A Comparative Study of Different Solvents on the Toxic and Antioxidant Properties of *Digitalis ferruginea* L. subsp. *ferruginea*

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**Abstract**

*Digitalis* plants have different biological actions, including antioxidant and antimicrobial activities. In addition, some members of this genus are used in traditional Turkish medicine as diuretics and tonics. The purpose of the present study is to compare the antioxidant, toxic activity and phenolic profile of two extracts of *D. ferruginea* L. subsp. *ferruginea*. The radical scavenging capacities in the hydromethanolic extract were higher than the hydroethanolic extract. The same extract was effective in total antioxidant (β-carotene, 83.75% and phosphomolybdenum 111.5 µg/mg) and metal chelating activities. The hydromethanolic extract (75.91 mgGAEs/g) exhibited higher phenolic content than hydroethanolic extract (70 mgGAEs/g). There were no statistical differences between the flavonoid and tannin contents of the extracts. The HPLC results determined major phenolics: 2,5 dihydroxybenzoic, vanillic and caffeic acid. In addition, this plant is also rich in polyphenolic content as well as toxic activity. The present data could provide significant information regarding its potential use in the pharmaceutical industry.

**Keywords:** *Digitalis ferruginea* L. subsp. *ferruginea*; HPLC; Toxicity; Antioxidant capacity.
**Digitalis ferruginea** L. subsp. *ferruginea*’nin Toksik ve Antioksidan Özellikleri Üzerine Farklı Çözücülerin Karşılaştırmalı Bir Çalışması

Öz

*Digitalis* bitkilerinin antioksidan ve antimikrobiyel aktiviteler dahil olmak üzere farklı biyolojik etkileri vardır. Ayrıca, bu cinsin bazı üyeleri geleneksel Türk tıbbında diüretik ve tonik olarak kullanılmaktadır. Bu çalışmanın amacı, *D. ferruginea* L. subsp. *ferruginea*’nin iki ekstresinin antioksidan, toksik aktivite ve fenolik profilini karşılaştırmaktır. Hidrometanolik ekstrakt’deki radikal süpürme kapasiteleri hidroetanolik ekstrakt’ından daha yüksekti. Aynı ekstrakt, toplam antioksidan (β-karoten, %83.75 ve fosfomolibdenum 111.5 µg/mg) ve metal şelatlama aktivitelerinde de etkili olmuştur. Hidrometanolik ekstrakt (75.91 mgGAEs /g), hidroetanolik ekstrakt’tdan (70 mgGAEs/ g) daha yüksek fenolik içerik sergilemiştir. Ekstraktların flavonoid ve tanen içeriği arasında istatistiksel fark bulunmamıştır. HPLC ile önemli fenolikler saptanmıştır: 2,5 dihidroksibenzoik, vanilik ve kafeik asit. Ek olarak, bu bitki aynı zamanda polifenolik içerik ve toksik aktivite bakımından da zengindir. Mevcut veriler, bu bitkinin ilaç endüstrisindeki potansiyel kullanımı hakkında önemli bilgiler sağlayabilir.

**Anahtar Kelimeler:** Digitalis ferruginea L. subsp. ferruginea; HPLC; Toksisite; Antioksidan kapasite.

1. **Introduction**

Generated in the body or by external factors, free radicals are highly reactive chemical species that can cause damage to cells, organelles, DNA, and other biomolecules. This action can result in diseases, including cancer, as well as cardiovascular and neurodegenerative conditions [1-3]. Remedies for ailments such as these are often prohibitively expensive and their cost could result in an unsurmountable financial burden for some patients. The discovery and development of effective therapies, and their being readily available and reasonably priced on the market, is therefore crucial for the general population. Moreover, natural antioxidants are a prerequisite for the prevention and/or cure of diseases caused by free radicals. Plants are a resource that contain a diversity of therapeutic molecules, and the development of these properties can result in novel remedies. Consequently, the study of such plants is highly beneficial in the development of novel therapeutic agents [1, 2, 4]. Many researchers are therefore searching for natural antioxidants that are potent but safe, in particular those sourced from medicinal plants. There are various plants among the Plantaginaceae family that have been shown to have different pharmacological actions, including antioxidant, antimicrobial, antidiabetic, anti-inflammatory and toxicological activities [5-8]. Moreover, some members of *Digitalis* genus (Plantaginaceae family) are used in traditional...
Turkish medicine as diuretics and tonics [5]. In this study, the antioxidant properties, toxic effects and total bioactive compounds of the hydroethanolic and hydromethanolic extracts of *D. ferruginea* L. subsp. *ferruginea* were evaluated and the phenolic compound of the hydromethanolic extract was determined.

