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PHYTOCHEMICAL CONTENTS AND ANTIOXIDANT ACTIVITIES OF FERN, *ASPLENIUM CETERACH* L. IN DIFFERENT ALTITUDES

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Abstract

Phytochemical contents and antioxidant activities of fern (*Asplenium ceterach* L.) distributed in different altitudes (22 stations) were compared and the relationships between altitude and plant chemical contents were studied. The highest 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was found in 22nd station (IC_{50} = 47.91 µg/ml) and the highest total phenolic content was found in 9th station (110.62 µgGAE/ml) whereas the maximum total flavonoid content was found in 20th station (232.67 µgCE/ml). High performance liquid chromatography (HPLC) analysis indicates that the maximum pterosin b (0.235 µg/ml), catechin (2.756 µg/ml) and quercetin (0.207 µg/ml) values were found in 21th station whereas the maximum chlorogenic acid (17.718 µg/ml) was obtained in 9th and caffeic acid (6,598 µg/ml) in 13th stations. It was observed that altitude is not potent alone, but it can be a factor in the occurrence of other ecological factors like soil properties, water, humidity, light and temperature.

Introduction

Plants are used in drug development and food research all over the world and thus the studies on plant chemistry and its relation to ecological factors are increasing day by day. Plant chemical content depends not only on plant genetic and other biotic factors but also on abiotic factors such as altitude, temperature, wind and light. Because altitude can affect such factors like light, wind and temperature; anatomical, morphological, physiological and biochemical properties of the plants which could be variable. Some reports have shown that secondary metabolite contents of the plants can increase (Dong *et al.* 2011, Mahdavi *et al.* 2013), or decrease (Giuliani *et al.* 2013, Uniyal 2013) with altitude or basically not (Verma *et al.* 2013). Since flavonoids and chlorogenic acids belonging to phenolic consituents of the plants have orto-hydroxylated constructions, they have abilities to absorb light and to scavenge free radicals. These properties can explain that flavonoid contents are higher in the higher altitudes which exposed to higher U.V. radiation (Dong *et al.* 2011). In a study on fern, *Pteridium caudatum* (L.) Maxon, it was shown that it has higher phenolic content and radical scavenging ability as it is grown in higher altitudes (Alonso-Amelot *et al.* 2004, 2007).

Since ferns, have secondary metabolites, they are used as folk medicines. Ferns contain sesquiterpenoids, flavonoids, cyanogenic glycosides, phenolic acids and pterosins (Alonso-Amelot *et al.* 1992, Zivkovic *et al.* 2010, Chen *et al.* 2015). Pterosins are carcinogens for human and animals (Gil da Costa *et al.* 2012) but these substances play a role in activation of protein kinases activated by adenosine monophosphate which adjusts blood glycose (Chen *et al.* 2015). Previously antioxidant activity of some Turkish medicinal plants was studied by Karadeniz *et al.* (2015).

Asplenium ceterach L. (golden herb) is a fern belonging to Aspleniaceae, which is used as a folk medicine in different regions of the world. The aim of the present study was to compare the effects of different altitudes on phytochemistry and antioxidant activity of *A. ceterach* L.

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Materials and Methods

Fresh fern (*Asplenium ceterach* L.) samples were collected from their natural distribution areas from 22 stations in different altitudes in Burdur and Antalya provinces, Turkey, in March-May 2016 (Table 1). Some of the plant samples were dried in shade and cool place and the other part of the samples were saved as herbarium material after drying and pressing and preserved as vaucher specimen. Plant names were verified from the sources such as Flora of Turkey and the East Agean Islands (Davis 1965-1985), Turkish Plant List (Güner *et al.* 2012), The Plant List, Euro Med Plant Base, The International Plant Names Index.

Table 1. Location, date, habitat and altitude of collected fern samples.

