



Research Article

# Cytotoxicity, Antioxidant Activity and Phenolic Content of Eight Fern Species from North of Iran

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## ABSTRACT

**Background:** Although ferns possess great potential because of some interesting medicinal properties, the phytochemical potential of ferns is relatively unexplored. Due to the lack of scientific evidence to support the traditional use of fern species in Iran, the present work focuses on evaluating the phenolic content, cytotoxicity and antioxidant properties of these plants. **Methods:** Toxicity of extracts was assessed by the brine shrimp test. Screening of antioxidant capacity of the rhizome and aerial parts of eight fern species was carried out using DPPH and ABTS radical scavenging assays. The total phenol content (TPC) of the methanol extracts were determined using the Folin-Ciocalteu method. **Results:** Compared to podophyllotoxin (with LC<sub>50</sub> of 30 µg/ml), *Athyrium filix-femina* and *Pteris cretica* demonstrated a significant cytotoxic activity (with LC<sub>50</sub> of 6.1 and 15.5 µg/ml, respectively). The methanol extract of *Polystichum aculeatum* was found to have significant antioxidant properties with IC<sub>50</sub> value of 0.45 ± 0.02 µg/ml. *Athyrium filix-femina* exhibited the strongest ABTS radical scavenging activity (29.85 ± 1.39 µmol Trolox/g plant). **Conclusion:** Rhizome parts generally had higher DPPH scavenging capacity and showed better ABTS scavenging activity than aerial parts. Results showed that these ferns could be used for discovery of new and biologically active natural compounds.

## Introduction

Medicinal plants are used for treatment of illnesses during history.<sup>1</sup> According to the report of World Health Organization, 80% of people trust in traditional medicine and most of this therapy includes utilizing of the plant extracts.<sup>2</sup> Ferns are an ancient family of plant kingdom. Preliminary fern fossils predate 360 million years ago, the beginning of the Mesozoic era.<sup>3</sup> Now about 12,000 species exist around the globe.<sup>3</sup> Ferns have been used for wide medicinal purposes and have great potential due to their medicinal characteristics, but their phytochemical and biological properties are comparatively unexplored.<sup>4</sup> They are traditionally used for the treatment of skin tumefaction, protect the liver and treat the hepatitis and also being used as antipyretics.<sup>5</sup> The ethnic groups and tribal societies are using ferns parts like pinnae, stem, fronds, rhizome and spores in several ways for the treatment of various illnesses from many years ago.<sup>6</sup> Approximately 300 species of ferns are used as medicinal plants in traditional Chinese medicine.<sup>7</sup> Ayurvedic medical system suggested the medicinal uses of fern plants for

the treatment of several diseases too.<sup>8</sup> The rhizome of *Osmunda regalis* has been traditionally employed in Spain, mainly for the treatment of bone fractures, joint disorders and rheumatic and arthritic pain.<sup>9</sup> Many indigenous ferns have been used commonly as folk medicines in Iran.<sup>10</sup> However, their active phytochemicals or biological effects have not been determined. In the northern forests of Iran, ferns are quite common in the areas used for animal grazing.<sup>11</sup> Although there is a wide application of ferns in Iran, but no systematic report regarding to their biological activities could be found. According to the last studies on the pteridophytes of Iran, occurrence of 52 species in 26 genera and 15 families are confirmed.<sup>12</sup> Especially, north of Iran represents a diverse range of habitats from sea level zones to high mountain cloudy forests. Ferns contain several classes of natural compounds with potential medicinal applications, but scientific knowledge of the phytoconstituents of these plants is limited.<sup>13</sup> Flavonoids, phenolics, alkaloids, steroids, triterpenes and polysaccharides are the isolated classes of constituents from fern species in the literature.<sup>14</sup>

