TEAC}$ results), and **I)** Fe^{2+} chelating levels based on sample concentration.

3.2. Chromatographic Analysis by LC-MS/MS

To interpret the antioxidant activities more accurately, the phenolic contents of the samples were determined by LC-MS/MS. The phenolic content of *Cspp*F sample was found to be higher than *Cspp*L sample (11.7199µg/mL and 21.3057µg/mL, respectively). Resveratrol as the lowest level (0.0004µg/mL and 0.0002µg/mL, respectively) and Rosmarinic acid as the highest level (7.2489µg/mL and 11.6250µg/mL, respectively) were detected in both samples and the concentration of some phenolics could not be determined (Table 2).

Table 2. LC-MS/MS results showing the phenolic concentrations of *Cspp*L and *Cspp*F as µg/mL

Phenolics	***Cspp*L**	***Cspp*F**	**Phenolics**	***Cspp*L**	***Cspp*F**
4-OH-Benzoic Acid	0.1714	0.3793	Keracyanin Chloride	0.0015	0.0029
Apigenin	0.0000	0.0000	Luteolin	0.0000	0.0000
Caffeic Acid	0.0600	0.1801	Myricetin	0.0000	0.0000
Catechin	0.0000	0.0000	Naringenin	0.0000	0.0000
Chlorogenic Acid	0.0000	0.0460	Naringin	0.0000	0.3507
Chrysin	0.0000	0.0000	p-Coumaric Acid	0.0872	0.1813
Curcumin	0.0000	0.0000	Peonidin-3-o-glucoside	0.0048	0.0036
Cyanidin-3-o-glucoside	0.0352	0.0357	Pyrogallol	0.0000	0.0000
Ellagic Acid	0.0000	0.0340	Quercetin	0.1075	0.3373
Epicatechin	0.2366	0.5592	Quinic Acid	0.0906	0.4604
Epigallocatechin Gallate	0.0000	0.0000	Resveratrol	**0.0004**	**0.0002**
Ferulic Acid	0.8641	1.8680	Rosmarinic Acid	**7.2489**	**11.625**
Fumaric Acid	1.2148	2.4987	Sinapic Acid	0.0000	0.0000
Galangin	0.0000	0.0000	Syringic Acid	0.0000	0.0071
Gallic Acid	0.0000	0.0000	Taxifolin	0.0000	0.0000
Hesperidin	1.5940	2.7309	Vanillic Acid	0.0015	0.0000
Isorhamnetin	0.0000	0.0000	Vanillin	0.0014	0.0056
Total Content in *Cspp*L	**11.7199**		**Total Content in *Cspp*F**	**21.3057**	

3.3. Anticholinergic Activity

The inhibition effects of the samples were tested on AChE and BChE enzymes and galantamine, known as the commercial inhibitor, was used as the standard. It was understood from the results that *Cspp*F was detected to have more effective inhibition compared to *Cspp*L (Figure 3). The samples showed a significantly higher inhibitory effect on AChE compared to galantamine (Figure 3 A, A_1, and A_2). However, its inhibitory effect on BChE was found to be quite close to galantamine (Figure 3 B, B_1, and B_2).