Station	Collection date	Province	Habitat	Altitude (m)
1	11.03.2016	Antalya	Humid rocky places in stream sides	605
2	14.04.2016	"	Rocky places in mixed *Juniperus excelsa* forest	1436
3	15.04.2016	"	Rocky places in mixed black pine forest	1200
4	15.04.2016	"	Rocky places in maquis	646
5	21.04.2016	Burdur	Rocky places in maquis	807
6	21.04.2016	"	Rocky places in *Quercus coccifera* maquis	906
7	21.04.2016	"	Rocky places in mixed *Cedrus libani* forest with black pine	1488
8	22.04.2016	"	Rocky places in black pine forest	1880
9	22.04.2016	"	Rocky places in mixed *Cedrus libani* forest	1479
10	22.04.2016	"	Rocky places in maquis	1155
11	5.05.2016	Antalya	Rocky places in red pine forest	692
12	5.05.2016	"	Rocky places in red pine forest	397
13	6.05.2016	"	Rocky places in red pine forest	181
14	6.05.2016	"	Humid rocky places in *Juniperus excelsa* forest	1330
15	6.05.2016	"	Rocky places in *Quercus coccifera* maquis	10
16	14.05.2016	"	Rocky places in maquis zone	1006
17	12.03.2016	"	Rocky places in red pine forest	536
18	14.04.2016	"	Rocky places in red pine forest	744
19	21.04.2016	Burdur	Rocky places in maquis	846
20	21.04.2016	"	Rocky places in maquis	807
21	21.04.2016	Burdur	Rocky places in maquis	1127
22	5.05.2016	Antalya	Rocky places in red pine forest	567

Air dried 0.5 g plant sample was homogenized in a blender and extracted within 100 ml methanol by using magnetic stirrer for a 4 hrs and filtered with Whatman No.1 filter paper. Extracts were used within two days to detect antioxidant capacity.

The method of Blois(1958) was used with some modifications to detect DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity. DPPH (50 µl, 1 mM) solution was added to methanol solution (200 µl) of the samples or the control at various concentrations. The reaction

mixture was shaken vigorously and the absorbance of remaining DPPH was measured at 517 nm after 30 min. Radical scavenging activity (inhibition percentage) was detected by comparing the absorbance with that of the blank containing only DPPH and solvent. Ascorbic acid was used as the positive control. All analyses were done in triplicates. Inhibition percentage was calculated by using the formula below:

Inhibition percentage = $[(Abs_{control} - Abs_{sample}) / Abs_{control}] \times 100$

Radical scavenging activity (inhibitory concentration) was expressed as IC_{50} of the extract. The method of Singleton and Rossi(1965) was used with some modifications to detect the total phenolic content of extracts by using the Folin-Ciocalteu reagent: 10 µl of sample or the standard (10 - 500 µg/mlgallic acid) and additionally 150 µl of diluted Folin-Ciocalteu reagent (1 : 4, reagent : water) was placed in each well of a 96-well plate and incubated at room temperature for 3 min. Following the addition of 50 µl of saturated sodium carbonate (7.5%) and a further incubation of 2 hrs at room temperature, absorbance was read at 725 nm. Total phenolic content was expressed as gallic acid equivalent (µgGAE/ml).

The method of Zhishen *et al.*(1999) was used to detect total flavonoid content. Briefly, 10 µl of 5% sodium nitrite was added to the 10 µl sample, after 5 min 10 µl 10% aluminum chloride, 150 µl 1 M sodium hydroxide and 50 µl ultra-pure water were added. Plate was mixed well. Then the absorbance was read at 510 nm in UV/vis spectrophotometer. The 70% methanol was used as control. Total flavonoid content was expressed as catechin equivalent (20 - 100 µgCE/ml).

HPLC analysis of phenolics (chlorogenic acid, caffeic acid, quercetin, catechin) and pterosin b were done in Mehmet Akif Ersoy University, Scientific and Technology Application and Research Center following the modified method of Gomes *et al.* (1999). System: Shimadzu Prominence, Detector: DAD (SPD-M20A), Column Oven: CTO-10ASVp, Pomp: LC20 AT, Autosampler: SIL 20ACHT, Computer Programme: LC Solution, Mobile Phase: A: %3 Formic acid B: Metanol.

All samples were analyzed in triplicates. Data are expressed as means ± standard deviations. Descriptive statistical analysis with graphics of inhibition percentage and of the linear regression curve were made by using Microsoft Office Excel 2007 program. To determine the relationship between two variables Pearson correlation coefficients (r) were also calculated. Statistical analysis was performed using SPSS (IBM SPSS Statistics 17 Portable) and Minitab (Minitab 18) programs.