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Bioactive ingredients of ferns show diverse biological properties, which could be classified as antioxidant, antibacterial, anti-tumor, and anti-inflammatory activities.<sup>13</sup> A broad range of human diseases and pathological conditions are associated with free radical damage. Numerous studies have also established the relationships between consumption of antioxidant-rich food and prevention of human diseases.<sup>15</sup> Natural antioxidants have an important role in the inhibition of different diseases, so there is an increasing interest in the antioxidant potential of natural products.<sup>16</sup> Antioxidant compounds could prevent oxidative damages at low concentrations.<sup>17</sup> The antioxidant crudes were estimated in connection with their phenolic compounds.<sup>17</sup> Antioxidant compounds, including phenolic compounds have miscellaneous biological activities, such as anti-carcinogenic anti-inflammatory, and antibacterial effects, because of their antioxidant effect.<sup>18</sup> Phenolic compounds are common metabolites in plants which have biological activities such as anti-inflammatory, anti-cancer and antioxidant.<sup>19</sup> Several extracts from ferns exhibited remarkable antioxidant capacity, comparable with vitamin C.<sup>8</sup> Flavonoids and other

phenolic compounds have been demonstrated to be potent antioxidants. Hence, one of the functional properties of ferns that are pertinent to human health is their antioxidant activities.<sup>15</sup> A literature review indicated that there is no phytochemical and pharmacological study on Iranian fern species. So, this paper attempts to create a comprehensive tool including data for some species of ferns growing in Iran. Our goal in this work was the investigation of antioxidant power of these ferns using the total phenol content, DPPH and ABTS scavenging activity and to correlate TPC and antioxidant potential of the methanolic extracts of leaves and rhizomes. In this research 8 species from 5 families belonging to polypodiaceae (also known as leptosporangiate ferns) were studied (Table 1). All of the ferns studied here, including *Polypodium interjectum*, *Polystichum woronowii*, *Polystichum aculeatum*, *Asplenium scolopendrium*, *Asplenium adiantum-nigrum*, which are evergreen plants, *Dryopteris affinis* and *Pteris cretica* evergreen to subevergreen and *Athyrium filix-femina* a deciduous fern, are cultivated as ornamental plants because of their beautiful landscape.<sup>20,21</sup>

**Table 1.** List of selected Fern species.

No	Taxon Name	Common Name	Family	Voucher Number
1	<i>Polypodium interjectum</i> Shivas	Intermediate polypody	Polypodiaceae	MPH-1967
2	<i>Polystichum woronowii</i> Fomin	-	Dryopteridaceae	MPH-1968
3	<i>Polystichum aculeatum</i> (L.) Schott	Hard shield fern	Dryopteridaceae	MPH-1969
4	<i>Dryopteris affinis</i> (Lowe) Fraser-Jenk	Golden-scaled male fern	Dryopteridaceae	MPH-1970
5	<i>Athyrium filix-femina</i> (L.) Roth	Lady fern	Woodsiaceae	MPH-1971
6	<i>Asplenium scolopendrium</i> L.	Hart's tongue fern	Aspleniaceae	MPH-1972
7	<i>Asplenium adiantum-nigrum</i> L.	Black spleenwort	Aspleniaceae	MPH-1973
8	<i>Pteris cretica</i> L.	Cretan Brake	Pteridaceae	MPH-2010

## Materials and Methods

### Chemicals

2,2'-azin-obis(3-ethylbenothiazoline-6-sulphonic acid) diammonium salt, Gallic acid, 1,1-diphenyl-2-picrylhydrazyl, potassium persulphate, ethanol, Folin-Ciocalteu reagent, sodium carbonate, butylated hydroxy toluene (BHT) and methanol were purchased from Merck (Germany). Podophyllotoxin and sea salt were obtained from Sigma-Aldrich (Germany).

### Collection and identification of plants

The selected ferns for the present study were collected from the wild forest of different localities in Zirab region at the province Mazandaran, north of Iran (latitude 36°16' N, longitude 52°96' E) in October 2012. In this region the average temperature is about 16.5° C and average rainfall is about 725 mm during one year. Plants were authenticated by Dr. Sonboli, Taxonomist at herbarium of Medicinal Plants and Drugs Research Institute (MPH) in Shahid Beheshti University of Tehran.

### Preparation of plant extract

Plant bodies were cleaned to remove any residuals and

then shade dried at room temperature for a period of 10 days. All dried plants were then powdered. Then the extraction of samples (10g) were carried out using methanol (Merck). For each extraction, maceration of plant parts (rhizome and leaves) was done by 200 ml methanol at 25 °C for 24 hours with shaking. The extracts filtered through paper filter (Whatman No.1) and the filtrates were collected. The solvent of the crude extracts was removed in vacuo at 40 °C, to obtain dry extracts.