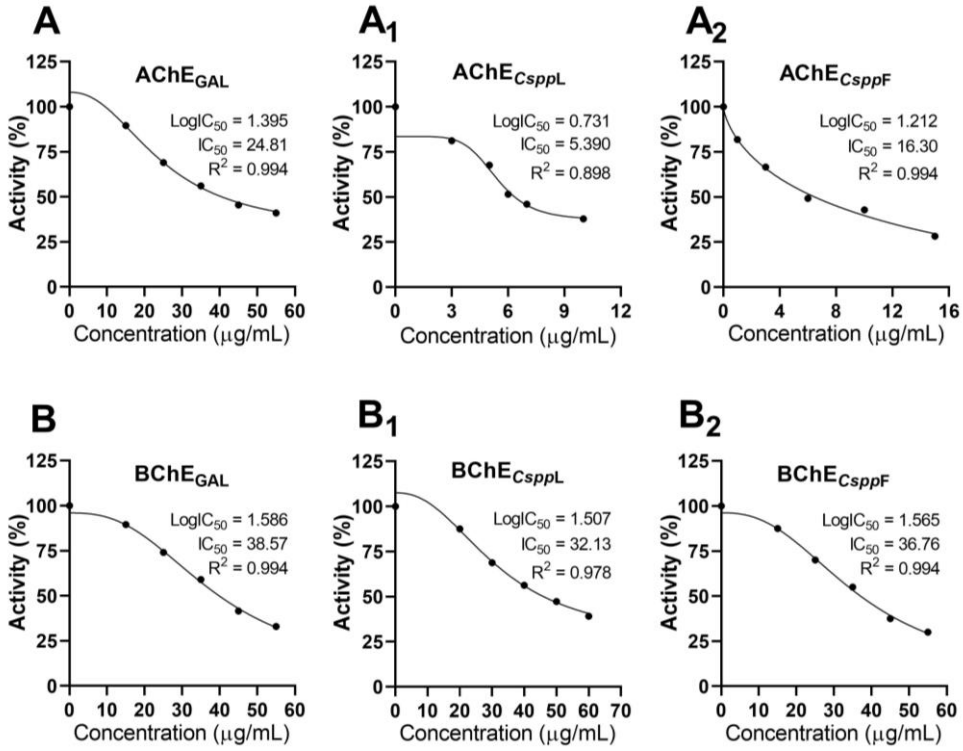

Figure 3. Inhibition effects of standard and samples on AChE and BChE enzymes. **A)** The inhibition effect of Galantamine on AChE, **A_1**) The inhibition effects of *Cspp*L on AChE, **A_2**) The inhibition effects of *Cspp*F on AChE, **B)** The inhibition effect of Galantamine on BChE, **B_1**) The inhibition effects of *Cspp*L on BChE, **B_2**) The inhibition effects of *Cspp*F on BChE. Galantamine (GAL) is a well-known inhibitor of AChE and BChE enzymes and was used as a standard inhibitor.

3.4. DNA Protection Activity

The nucleic acid protective effects of *Cspp* samples were tested on pUC18 plasmid DNA. Following the treatments given in Table 1, the samples were incubated at 36 ºC for 24 hours. The samples were then loaded into gel electrophoresis and the fate of plasmid DNA was visualized (Figure 4). Following 24 hours of incubation, the DNA forms disappeared in wells treated with H_2O_2 alone and H_2O_2+DMSO (Figure 4 well 2 and well 3, respectively), and plasmid DNA was not affected by DMSO alone (Figure 4, well 4). Accordingly, plasmid DNA forms were not affected by the different concentrations of *Cspp* samples. Interestingly, form II was preserved by the 0.25mg/mL *Cspp*L (Figure 4, well 6). However, this effect could not be displayed in other wells. In conclusion, *Cspp* could not protect the plasmid DNA against the scavenging effect of H_2O_2, and no significant effect on the stabilization of form I and form II was also observed when applied alone (Figure 4).

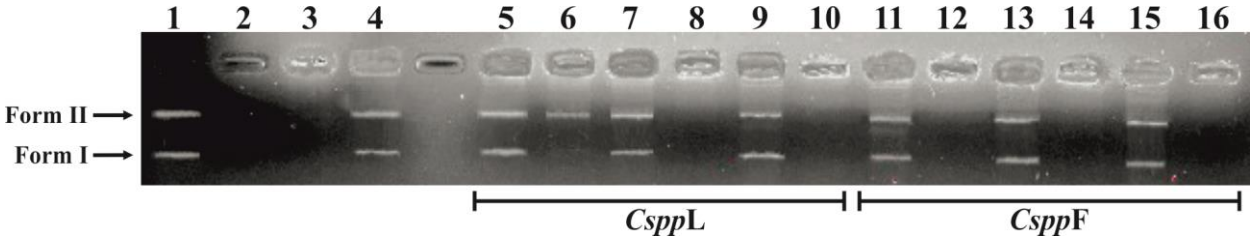

Figure 4. Electrophoresis image showing the effect of samples on pUC18 DNA in the environment with H_2O_2 and H_2O_2 free. The effect of *Cspp*L was visualized in wells 5-10 and the effect of *Cspp*F was visualized in well 11-16. H_2O_2 free samples were loaded into wells 5, 7, 9, 11, 13 and 15 and the samples with H_2O_2 were loaded into wells 6, 8, 10, 12, 14, and 16.