Results and Discussion

The highest DPPH radical scavenging activity and the lowest IC_{50} value (IC_{50} = 47.91 µg/ml) determined in the plant extracts collected from 22nd station are presented in Table 2. The lowest IC_{50} indicated the highest radical scavenging activity, it means the extract is effective in lower concentrations (50% of the radical can be scavenged by the extract). IC_{50} values of the plant extracts collected from different stations showed significant differences ($p \leq 0.05$). Tukey multiple range test showed that the extract collected from 22th station is significantly different from the others ($p \leq 0.05$). One way ANOVA showed that IC_{50} values of the extracts collected from the stations in Burdur and Antalya provinces were not found significant ($p > 0.05$). Pearson correlation test showed that the weak correlation ($r = 0.211$) was obtained between altitude and IC_{50} values which was not significant ($p > 0.05$).

The highest total phenolic content was obtained in the plant extracts collected from 9th station (110,62 µg GAE/ml). Total phenolic contents of the plant extracts collected from different stations showed significant differences ($p \leq 0.05$) (Table 2). Tukey multiple range test showed that the extract collected from 9th station is not significantly different from others ($p > 0.05$). One way ANOVA showed that total phenolic contents of the extracts collected from the stations in Burdur

and Antalya provinces were not significantly different ($p > 0.05$). Pearson correlation test observed that negatively weak correlation ($r = –0.106$) between altitude and total phenolic content was obtained not significant ($p > 0.05$).

Table 2. DPPH radical scavenging activity, total phenolic and flavonoid contents and HPLC analysis of fern extracts collected from different stations.

	Antioxidant activity			HPLC analysis (µg/ml)				
Station	DPPH RSA (IC_{50}) (µg/ml)*	Total phenolic content (µgGAE/ml) *	Total flavonoid content (µgCE/ml) *	Pterosin B	Cate-chin	Chloro-genic acid	Caffeic acid	Quer-cetin
St1	184.56 ± 3.15	66.41 ± 3.28	178.89 ± 12.41	0.094	1.327	0.085	0.193	0.125
St2	230.25 ± 4.99	68.90 ± 3.54	99.11 ± 11.19	0.051	1.979	10.371	0.008	0.129
St3	249.87 ± 5.77	62.00 ± 3.68	119.11 ± 6.33	0.056	1.52 9	6.405	0.057	0.143
St4	237.35 ± 5.87	75.59 ± 6.99	90.44 ± 8.22	0.046	1.759	10.453	0.097	0.122
St5	294.66 ± 3.4	50.00 ± 3.44	160.89 ± 21.48	0.098	1.176	17.042	5.868	0.14
St6	179.79 ± 2.83	62.28 ± 3.40	87.56 ± 12.80	0.09	0.874	13.4	0.293	0.143
St7	209.7 ± 9.86	62.62 ± 9.56	117.56 ± 8.77	0.116	0.492	13.758	0.728	0.132
St8	213.59 ± 4.74	74.55 ± 9.85	161.56 ± 20.73	0.063	0.983	13.103	0.65	0.136
St9	204.96 ± 6.64	110.62 ± 10.70	125.33 ± 12.83	0.079	0.718	17.718	1.452	0.125
St10	223.56 ± 6.62	97.10 ± 8.27	208.22 ± 15.64	0.084	0.645	8.938	1.48	0.121
St11	223.86 ± 5	49.93 ± 3.33	74.89 ± 7.26	0.062	0.549	13.666	3.258	0.165
St12	192.03 ± 3.74	70.90 ± 7.21	116.44 ± 23.67	0.073	0.438	7.719	4.081	0.163
St13	223.48 ± 0.7	104.48 ± 7.47	104.22 ± 9.32	0.058	2.203	15.143	6.598	0.148
St14	221.98 ± 9.57	67.45 ± 7.92	143.78 ± 10.73	0.053	0.519	11.462	2.478	0.164
St15	197.95 ± 3.9	94.83 ± 7.88	198.44 ± 7.92	0.064	0.292	8.183	2.424	0.123
St16	180.12 ± 5.25	60.21 ± 1.48	156.22 ± 20.91	0.034	0.15	6.933	0.332	0.142
St17	127.21 ± 3.37	48.83 ± 5.71	100.22 ± 12.98	0.066	0.179	5.31	2.434	0.172
St18	100.06 ± 4.65	76.41 ± 8.02	158.22 ± 10.70	0.049	0.12	6.043	2.086	0.124
St19	165.55 ± 5.77	63.17 ± 4.17	185.33 ± 22.53	0.04	0.234	2.33	1.818	0.141
St20	138.46 ± 7.39	56.00 ± 4.06	232.67 ± 18.53	0.058	0.711	11.513	3.42	0.141
St21	143.44 ± 5.49	43.10 ± 6.10	167.56 ± 14.92	0.235	2.756	11.556	1.618	0.207
St22	47.91 ± 15.03	88.14 ± 22.42	108.00 ± 4.44	0.041	0.35	6.051	1.617	0.122

*Means of three replicates ± standard deviation, RSA: Radical scavenging activity, GAE: Gallic acid equivalent and CE: Catechin equivalent.