### DPPH radical scavenging activity

DPPH test confirms the radical inhibition potential of the extract by measuring the scavenging potential. 1,1-diphenyl-2-picrylhydrazyl radical scavenging capacity of the sixteen samples were measured according to the method described by Xu.<sup>22</sup> 50 µl of various concentrations (5, 10, 20, 40, 80 µg/ml) of extract solution in MeOH were added to 200 µl of 100 µM solution of DPPH in methanol, using butylated hydroxytoluene (BHT) as standard compound. After incubation of reaction mixture for 30 minutes at 25 °C in the darkness, and the decrease in the absorbance was determined immediately after mixing at 517 nm with a

microplate reader spectrophotometer (BioTek XS2 model). The control contained 50  $\mu$ l of MeOH in place of the test sample, and the blank contained pure methanol instead of DPPH solution. Experiments were carried out in quadruplicates. The inhibition in percent for each concentration was calculated according to the equation below:

$$\% \text{inhibition} = [1 - (A_s - A_b)/A_c] \times 100 \text{ Eq(1)}$$

Where  $A_s$  is the absorbance of the samples,  $A_b$  is related to the blank and  $A_c$  is for control. Lower observed absorbance values are related to higher antioxidant potential.  $IC_{50}$  value is a concentration that could inhibit 50% of DPPH radicals in the reaction mixture and is obtained from calibration curve.

#### **Trolox equivalent antioxidant capacity (TEAC) assay**

The Trolox equivalent antioxidant capacity test is a common and reliable method for the determination of antioxidant capability of compounds or extracts to inhibit ABTS radicals. The TEAC test could determine antioxidant activities of hydrophilic and lipophilic natural products. The TEAC assay was performed using the method reported in the literature.<sup>23</sup> The ABTS radical cation was made by mixing a solution of 7 mM ABTS in ethanol and 2.45 mM  $K_2S_2O_8$  in a ratio of 1:1. The reaction was completed after incubation in the dark for 12 h at 25 °C and the solution was used during 1 day. For measurements, the solution was diluted to have an absorbance of 0.70 at 734 nm. 3.8 ml of the radical solution and 100  $\mu$ l of plant samples were mixed for 1 min. Then after 1 min of incubation the absorbance was recorded at 734 nm. The percentage of inhibition was calculated and Trolox was used as standard compound. The results were imported as  $\mu$ mol Trolox/g dry mass of extract.

#### **Total phenolic content determination**

The TPC of the methanolic extracts were estimated by Folin-Ciocalteu assay using the method of Zhou.<sup>24</sup> 2.5 ml of plant sample was mixed with equal volume of Folin-Ciocalteu solution. Then 50  $\mu$ l of  $Na_2CO_3$  (35%) was mixed with the medium and it was made up to 250 ml. The reaction was mixed thoroughly for 30 min at 25 °C. The absorbance of the tested solutions was determined against a blank at 765 nm using a spectrophotometric micro plate reader. TPC values were expressed as mg equivalents of gallic acid per gram of dry weight of extract (mg GAE/g of extract). Gallic acid was used as the standard reagent and different concentrations of this reagent ( $\mu$ g/ml), were used to create a calibration curve. All measurements were performed in triplicates.

#### **Brine shrimp toxicity**

The assays were performed according to the method described in the literature with some modifications.<sup>25</sup> *Artemia salina* eggs were hatched in an erlenmeyer flask containing artificial seawater (250 ml, 3.8% w/w sea salt) at 30 °C for 48 hours at room temperature under

steady aeration. The extracts were dissolved in dimethyl sulfoxide (DMSO). Ten larvae were collected with a pipette and put to serially diluted solutions (500.0–7.8  $\mu$ g/ml extract) in the test tubes. After 24 h, a magnifying glass was used for counting the number of killed larvae. The mean of mortality percentage derived from triplicate experiments was plotted against the logarithm of drug concentrations using software.  $LC_{50}$  values were determined using the obtained equation by antilogarithm. Podophyllotoxin was used as the standard drug and DMSO was used as negative control. Final DMSO concentration was 0.2%.