2. Materials and Methods

2.1. Plant material and preparation of the extracts

The plant material was collected from Denizli, Turkey, in August 2018 and it was identified and stored with voucher specimens (*D. ferruginea* L. subsp. *ferruginea*; Herbarium No: 2018-5-2) at the private herbarium of Dr. M. Çiçek. To obtain the hydroethanolic and hydromethanolic extracts, 30 g of dried plant material (aerial parts) was added to 300 mL of a solution containing ethanol: water (70:30, w:w) and methanol: water (70:30, w:w) respectively and shaking at 50°C for 6 h in a temperature controlled shaker. The combined extracts was evaporated using a rotary evaporator under vacum at 40-50°C. The samples were lyophilized and kept at -20°C. The details of preparation of the extracts followed those given by Kaska et al. [9].

2.2. Total bioactive compounds and quantification of phenolic compounds by HPLC

With reference to Kaska et al. [10], the total phenolic, flavonoid and tannin content (by the standard Folin-Ciocalteu method), flavonoids (by AlCl$_3$ method) and tannin (by the vanillin–HCl method) were determined. The results were expressed as mg of Gallic acid (mg GAE/g sample), Quercetin (mg QEs/g sample) and Catechin (mg CEs/g sample) equivalents for phenolic, flavonoid and tannin content respectively.

The phenolic profile of the hydromethanolic extract of *D. ferruginea* L. subsp. *ferruginea* was determined using previously described method Caponio et al. [11] using reversed-phase high performance liquid chromatography. The details of this method were given in Kaska and Mammadov [12].

2.3. Antioxidant activity

The radical scavenging (DPPH (2,2'-Diphenyl-1-picrylhydrazyl radical) and ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)), total antioxidant (phosphomolybdenum and β-carotene/linoleic acid) and metal chelating activities were evaluated by the method described by Kaska et al. [10] and EDTA (Ethylendiaminetetraacetic acid), BHT (Butylated hydroxytoluene) and TROLOX (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) were used as standards for these methods. The ferric ion reducing antioxidant power procedure followed are those given by
Kaska and Mammadov [12] and results were expressed as mg of ascorbic acid equivalents (AA) per milliliter of extract.

2.4. Brine shrimp lethality assay

The brine shrimp lethality bioassay was used to investigate the toxicity of *D. ferruginea* L. subsp. *ferruginea* [13]. The details of determining the toxicity were given in Kaska and Mammadov [12]. The EPA Probit Analysis Program was used for data analysis.

2.5. Statistical analysis

The experimental results were analyzed using the MINITAB Statistical Package Program and the results expressed as mean ± SE (Standard Error). To see how the groups differed from each other, the variations between the different extracts were tested with Analysis of Variance (ANOVA) and Tukey (*p* < 0.05), and the different groups were shown with different letters in the same column. If there were only two groups then a t-test was used.

3. Results and Discussion

3.1. Total phenolic, flavonoid, tannin content and phenolic composition

The content of the bioactive compound in the different *D. ferruginea* L. subsp. *ferruginea* extracts are existed in Table 1.

| Sample            | Total phenolic content (mg GAEs/g) | Total flavonoid content (mg QEs/g) | Total tannin content (mg CEs/g) |
|-------------------|------------------------------------|-----------------------------------|----------------------------------|
| Hydroethanolic    | 70 ± 1.2 b                         | 37.94 ± 2 a                       | 35.43 ± 1.7 a                    |
| Hydromethanolic   | 75.91 ± 1.9 a                      | 34.19 ± 0.26 a                    | 34.55 ± 2.4 a                    |

The statistical differences were given as different letters (*p* < 0.05).

The procedure was based on the Folin-Ciocalteu reagent. This is frequently used for ascertaining and quantifying total phenols, as it assesses the capacity of phenols to react with oxidizing agents [14]. In the present study total phenolic content was determined using the Folin–Ciocalteu method [15]. There were statistical differences (*t* = 2.71, df = 11, *p* < 0.05) in the total phenolic contents of the hydroethanolic and hydromethanolic extracts of *D. ferruginea* L. subsp. *ferruginea*. 

