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Cardenolides in *Digitalis trojana* Ivanina, an endemic plant of Ida mountain, Turkey

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ABSTRACT

Cardiac glycosides are used as positive inotropic agents in the treatment of heart rhythm disorders. Digitalis species have been used as a source of these components for many years. Digitalis trojana Ivanina is an endemic plant of Ida Mountain (Kazdagı), Çanakkale, Turkey. In this study, we report the cardenolide content changes of Digitalis trojana Ivanina which was collected from Kazdagı at different altitudes (836m, 961m, 1041m and 1191 m). The content of lanatoside C, digoxin, digitoxin and gitoxigenin in the plant samples were analysed by HPLC. According to the results of analyses, cardenolide contents of D. trojana (lanatoside C, digoxin, digitoxin and gitoxigenin) showed variations by altitude (836m, 961m, 1041m and 1191 m) and month (May and July). It was determined that these cardenolides were higher in plants collected in July than in May and the highest cardenolide concentration was found in the samples collected at 1047 m. both in May and July.

Key words: *Digitalis trojana* Ivanina, Ida Mountain, Plantaginaceae, Cardenolide, Secondary metabolite.

INTRODUCTION

Cardiac glycosides (CGs) or cardenolides are class of natural products and a pharmacologically important group of plant secondary metabolites. They have been used as the most effective heart drugs for treating congestive heart failure, cardiac arrhythmias and atrial fibrillation [1]. They also exhibit a wide spectrum of biological activities, including anti-carcinogenic, acaricidal and antibacterial properties. Recent studies especially focused on the anti-carcinogenic effects of digoxin and digitoxin particularly for cancer treatments [2, 3].

These cardenolides are found in a diverse group of plants including *Digitalis*, *Strophanthus*, *Scilla maritime* and *Nerium oleander*. *Digitalis* species in the family Plantaginaceae are a medicinally important group because of these cardenolides applied in human medicine; they have been used therapeutically for the treatment of cardiac insufficiency for more than 1500 years. Several *Digitalis* species (e.g. *D. lanata*, *D. purpurea*,) are potent sources of these glycosides [1]. Because cardenolides have been of commercial interest for the past two decades, the studies have focused on several *Digitalis* species as part of a general strategy for the evaluation cardenolides for commercially used in pharmaceutical industry and the enhancement of the yield of valuable cardenolide and [4-7].
Digitalis trojana Ivanina is an endemic plant of Ida Mountain (Kazdagı), Çanakkale, Turkey. In this study, we report the cardenolide content changes of *Digitalis trojana* Ivanina which was collected from Kazdagı. Our main aims were: (1) to determine cardenolide content of *D. trojana* as secondary metabolites of commercial value for the pharmaceutical industry and (2) to compare cardenolide contents of plants that were collected from different altitudes in May and July.

MATERIALS AND METHODS

Plant material

D. trojana plants were collected at different altitudes (836m, 961m, 1041m and 1191 m) in different vegetation phases from the Kazdagı National Park, Balıkesir, Turkey in May 2011 and July 2011. Plant samples were identified by Dr. Ersin Karabacak, Department of the Biology, Çanakkale Onsekiz Mart University, Çanakkale, Turkey. A voucher of the studied plant was deposited in the Çanakkale Onsekiz Mart University Herbarium (Çanakkale, Turkey) under number 938 (May), 939 (July).

Cardenolide solid phase extraction

Solid phase extractions of cardenolides were performed as described by Wiegrebe and Wichtl, 1993 [8], Roca-Pėrez et al. 2004 [9] with some modifications. 500 mg of basal leaves were ground to a fine powder in liquid nitrogen using a pestle. The leaf powder was treated with 12 ml of methanol (70%) and this solution was incubated in a boiling water (95 °C) bath for 10 min and then rapidly cooled to room temperature. The extract was centrifuged for 5 min at 11000 rpm. The supernatant was transferred into a new tube. After the addition of 2 ml of a solution of lead acetate (15%), the extract was mixed well and then 2 ml of a solution of monosodium phosphate (4%) was added and the extract was mixed again. The extract was centrifuged for 5 min at 11000 rpm. The supernatant was transferred into a new tube and the extract was diluted with water to 24 ml. Solid-phase extraction (SPE) cartridges (C18, Agilent, Germany) were used to purify the aqueous extracts. The cartridges were conditioned with 4 ml of methanol and 4 ml of H_2O. All of the extract was passed through the cartridges, followed by washing with 2 ml of water. Cardenolides were eluted with 2 ml of methanol and transferred to the auto sampler vial for HPLC analyses.

HPLC determinations

Cardenolide separation and analyses were carried out by HPLC as described by Roca-Pėrez et al. 2004 [15]. The extracts were analysed in an Agilent Chromatograph (1200 series) coupled to a 10 µl injector. Cardenolides were separated at 40°C on an ACE C18 250-4 column (5 µm) and detected at 230 nm under a flow rate of 1 ml min^{-1}. A gradient elution of H_2O and CH_3CN was employed: initial= 20% CH_3CN; 35 min=32% CH_3CN; 45 min=40% CH_3CN; 55 min=50% CH_3CN; 59 min=55% CH_3CN; 65 min=60% CH_3CN. The identities and amounts of the cardenolides (Lanatoside C, Digoxin, Digitoxin and Gitoxigenin) were checked by co-chromatography with commercially available standards (Sigma). Four replicates were analysed per experimental sample. Arrival times and the peak areas of the standards were determined by results of HPLC. All the samples were passed through the column and the peak areas were reported for each sample. Amounts of lanatoside C, gitoxigenin, digoxin and digitoxin were calculated by using the peak area of the chromatogram of the standards. Final data are presented as µg of cardenolides g^{-1} dry wt.