#### **Statistical analysis**

Experiments were done in triplicates and results are shown as mean  $\pm$  SD (standard deviation). Analysis of correlations were determined by a bivariate correlation test using IBM SPSS Statistics V21.

## **Results**

#### **DPPH radical scavenging activity**

DPPH radical scavenging test could be used for evaluation of the antioxidant capacity of pure compounds and extracts in a short period of time. The lower  $IC_{50}$  value leads to the stronger radical scavenging capacity. Regarding to  $IC_{50}$  values as shown in Table 2, between all examined extracts, the rhizome extract of *P. aculeatum* with the lowest  $IC_{50}$  ( $0.45 \pm 0.02$   $\mu$ g/ml), exhibited an excellent DPPH radical scavenging activity, followed by *D. affinis* rhizome with  $IC_{50}$  value of  $4.60 \pm 0.12$   $\mu$ g/ml. Though the aerial parts extract of *A. scolopendrium* with the highest  $IC_{50}$  value ( $112.26 \pm 4.73$   $\mu$ g/ml) showed the weakest DPPH radical scavenging power. However the standard reference BHT showed  $IC_{50}$  value of  $9.96 \pm 0.45$   $\mu$ g/ml which is significantly higher than those of  $IC_{50}$  of rhizome extracts from *P. aculeatum* and *D. affinis*. Other samples exhibited good to moderate DPPH radical inhibition capacity. In general, rhizome extracts were stronger radical scavengers than the aerial part extracts in all of the ferns.

#### **ABTS radical scavenging assay**

Table 2 shows the ABTS radical scavenging activities of ferns 1–8 via TEAC values. Generally, these fern species had high free radical scavenging activity. TEAC values ranging from  $3.24 \pm 0.24$  to  $29.85 \pm 1.39$   $\mu$ mol Trolox/g. Among the tested extracts, *Athyrium filix-femina* was the strongest ABTS radical cation scavenger with TEAC value about 29.85  $\mu$ mol Trolox/g, followed by *Polystichum aculeatum* aerial part extract ( $22.45 \pm 2.14$   $\mu$ mol Trolox/g) and *Athyrium filix-femina* rhizome extract ( $21.78 \pm 1.02$   $\mu$ mol Trolox/g). Both aerial and rhizome extracts of *Asplenium adiantum-nigrum* showed the weakest free radical scavenging activity ( $4.02 \pm 0.29$  and  $3.24 \pm 0.24$   $\mu$ mol Trolox/g respectively) among the tested ferns. Rhizome extracts showed higher ABTS free radical inhibitory activity than the aerial parts extracts in all of the plants.

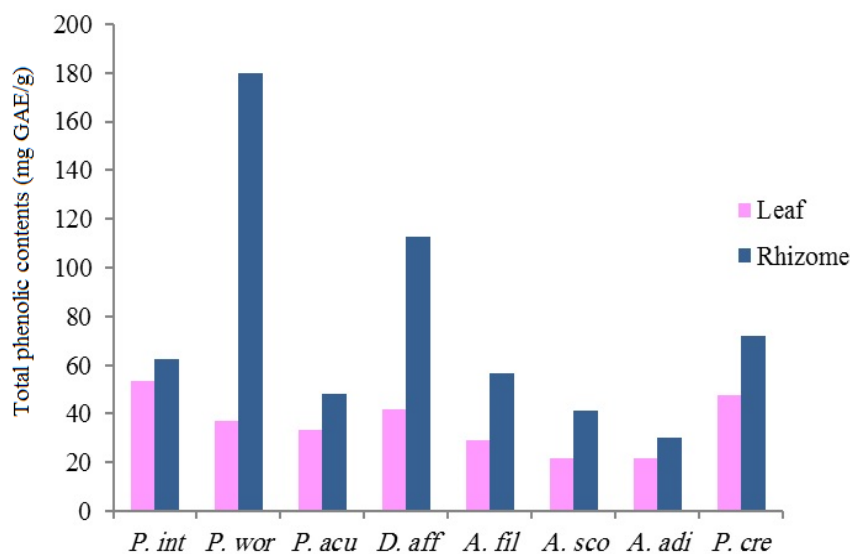
**Table 2.** DPPH and ABTS radical scavenging activity of selected fern species.