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In this study, an AlCl$_3$ colorimetric method was employed to explore the factors influencing the determination of total flavonoids in *D. ferruginea* L. subsp. *ferruginea*. The AlCl$_3$ colorimetric assay is a simple, feasible, reproducible and stable method [16]. There were no statistical differences in the total flavonoid (t = 1.86, df = 7, p > 0.05) and the tannin contents (t = 0.30, df = 12, p > 0.05) of the hydroethanolic and hydromethanolic extracts.

This study determined that total phenolic, flavonoid and tannin content of the extracts varied in accordance with the solvent. Similar to these results, the studies of Kaska et al. [10] found that the total phenolic, flavonoid and tannin content differed according to the solvents used on *Nepeta cadmea*.

The results for phenolic compositions of the hydromethanolic extract of *D. ferruginea* L. subsp. *ferruginea* by HPLC analysis are presented in Table 2.

### Table 2: Phenolic components in the hydromethanolic extract of *D. ferruginea* L. subsp. *ferruginea*

| No | Phenolic component            | Approximate Rt (min) | µg/g* |
|----|-------------------------------|----------------------|-------|
| 1  | Gallic acid                   | 6.8                  | 3.55  |
| 2  | 2,5 dihydroxybenzoic acid     | 17.2                 | 20690.02 |
| 3  | Chlorogenic acid              | 18.2                 | 1222.44 |
| 4  | 3,4 dihydroxybenzoic acid     | 10.7                 | 26.23  |
| 5  | 4-hydroxybenzoic acid         | 15.7                 | 54.39  |
| 6  | Cinnamic acid                 | 71.1                 | 208.10 |
| 7  | Quercetin                     | 70.4                 | 138.49 |
| 8  | *p*-coumaric acid             | 26.1                 | 195.37 |
| 9  | Ferulic acid                  | 30.1                 | 296.63 |
| 10 | Caffeic acid                  | 22.7                 | 2761.59 |
| 11 | Vanilic acid                  | 19.2                 | 5728.93 |
| 12 | Epicatechin                   | 21.3                 | 1074.11 |
| 13 | Rutin                         | 45.6                 | 34.14  |

* based on dry weights

Some of the phenolics determined in this study, such as caffeic, chlorogenic, ferulic and *p*-coumaric acid were obtained from Plantaginaceae plants used in previous studies [7, 8, 17].

Phenolic compounds comprise a broad range of chemical substances, with varied chemical structures and disparate biological activities, involving as many as 8000 diverse compounds [18, 19]. Phenolic compounds can function as hydrogen donors or to chelate metal ions (viz. iron and
copper) by impeding the oxidation of low-density lipoproteins (LDL). These features of phenolic compounds are related to a reduction in the risk of contracting neurodegenerative diseases, for example cardiovascular diseases [20] and gastrointestinal cancers [21]. In plant foods phenolic compounds are pervasive and hence significant amounts are ingested on a daily basis. Furthermore, the antioxidant activities of phenolic compounds are nowadays becoming increasingly well-known, and researches on the use of natural substances or food ingredients including phenolic antioxidants are continuing to be of great interest to the food industry [22]. For this reason, the plants’ phenolic compounds analyses are very important in the understanding of these plant’s medicinal value.

3.2. Antioxidant capacity

The antioxidant capacity by phosphomolybdenum assay can be assessed by the reduction of molybdenum to a green molybdenum complex by the antioxidant compounds present in the plant extracts [23].

The antioxidant capacity with a phosphomolybdenum assay of extracts are given in Table 3. These findings indicate that hydroethanolic and hydromethanolic extracts from *D. ferruginea* L. subsp. *ferruginea* possess antioxidant capacities. The antioxidant activities were found to be statistically different between the hydroethanolic and hydromethanolic extracts \( t = 11.81, \text{df} = 9, \ p < 0.001 \). As previously reported by Kaska and Mammadov [12] and Nickavar and Esbati [24], these findings also showed that the high antioxidant capacities of the hydromethanolic extract is due to the presence of the high phenolic content.

