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Three seasonal comprehensive evaluation process of *Digitalis trojana* Ivan's phenolics

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ABSTRACT

Digitalis trojana Ivan, a member of *Digitalis* genus, has highly bioactive properties due to efficient phenolics. These compounds have shown to convert into each other in a limited time. Seasonal fluctuations of this plant leaves have been studied during the summer season using the high performance liquid chromatography–tandem mass spectrometry. Caffeic acid in June (2.48 mg/g extract), *p*-coumaric acid in August (6.69 mg/g extract) and *trans*-ferulic acid in July (4.85 mg/g extract) were the main compounds in the non-hydrolysed extracts. These compounds were heavily found in the hydrolysed extracts in August (14.63, 18.29 and 5.70 mg/g extracts, respectively). In addition, analysed flavonoids were found to be highest in July. Total phenolic contents were measured spectrophotometrically by using Folin-Ciocalteu's phenol reagent. The spectrophotometrically results were lowest (e.g. 43.50 mg GAE/g extract) in July while those of the hydrolysed extracts were highest (e.g. 167.19 mg GAE/g extract) in June.

1. Introduction

Plants have been a reliable sources of bioactive compounds used as remedies for a wide range of diseases. Secondary metabolites of plantsare not only indispensable for the defence against many organisms such as herbivores and pathogens but they also possess antioxidant, antimicrobial, and antitumor bioactivities (Hartmann, 1996; Ng et al., 2000; Prajda et al., 1995). However, they are not always safe to use since they might have natural cytotoxic compounds. Therefore the secondary metabolites of plants have to be identified carefully for possible toxic potentials. Since the synthesis of secondary metabolites in plants is influenced by the environmental conditions such as soil, temperature, UV light, moisture and altitude,harvest time and conditions should be arranged based on the abundance of metabolites of interest (Raal et al., 2015; Skrzypczak-Pietraszek and Pietraszek, 2012; Vagiri et al., 2015; Wu and Lay, 2013).

The genus *Digitalis,* in the family of Scrophulariaceae, harbours poisonous plants that has a long history of use in the treatment of heart diseases including myocardial infarction, cardiac dysfunction, oedema, angina, cardiac hypertrophy, and arterial hypertension due to rich content of cardiac glycosides as a type of secondary metabolites (Frohne and Pfander, 2005; Pellati et al., 2005). A number of studies investigated the cardiac glycosides of the *Digitalis* genus, particularly digoxinthat is used in therapy (Dinan et al., 2001). Many previous studies were concerned with the isolation of chemical constituents of plants belonging to this genus. Examples include the isolation of phenylethanoid and phenylpropanoid glycosides of caffeic and ferulic acids such as lugrandoside, ferruginoside A–B, purpureasides A–C, calceolarioside A, plantamajoside, poliumoside, and forsythiaside from *Digitalis purpurea*, *Digitalis lutea*, *Digitalis grandiflora*, *Digitalis ferrugine* and *Digitalis thapsi* (Baudouin et al., 1988 Çalış et al., 1999; Lee et al., 2006; Matsumoto et al., 1987; Taskova et al., 2005; Zhou et al., 1998). In Turkey, there are four endemic specious belonging to *Digitalis* genus (Davis, 1978). These include *Digitalis trojana* Ivan, *Digitalis davisiana* Heywood, *Digitalis cariensis* Boiss. Ex Jaub. Et Spach, and *Digitalis lamarckii* Ivan. *Digitalis trojana* Ivan is a biennial plant found in Çanakkale and Balikesir at 90–800 M altitude (Davis, 1978).

In this work, phenolic acid and flavonoid contents of *Digitalis trojana* Ivan were determined for the first time. The seasonal fluctuations of these compounds under natural environmental con-

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ditions were investigated during three consecutive months, which includes the flowering period (June–August).

2. Materials and methods

2.1. Chemicals and solvents

All analytical grade phenolic standards were purchased from Sigma-Aldrich. HPLC grade acetonitrile and methanol used in LC-UV experiments and sample preparation were from Sigma-Aldrich. LC–MS grade acetonitrile and water used in LC–MS experiments were from Biosolve LTD (Netherlands). Diethyl ether, ethyl acetate, HCl and glacial acetic acid were purchased from Merck (ACS, ISO, Reag. Ph. Eur for analysis, Darmstadt, Germany). HPLC syringe filters (13 mm, 0.2 μm, PVDF) were purchased from Whatman (Clifton, NJ, USA) for LC-UV and LC–MS experiments.

2.2. Sample preparation

Arial parts of *Digitalis trojana* Ivan samples were collected from 345 M altitude on Kazdağı (Edremit, Balıkesir, Turkey) in June (DT6), July (DT7), and August (DT8) 2009. The plant was air-dried after which the leaves were separated and dry leaves were crushed in a coffee blender. Initially, 0.800 g leaf powder was extracted with 15 mL methanol under the condenser in the ultrasonic bath at 60 °C for 3 h. Extracts were centrifuged and residues were washed with methanol until exhaustion. Methanol extracts were concentrated to 13.3 mL using evaporator. 4 mL of the extracts were coded as A and kept at 4 °C for spectrophotometric analysis. 6 mL of the extracts were evaporated until dry and 2 mL methanol and 4.25 mL of 2.95 M HCl solution were added to the dried extract adjusting to 2 M HCl for acidic hydrolysis for 2 h at 90 °C. After hydrolysis, liquid-liquid selective extraction was carried out with 3 × 2 mL diethyl ether and 4 × 2 mL ethyl acetate consecutively. The organic phases were pooled and evaporated until dry. These hydrolysed and dried residues were dissolved with 5 mL methanol and coded as B. The last 3.3 mL of the extract was evaporated and a pH 2 aqueous solution was added to the dried residue and liquid-liquid selective extraction was carried out as stated above. The organic phases were pooled and evaporated until dry. These were dissolved in 2.75 mL methanol and coded as C. Extracts were diluted with 70% water and filtered through Whatman 0.2 μm PVDF filters prior to HPLC-UV–MS/MS experiments.