Fern species	DPPH ( $\mu\text{g/ml}$ )		ABTS ( $\mu\text{mol Trolox/g}$ )	
	leaf	rhizome	leaf	rhizome
<i>Polypodium interjectum</i>	54.52 $\pm$ 1.56	31.00 $\pm$ 0.84	7.09 $\pm$ 0.31	10.35 $\pm$ 0.56
<i>Polystichum woronowii</i>	19.38 $\pm$ 0.24	15.26 $\pm$ 0.49	12.25 $\pm$ 0.64	20.90 $\pm$ 0.89
<i>Polystichum aculeatum</i>	41.39 $\pm$ 2.56	0.45 $\pm$ 0.02	18.19 $\pm$ 1.34	22.45 $\pm$ 2.14
<i>Dryopteris affinis</i>	71.83 $\pm$ 3.89	4.60 $\pm$ 0.12	13.29 $\pm$ 1.20	22.35 $\pm$ 1.03
<i>Athyrium filix-femina</i>	34.17 $\pm$ 1.12	12.12 $\pm$ 0.57	18.78 $\pm$ 1.02	20.85 $\pm$ 1.39
<i>Asplenium scolopendrium</i>	89.15 $\pm$ 6.99	9.63 $\pm$ 0.33	12.05 $\pm$ 0.57	17.74 $\pm$ 1.01
<i>Asplenium adiantum-nigrum</i>	71.33 $\pm$ 2.02	46.61 $\pm$ 1.21	7.24 $\pm$ 0.24	8.02 $\pm$ 0.29
<i>Pteris cretica</i>	112.26 $\pm$ 4.73	51.18 $\pm$ 2.75	3.06 $\pm$ 0.59	12.24 $\pm$ 0.90
Butylated hydroxy toluene (BHT)	9.96 $\pm$ 0.45	-	-	-

### Total phenol content

TPCs of eight ferns extracts were determined using the Folin–Ciocalteu test, which is based on the electron transfer from phenolic natural products to the Folin reagent in alkaline pH. This method and is very simple and widely used.<sup>26</sup> As shown in Figure 1, the TPC varied from 21.85  $\pm$  3.12 to 180.00  $\pm$  12.5 mg GAE/g. *Polystichum woronowii* (180.00  $\pm$  12.5 mg GAE/g)

exhibited the highest amount of phenolic compounds, followed by *Dryopteris affinis* (112.54  $\pm$  8.09 mg GAE/g) and *Pteris cretica* (71.86  $\pm$  5.34 mg GAE/g). Among the tested extracts, *Asplenium adiantum* (21.85  $\pm$  3.12 mg GAE/g) had the lowest TPC. All of the tested fern species exhibited high content of phenolic compounds.

**Figure 1.** Total phenolic contents of rhizome and leaves of ferns.

### Brine shrimp toxicity assay

The LC<sub>50</sub> values of methanolic extracts against brine shrimp larvae are shown in Table 3. The LC<sub>50</sub> values for the tested 16 extracts were lower than 500  $\mu\text{g/ml}$ . Several extracts demonstrated high toxicity against brine shrimp larvae compared to the positive control, podophyllotoxin (50% lethality at 30  $\mu\text{g/ml}$ ). The lethality rate of larva was found to be dose dependent. Leaf extracts of *Athyrium filix-femina* and *Pteris cretica* had LC<sub>50</sub> values of 6.1 and 15.5  $\mu\text{g/ml}$ , respectively, and were considered to be highly toxic. Other extracts

considered to have moderate toxicity (Table 3).