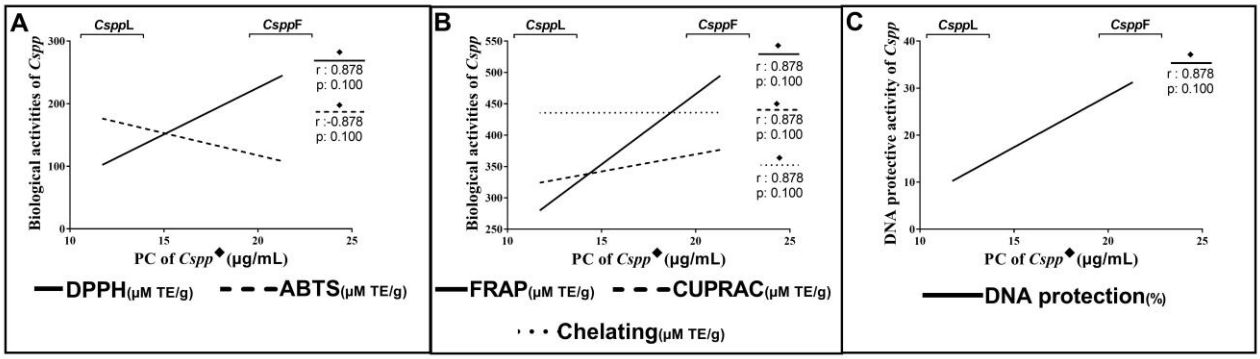

Figure 5. Correlation of biological activities depending on phenolic content in leaf (*Cssp*L) and Flower (*Cspp*F) samples. **A)** Correlation between phenolic content and radical scavenging activities in *Cspp* samples, **B)** Radical scavenging activities depending on the phenolic content of the samples, **C)** Heavy metal reduction activities depending on the phenolic content of the samples. (♦); was used as the symbol for phenolic content (PC). The x-axis represents the change in phenolic content, and the y-axis represents the change in biological activities. r and p values were shown on correlation graphs. The biological changes in each of the 3 graphs was shown with different line graphs. The closeness of r to 1 indicates the same directional correlation and the closeness of r to -1 indicates the opposite directional correlation.

3.5. Correlation of Phenolics and Biological Activities

The comparative interpretation of phenolic content and biological activities is essential to better understand the heavy metal and radical elimination based on phenolics. The results of phenolics and biological activities were compared by nonparametric Spearman's correlation test. The p and r values were calculated and displayed by a line graph (Figure 5). Accordingly, it was generally observed that the biological activities of flower samples were higher than leaf samples. These results are consistent with phenolic contents. However, the opposite situation was detected in ABTS radical scavenging activity. The band intensities were evaluated using the ImageJ 2.0 software program for Windows and these values were expressed as % protective activity.