2.3. Determination of phenolic acids by HPLC-UV

HPLC-UV analyses were performed on a Shimadzu HPLC VP Series system equipped with a Shimadzu SPD-10AVPUV–vis dual wavelength detector. The instrument was controlled with Class VP chromatography software. An Agilent Zorbax Eclipse XDB-C18 column was used (4.6 × 150 mm, 5 μm) with gradient programme using two solvent systems, (A: 80% acetonitrile (ACN) in water, B: 2% acetic acid in water). A gradient elution that was modifiedfrom the method developed by De Villiers et al. (2004) was applied: 0–2 min, 95% B; 2–8 min, 95–90% B; 8–11 min, 90–85% B; 11–13 min, 85–75% B; 13–17 min, 75–70% B;17–30 min, 70–65% B; 30–33 min, 65–0% B; 33–38 min, 0% B; 38–40 min 95% B; 40–48 min 95% B. The injection volume was 50 μL and the column was at 30 °C in column oven. A flow rate of 1 mL/min was used and detection was performed at 280 and 315 nm. External calibration curves of standards were performed in the concentrations of 2.5, 5, 10, 20, 50, and 100 ppm. Limit of detection (LOD) and limit of quantification (LOQ) were calculated according to the signal/noise (S/N) level of 3 and 9 respectively (Table 1).

2.4. Determination of phenolic compounds by HPLC-UV–MS/MS

Two different kinds of Mass Spectrometers (MS) were used for sample analyses. A Quattro electrospray ionization (ESI) triple quadrupole mass spectrometer (Micromass) connected to HPLC-UV system (Waters) was used for HPLC-UV–MS/MS analyses. Gradient elution was used for liquid chromatographic (LC) separation. Mobile phase A: 2% acetic acid in water, mobile phase B: 70% ACN in water, and, for the cleaning stage, mobile phase C: 100% ACN. Gradient was 0–3 min, 95% A; 3–8 min, % 95–92 A; 8–15 min, % 92–82 A; 15–20 min, % 82–80 A; 20–25 min, % 80–62 A; 25–35 min, % 62–25 A; 35–40 min, % 0 A, %0 B, 100% C; 40–50 min, 95% A; 50–65 min 95% A. Injection volume was 20 μL and column temperature was 25 °C. 17 Phenolic compounds, which were gallic acid, protocatechuic acid, *p*-hydroxy benzoic acid (*p*-OH benzoic acid), catechin, vanillic acid, chlorogenic acid, epicatechin, caffeic acid, syringic acid, *p*-coumaric acid, *trans*-ferulic acid, rutin, quercetin, luteolin, apigenin, kaempferol and rhamnetin were used to identify the phenolic compounds in methanol extracts of three types of extracts of the plant. Optimized negative ESI spectra were obtained using the following conditions: capillary voltage, 2.8 kV; cone voltage, 25 V; desolvation gas (N_2) flow of 400 L/h; cone gas flow of 50 L/h; desolvation temperature of 350 °C, capillary temperature of 110 °C; collision energy of 1. The spectra were recorded in the range of m/z 100–900.

An LCQ ion trap (IT) electrospray ionization-mass spectrometry (ESI–MS) instrument (Thermo Electron, Brussels, Belgium) with HPLC-UV system (Waters) was also used for HPLC-UV-IT–MS^2 analyses. Mobile phase A: 0.2% formic acid in water, mobile phase B: 70% ACN in water, and, for the cleaning stage, C: 100% ACN. Gradient was 0–2 min, 85% A; 2–10 min, 85–70%A; 10–20 min, 70–60%A; 20–40 min, 60–50% A; 40–45 min, 50–40%A; 45–50 min, 0% A, 0 B%, 100% C; 56 min,0%A, 0%B, 100% C; 56–60 min 85% A; 60–75 min 85% A. Injection volume was 20 μL and column temperature was 25 °C. Ultrahigh pure helium was used as the collision gas and high purity nitrogen (N_2) as the nebulizing gas. Optimized negative ESI spectra were obtained using the following conditions: Ion spray voltage of 4 kV; sheath gas (N_2), 70 arbitrary units; auxiliary gas (N_2) of 30 units; capillary temperature of 180 °C; capillary voltage of −32 V. For full scan MS analysis, the spectra were recorded in the range of m/z 150–800. The collision-induced dissociation (CID) energy was adjusted to 35%.

A Luna C18 column (250 × 2.00 mm i.d., 5 μm) Phenomenex, Torrance (USA) was used for all LC-UV–MS analyses. Since each LC–MS system has different gradient elution programme, retention times of the commonly detected compounds differ.

2.5. Determination of total phenolic content

Total phenolic contents (TPC) were analysed with the spectrophotometric method of Folin-Ciocalteu's phenol reagent, using gallic acid and catechinas the standard phenolic compounds (Singleton and Rossi, 1965). Briefly, 20 μL sample (1 mg/mL), 400 μL of 0.5 Folin-Ciocalteu regent and 680 μL of distilled water were mixed and vortexed. Following 3 min of incubation, 400 μL of Na_2CO_3 (10%) solution was added and vortexed. Absorbance was measured at 760 nm after 2 h. The quantity of TPC was calculated as mg of gallic acid equivalents (GAE) and mg of catechin equivalents (CE) per g of extract obtained from theregression equation of calibration curves of gallic acid and catechin in the concentration range between 0.015 and 0.5 mg/mL (r^2 = 0.999).