### Discussion

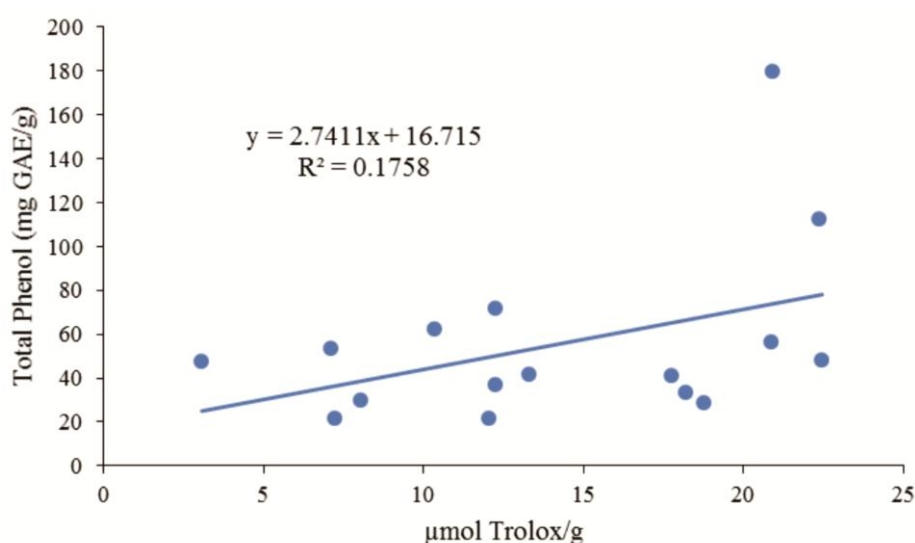
All the eight plant species reported in the current study, exhibited moderate to high cytotoxicity (6–450  $\mu\text{g/ml}$ ). The results indicated that these plants could be toxic for human, so caution must be taken when using these ferns. In general, the toxicity of the leaf extracts was significantly higher than those of rhizome extracts. Total phenolic content values of all sixteen extracts in this

study, were all higher compared with 31 Chinese medicinal ferns.<sup>7</sup>

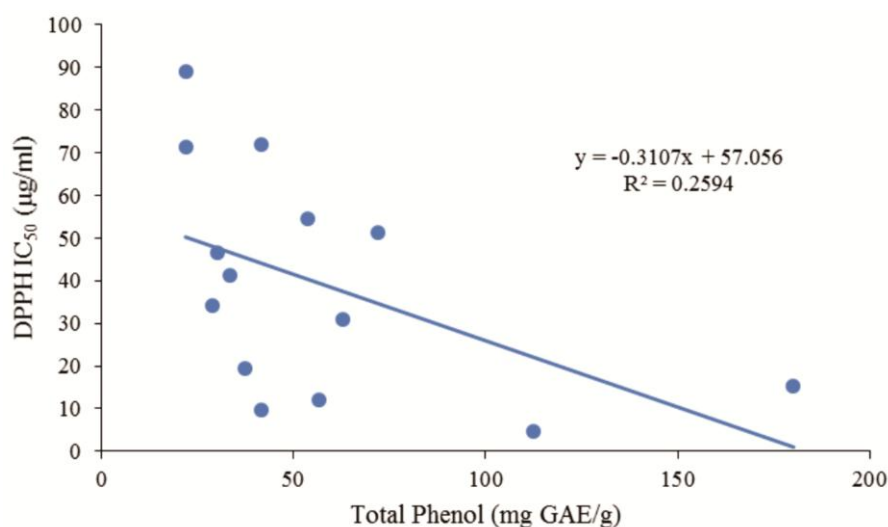
**Table 3.** Cytotoxic activity of Iranian Ferns ( $\mu\text{g/ml}$ ).

Tested Plant	LC <sub>50</sub> (24 h)	
	Rhizome	Leaves
<i>Polypodium interjectum</i>	482.0	205.7
<i>Polystichum woronowii</i>	307.1	52.2
<i>Polystichum aculeatum</i>	410.5	182.3
<i>Dryopteris affinis</i>	323.9	85.5
<i>Athyrium filix-femina</i>	45.8	6.1
<i>Asplenium scolopendrium</i>	405.4	112.0
<i>Asplenium adiantum-nigrum</i>	366.1	158.9
<i>Pteris cretica</i>	65.8	15.5
Podophyllotoxin	30	-