| Sample            | DPPH (IC\(_{50}\), µg/mL) | ABTS (IC\(_{50}\), µg/mL) | Phosphomolybdenum (µg/mg) | Power reducing (mg/mL) |
|-------------------|---------------------------|---------------------------|---------------------------|------------------------|
| hydroethanolic    | 292.47 ± 9.10 a           | 308.43 ± 16.5 a           | 60.94 ± 1.7 b             | 0.38 ± 0.02 b          |
| hydromethanolic   | 197.54 ± 23.9 b           | 222.80 ± 18.4 b           | 111.5 ± 3.9 a             | 0.31 ± 0.03 b          |
| TROLOX            | 20.43 ± 1.12 c            | 26.44 ± 1.08 c            | nt                       | nt                     |
| BHT               | 31.76 ± 1.72 c            | 27.10 ± 0.36 c            | nt                       | 1.15 ± 0.02 a          |
| EDTA              | nt                        | nt                        | nt                       | nt                     |

TROLOX, BHT and EDTA: standard antioxidants.
The statistical differences were given as different letters \( p < 0.05 \). nt: not tested

In this study, according to the results of the inhibition of linoleic acid oxidation for hydroethanolic and hydromethanolic extracts, similar to that of the phosphomolybdenum method,
the hydromethanolic extract from *D. ferruginea* L. subsp. *ferruginea* (85.57±0.62%) showed stronger antioxidant capacities than those in the hydroethanolic extract (72.30±2.20%) (Fig. 1).

In addition, the hydromethanolic extract’s activity was found to be significantly different from that of the BHT (93.71±0.23%) and TROLOX (94.81±1.15%) antioxidant activities ($F_{3,28} = 65.42\ p < 0.001$). These findings demonstrate that hydroethanolic and hydromethanolic extracts were found to effectively inhibit linoleic acid oxidation, showing that they have strong antioxidant properties.

![Figure 1](image_url)

**Figure 1**: The $\beta$-carotene/linoleic acid activity (%) of *D. ferruginea* L. subsp. *ferruginea*

When the hydromethanolic and hydroethanolic extract of *D. ferruginea* L. subsp. *ferruginea* were compared with the methanol extract from *D. ferruginea* L. subsp. *ferruginea* (78.59%) [7], the $\beta$-carotene/linoleic acid activity of the hydroethanolic extract was lower, while the hydromethanolic extract’s antioxidant activity was higher than those of the methanol extract of this species. It is known that the antioxidant efficacy of the resulting extracts is strongly affected by the type of solvent used and is a polarity of this solvent [25]. This is because the type of solvents and polarity may modify the single electron transfer and the hydrogen atom transfer, both of which are crucial to the measurement of antioxidant capacity [26]. So it is very important to investigate the plants' antioxidant activities using a of variety solvents.

DPPH and ABTS radical-scavenging assays offer a redox-functioned proton ion for unstable free radicals and perform a vital role in the human body for the stabilization of detrimental free radicals [27].
Moreover, ABTS and DPPH assays are commonly used for evaluating the volume of antioxidants in natural products; both are spectrophotometric techniques, which are based on the quenching of stable colored radicals (ABTS or DPPH). They reveal the radical scavenging ability of antioxidants, even when found in complex biological amalgams, such as plant or food extracts [28]. It is known that in the ABTS and DPPH assay, when antioxidant activity transpires, the capacity to eradicate hydroxyl radicals or superoxide radicals through physiologic action or oxidation is calculated with a high index indicating a powerful antioxidant capacity [27].

DPPH is one of the most stable free radicals and is frequently used in the evaluation of radical scavengers in natural foods. The DPPH assay method is very straightforward and fast for the manual analysis of antioxidant contents [29].

In the current study, the DPPH radical scavenging activity of different extracts of *D. ferruginea* L. subsp. *ferruginea* is expressed in terms of IC$_{50}$ (µg/mL) values (Table 3). A reduction in the IC$_{50}$ value signifies a higher level of antioxidant activity. The DPPH free radical scavenging activity of the hydroethanolic and hydromethanolic extracts were significantly different from each other and IC$_{50}$ values of these extracts were found to be different from the BHT and TROLOX IC$_{50}$ values ($F_{3,28} = 106.24$ p $< 0.001$).

Hydrogen donating ability is related to the effect of antioxidants on DPPH [30]. The results obtained in this study suggested that extracts from the *D. ferruginea* L. subsp. *ferruginea* contained a large amount of radical scavenging compounds with hydrogen donating abilities.

The ABTS radical-scavenging measurement method, frequently used when calculating antioxidant activity, exploits the fact that ABTS free radicals become stable by taking a hydrogen ion from the antioxidant, thereby relinquishing their blue colors [27].

In the ABTS radical scavenging assay, the IC$_{50}$ values for hydroethanolic and hydromethanolic extracts are shown in Table 3.