The highest total flavonoid content was determined in the plant extracts collected from 20th station (232.67 µgCE/ml). Total flavonoid contents of the plant extracts collected from different stations showed significant differences ($p \leq 0.05$) (Table 2). Tukey multiple range test showed that the extract collected from 20th and 10th stations are significantly different from the others ($p \leq 0.05$). One way ANOVA observed that total flavonoid contents of the extracts collected from the stations in Burdur and Antalya provinces were significantly different ($p \leq 0.05$). Pearson correlation test showed that very weak correlation ($r = 0.017$) between altitude and total flavonoid content was not found significant ($p > 0.05$).

HPLC analysis presented in Table 2 shows that the highest pterosin b, catechin and quercetin were found in the plant extracts collected from 21th station whereas the highest chlorogenic acid

and caffeic acid were found in 9th and 13th stations, respectively. Pearson correlation test showed that negatively weak correlation ($r = –0.531$) was obtained between altitude and the amount of caffeic acid was significant ($p \leq 0.05$). There was weak correlation between pterosin b and quercetin ($r = 0.568$) and catechin ($r = 0.512$) ($p \leq 0.05$).

It was observed that plant extracts from 22nd, 9th, 21st, 20th and 13th stations have the highest radical scavenging activity and phenolic contents. Among them only 13th station was in Antalya while others were in Burdur province.

It was found that altitude does not affect the phenolic and pterosin b content of the plants which corroborates with the findings of Verma *et al.* (2013). Dong *et al.* (2011) and Uniyal (2013) also studied on the increase/decrease of some secondary metabolites which depended on the altitude.

Altitude did not affect plant phenolic and pterosin content in the present study. *A. ceterach* can be grown on rock cracks and the bottom of the rocks. Therefore, the chemical content of fern can be explained with soil properties, humidity, temperature, shade and sunny situations while having no relationship with altitude. Burdur and Antalya are neighboring cities and a part of Burdur province and the most of the Antalya province belong to Mediterranean Floristic Region, hence a comparison among stations would have been a better approach. Burdur province receives less rainfall in a year when compared to Antalya province. Annual precipitation averages of Burdur and Antalya provinces are 443 mm (Özçelik and Çinbilgel 2016) and 1062.4 mm, according to official statistics of Ministry of Forest and Water Affairs, Meterorology General Directorate, respectively.

As Zivkovic *et al.* (2010) stated that chlorogenic and caffeic acids are the main phenolics of *A. ceterach.* It was found that these phenolicswere in higher amounts compared to other phenolics in the plant extracts and a moderate negative correlation ($r = –0.531$) was obtained between altitude and caffeic acid ($p \leq 0.05$). It means that when the height increased, caffeic acid content decreased in plant extracts. Phenolic substances protect plants from drought stress, therefore increase in polyphenol oxidase enzyme activity indicates increase in phenolic content (Zivkovic *et al.* 2010).

According to field observations, sizes of fern plant were smaller in the direct sunlight than in shade because shade and bottom of rocks were rich in organic material and water. Directions were also considered to affect plant mass. Plant sizes were smaller when grown in south and west sides, but when south and west side plants were in the bottom or shade, the effects of directions were less.

There was no correlation between altitude and the other plant compounds except modarate correlation between pterosin b and quercetin ($r = 0.568$), catechin ($r = 0.512$). It means plant extracts in which pterosin b content is higher, have higher quercetin and catechin. Pterosins (ptaquilosides) are the fern toxins, detected in *Pteridium aquilinum* and carcinogenic for humans and animals (Gil da Costa *et al.* 2012). These toxic compounds seem to be a part of defence system of plants against predators or pathogens. Pterosin b was found in nanogram levels in *A. ceterach* extracts.

On conclusion, it may be said that altitude is not potent alone, but it can be a factor in occurrence of other ecological factors like soil properties, water, humidity, light and temperature.

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