All of the tested plants showed strong antioxidant activities. *Dryopteris affinis*, *Polystichum aculeatum* and *Asplenium scolopendrium* displayed higher or equal antioxidant capacities in comparison with the standard reagent, BHT. Our results on the antioxidant power showed potential use of these ferns as their activities were comparable with the standard antioxidant BHT, while exceeding those for some medicinal ferns: fern *Angiopteris evecta* IC<sub>50</sub> > 90  $\mu\text{g/ml}$ ;<sup>27</sup> nine medicinal plants used in the Indian traditional system, IC<sub>50</sub> 83-560  $\mu\text{g/ml}$ <sup>28</sup> and six medicinal ferns used in Chinese traditional medicine, IC<sub>50</sub> 27-400  $\mu\text{g/ml}$ .<sup>29</sup> A very weak correlation between the TPC and TEAC value ( $R^2 = 0.1758$ ) demonstrated that phenolics are not responsible for ABTS radicals scavenging potential of these Ferns (Figure 2).



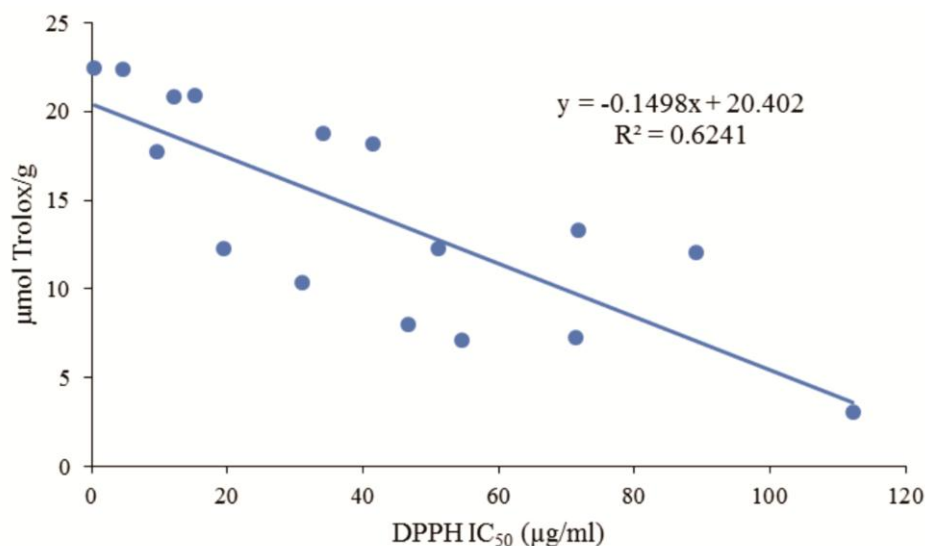
**Figure 2.** Correlation between the TEAC value and total phenolic content.



**Figure 3.** Correlation between the total phenolic contents and DPPH values.

Very weak correlation ( $R^2 = 0.2594$ ) between the TPC and DPPH values showed that phenolic compounds are different from those which are responsible for DPPH scavenging in these plants (Figure 3). In a recent study, high total phenolic content value found in *Blechnum orientale*, a medicinal fern, implied the role of phenolic compounds in contributing antioxidant activities.<sup>30</sup> Correlation analyses suggested that phenolic derivatives

could not be the key constituents responsible for the antioxidant activity of studied fern extracts. This is not in agreement with the findings of some of earlier studies on some medicinal plants.<sup>31</sup> A good correlation was observed between DPPH and TEAC values ( $R^2 = 0.6241$ ) indicating that compounds which reduce oxidants, could be responsible for ABTS scavenging capability of the tested ferns too (Figure 4).



**Figure 4.** Correlation between DPPH and TEAC values.

### Conclusion

Our work provides useful knowledge on cytotoxicity, radical scavenging properties and contents of phenolic about eight fern species, traditionally used in Iran. The methods used in this work are easy, simple and reliable which their results are reproducible. In this report, we have shown for the first time, Iranian fern species exhibited strong DPPH and ABTS radical scavenging activity in vitro and had the high phenolic compounds content. Our observations implied that differences in the antioxidant activities among the extracts cannot be adequately explained by quantitative difference in their total phenolic contents. We hope this study could be a start point for advanced biological and phytochemical investigations of Iranian Fern species.

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### Conflict of Interest

The authors report no conflicts of interest.

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