The hydroethanolic and hydromethanolic extracts investigated in present study demonstrated a scavenging capacity. These samples were significantly different from each other and the IC$_{50}$ values of the all extracts were found to be statistically different from the BHT and TROLOX IC$_{50}$ values ($F_{3,28} = 132.55$ p $< 0.001$).

Free radicals cause a loss of fluidity in the membranes lipids, a denaturating of the proteins and cell lysis. In addition, they produce mutagenesis and carcinogenesis, by modifying the bases in nucleic acids Antioxidants protect the body from free radicals, [31, 32]. Natural antioxidants in the plants are of a greater benefit when compared with synthetic ones. This is because, due to
their natural origin, the use of natural antioxidants from plants does not induce side effects [33, 34]. As a result, the investigation of biological activity in plants as a potential source of natural antioxidants are very important. The hydroethanolic and hydromethanolic extracts of *D. ferruginea* L. subsp. *ferruginea* tested in this study showed a radical scavenging capacity.

Metal ion chelating capacity is noteworthy, as it decreases the concentration of transition metal, which catalyzes lipids through oxidation [35]. Consistent with the results, the plant extracts are not as beneficial as the standard EDTA; nevertheless, the reduction of the concentration – dependent color formation in the presence of the extracts – reveals iron chelating activity. Figure 2 shows the comparison of hydroethanolic (29.79 ± 0.7 %) and hydromethanolic (39.06 ± 1.27%) extracts from *D. ferruginea* L. subsp. *ferruginea* with EDTA (88.27 ± 1.18%) for metal ion chelating activity. The Metal ion chelating activities were found to be statistically different between the hydroethanolic, hydromethanolic extracts and EDTA (*F*₂,₂₁ = 835.07 *p* < 0.001).

![Figure 2: The Metal chelating activity of *D. ferruginea* L. subsp. *ferruginea*](image)

The ferric ion reducing method is based on the reduction of ferric (Fe³⁺) to ferrous (Fe²⁺), in the presence of antioxidants. Substances exhibiting a reduction potential respond to potassium ferricyanide, forming potassium ferrocyanide that also reacts with FeCl₃ to create a deep Prussian blue complex that has maximum absorbance at 700 nm. The quantity of the complex created is directly relative to the reducing power of the test sample [36].

In this study, hydroethanolic and hydromethanolic extracts from *D. ferruginea* L. subsp. *ferruginea* were investigated for reducing capacity and there were no statistically differences among the reducing activities of hydroethanolic and hydromethanolic extracts (*F*₂,₂₁ = 468.98,
The reducing power is a very important feature for the estimation of the antioxidant activity [37]. This antioxidant activity is attributed to the presence of natural antioxidants such as phenolic compounds in *D. ferruginea* L. subsp. *ferruginea*.

### 3.3. Brine shrimp lethality assay

The Brine Shrimp Lethality Assay is a valuable method for an assessment of the toxic potential of various plant extracts. This assay is also easily mastered, is of little cost, and utilizes only a small amount of the test material. In addition, applied with *Artemia salina* nauplii. The Brine Shrimp Lethality test turned out to be significantly correlated with several other animal models. The preliminary toxicity data obtained by conducting this assay gives LC$_{50}$ values for further toxicity studies [38-40].

The lethality of the extracts of *D. ferruginea* L. subsp. *ferruginea* to brine shrimp was determined on *A. salina* after 24 hours of exposure to the test solutions, by following the procedure of Meyer et al. [13]. The hydroethanolic and hydromethanolic extracts showed potential toxic activity, having an LC$_{50}$ value of 281.497 and 107.437 µg/ml respectively. The result obtained from the brine shrimp lethality bioassay of *D. ferruginea* L. subsp. *ferruginea* can be used as a guide for the isolation of toxic components from the hydroethanolic and hydromethanolic extracts of this plant.

### 4. Conclusions

Testing the antioxidant properties of natural products has attracted growing interest in recent years. This is largely due to antioxidants being able to neutralize harmful free radicals. According to the findings, hydroethanolic and hydromethanolic extracts of *D. ferruginea* L. subsp. *ferruginea* were found to be a valuable antioxidant in various *in vitro* methods. In addition, this plant exhibited toxic effects and has various polyphenolic compounds that possess beneficial properties. The findings shown here will supply new data for further investigations of this species. The results of this study could provide additional information for the potential use of this plant for the pharmaceutical industry. However, further research would be required before such uses could be proposed with confidence.

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