4. DISCUSSION AND CONCLUSION

ROS can be produced by heavy metal accumulation such as iron and copper. The high oxidation power of ROS affects cell physiology negatively [14, 29, 30]. It is well known that excessive ROS has a lethal potential for cancerization and some neurodegenerative disease formation [14, 17, 31]. Plants are known as good regulators to balance the ROS and the heavy metal, and the biogeography of the plants is the architect of their unique biological character. Feeble antioxidant activities were demonstrated compared to the standards (Figure 1 and Figure 2), and the phenolic results from LC-MS/MS supported the antioxidant activity results. Previous studies document that herbal samples show lower antioxidant activity than commercial antioxidants [32, 33]. A lower effect is a situation expected compared to the commercial pure antioxidants because of the other molecules in plants limiting the expected effects. Even if their low effects, plants are highly preferred due to the lack of side effects and holistic beneficial effects across a wide range. A study emphasized that plants have low radical scavenging activities but are still considered good radical scavengers [34]. In this case, radical scavenging and heavy metal reduction activities are at acceptable levels. The higher IC_{50} values and the lower TEAC values were calculated in the iron chelating activity results. Accordingly, it is possible that the samples may be able to chelate Fe^{2+} effectively depending on the incubation time (Figure 2I). Zulfqar [35] stated that some *Chenopodium* species accumulate heavy metals in their leaves by absorbing them from the soil and they reduce some antioxidant molecules such as carotenoids. The results of iron chelation tests support this situation. That is, the *Chenopodium* species may have absorbed the iron from the soil, and this may be the reason for the low activities observed in antioxidant studies. Low heavy metal reduction was observed in FRAP and CUPRAC assays. This may be a result of possible chelation in plant metabolism using its own secondary metabolites. Furthermore, chromatographic results support this data. Phenolics known as secondary metabolites have a significant role in herbal antioxidant capacity [36] and they offer many beneficial effects such as strengthening the cellular defense against cancer and Alzheimer's [4, 10, 14]. Plant biogeography has an impact on their unique biological characteristics [37]. Therefore, the determination of herbal biological activities is important for the development process of alternative therapy.

It is known that some Chenopodium species can absorb nickel, chromium, and cadmium from soil and accumulate in their leaves. Heavy metal accumulation may be a reason for the low phenolic amount in plant content. The chelation results are consistent with this idea. Previous studies emphasizing the high and protein in Chenopodium content suggests their nutritional value. However, nutrition and biological effects are different properties.

Plants may be natural candidates for preventing AD formation by neurotransmitter regulation and the inhibition of AChE and BChE enzymes. Based on this, the inhibition potential of the samples on AChE and BChE was tested to reveal their importance in Alzheimer's treatment, and the samples were shown to have a higher total inhibition effect on AChE and BChE compared to galantamine. Accordingly, the samples may be natural and strong inhibitor candidates, however, the background of the inhibition may be based on possible heavy metal accumulation in the plant body.

Antioxidant activity and chromatography results revealed the necessity of investigating the DNA protective effects of the samples. DNA protective results largely supported the antioxidant activity and phenolic contents. Interestingly, form II was preserved by *Cspp*L at 0.25mg concentration (Figure 4 well 6). Except for this, DNA forms were degraded by H_2O_2 in all wells. Comparative interpretation of biological activity results obtained from the different tissues is essential to better understand the relationship between phenolic content and biological activities. Apart from the chelating activity, a general compatibility was observed between the phenolic results and the biological activities. The chelation effect of the samples is highly similar to the standards (Figure 2). This strengthens the possibility of using phenolics for chelating purposes. The inverse relationship between chelation activity and phenolic content could be evidence of this.

In summary, when thinking about the purity of the standards, it is clear that the samples showed quite sufficient heavy metal reduction and radical scavenging activity. Iron chelation results support the possibility of heavy metal accumulation in the leaves. That is, rather than radical scavenging and heavy metal reduction, this result may be assumed as evidence indicating the possibility of effective chelation in leaves. That is, the low phenolic content may be associated with the use of some metabolites such as carotenoids in the heavy metal chelation and the high iron chelation results may also be proof of this. Based on the study results, the samples can be assumed as natural AChE and BChE inhibitors. The study data contains valuable data supporting the improvements in the treatment of AD with the regulation of neurotransmitters. Plants can influence the fate of DNA against carcinogenic molecules. low antioxidant activity and quantitative phenolic content greatly support the DNA protective activity results. Despite the increase in sample concentration, decreases in DNA protection activity were noticed. This reverse effect in DNA protective activity may be related to the predominance of toxicity based on metal accumulation and thus the masking of antioxidant effects.

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