Statistical Analysis

The data were statistically analyzed by analysis of variance (ANOVA) and means were compared for level of significance ($p<0.05$) by Tukey's test using Statistical Package for Social Sciences (SPSS 20.0, IBM).

RESULTS

In this study, we investigated the amount of the cardenolides (gitoxigenin, lanatoside C, digitoxin and digoxin) in *D.trojana*. The experiments were performed with *D.trojana* plants which were collected in different altitudes in May and July. In order to determine, quantitive analyses were performed by direct chromatographic comparison with standards under HPLC conditions allowed the amount of the five cardenolides (gitoxigenin, lanatoside C, digitoxin and digoxin) in the plant samples.

HPLC analyses showed that the cardenolide content of plants varied according to the altitude and month. The plant samples that were collected in May contain gitoxigenin, digitoxin and lanatoside C while digoxin was not detected in any samples (Table 1). A comparison of the profiles of plants collected at different altitudes and months were showed that lanatoside C existed in higher concentrations in the samples collected in May at 1041 m.

Table 1. HPLC results of the plant samples that were collected in May 2011

Altitude	Gitoxigenin (µg/g dry wt.)	Digoxin (µg/g dry wt.)	Lanatoside C (µg/g dry wt.)	Digitoxin (µg/g dry wt.)
836 m	6.7 ± 0.98^{b}	0	72 ± 11.31^{b}	0^{a}
961 m	0^{a}	0	123 ± 12.72^{c}	0^{a}
1041 m	7.5 ± 1.76^{b}	0	128 ± 13.43^{c}	0^{a}
1191 m	0^{a}	0	10.7 ± 1.99^{a}	4 ± 1.41^{b}

Means with the same letter within the columns are not significantly different at $p < 0.05$.

Gitoxigenin, lanatoside C, digoxin and digitoxin content have been identified in plants collected at different altitudes in July. Cardenolide content varied in the plant samples that were collected in July (Table 2). All cardenolides were detected in the samples collected at 1041 m in July. In addition the lowest cardenolide content was determined to be in the samples collected at 1191m in July.

Table 2. HPLC results of the plant samples that were collected in July 2011

Altitude	Gitoxigenin (µg/g dry wt.)	Digoxin (µg/g dry wt.)	Lanatoside C (µg/g dry wt.)	Digitoxin (µg/g dry wt.)
836 m	20±4.78^{c}	0^{a}	129±10.36^{c}	32± 7.6^{b}
961 m	0^{a}	0^{a}	96±4.24^{b}	16±4.94^{ab}
1041 m	13± 2.12^{bc}	25±3.53^{b}	188± 14.16^{d}	28±5.65^{b}
1191 m	10.4±0.98^{b}	0^{a}	27±2.12^{a}	0^{a}

Means with the same letter within the columns are not significantly different at $p < 0.05$.

A comparison of the cardenolide profiles of the plant samples collected in May and July determined that gitoxigenin, lanatoside C, digoxin and digitoxin content (mg/g dry wt.) was higher in plants collected in July than in May. Cardenolides were found in higher concentrations in the plants samples collected at1047 m. both in May and July.

DISCUSSION

Recent research coupled with the results of our own survey discovered that the amount and content of cardenolide was low in the spring season (May) due to plants giving emphasis to the production of primer metabolite, while in the summer season (July) cardenolide is higher because environmental factors are more dominant and plants have completed their development. Which arise probably of different climate characteristics in different seasons in the region, since the metabolism is influenced in many ways by those conditions.

The secondary metabolite production in plant tissues is influenced and promoted by biological and environmental factors as well as biochemical, physiological and ecological processes. These factors such as seasonality, altitude, plant development, temperature, water availability, humidity, UV radiation and pathogenic attacks are considered to be the factors that most affect the occurrence and accumulation of plant secondary metabolites in the tissues. Because of these factors, the quantity and quality of secondary metabolites in plants may vary over time and seasonal or daily variation [10-12]

Cardenolides contents of natural and *in vitro* cultured plants has previously been reported for other *Digitalis* species and our results are quite similar to those in the report. Roca-Perez et al. 2004 [9] reported productivity variations and seasonal fluctuations of cardenolides in 10 natural populations of *D. obscura* distributed in three bioclimatic belts of Spain.They found that the lowest production was recorded in plants collected in May, followed by a fast cardenolide accumulation in summer, a decreasing phase in autumn, and a stationary phase in winter. In this study it was reported that cardenolide contents changed in the course of the four seasons as a multiple response to distinct plant and environmental factors. In one report [13] the amounts of digitoxigenin and gitoxigenin in wild Sardinian *Digitalis purpurea* L. was analyzed by HPLC and the results showed that the samples, stemming from six different locations showed a great variability in glycoside content and the report pointed out the different morphological characters showed that correlations between morphological variations and glycoside content was poor.Gürel et al. 2011 [14] studied digoxin and lanatoside C content of *D. davisiana* Heywood collected at different periods of vegetation in the same year (June, July and September). They reported that digoxin was present in leaf extracts of *D. davisiana* collected at different vegetation periods, whereas lanatoside C content was present as either trace amounts in June and July or in very low amounts in September and they determined that highest amounts of digoxin (246.58 mg/kg dry wt) were found in leaf samples collected in July, which coincides with the plant's flowering stage in the collection region and it was point out that seasonal variations seem to affective production of digoxin in leaves.

CONCLUSION

Our study revealed the cardenolide content of *D. trojana* from different altitudes in May and July and the cardenolides content of these plants are not enough for commercial value for the pharmaceutical industry. Because of these, strategies, based on *in vitro* culture methods, have been extensively studied to improve the production of valuable cardenolides in *D. trojana*.

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