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Anthocyanins profiling of *Thymus praecox* opiz subsp. *caucasicus* var. *caucasicus*

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ABSTRACT

Thymus praecox Opiz subsp. *caucasicus* var. *caucasicus* (Anzer tea) was studied to determine its anthocyanin content; anthocyanin stability in response to acidic hydrolysis and temperature changes. In this study, anthocyanin profiling of the flowers of *T. praecox* Opiz subsp. *caucasicus* var. *caucasicus* (Anzer tea) were determined for the first time by high performance liquid chromatography coupled with ultraviolet detector (HPLC-UV). The effect of acidic hydrolysis on the extracts also was examined. While eleven peaks were observed before hydrolysis, they dropped to three peaks including that of cyanidin post-hydrolysis. Beside, cyanidin and pelargonidin were quantified in plant extracts. Since, the amount of cyanidin was greater than the cyanidin equivalent to the other two peaks (1.9 and 8.9 times) in the hydrolyzed extract, it can be concluded that the plant has mainly cyanidin derivatives such as glycosides. The stability of standard cyanidin in methanol with 1% HCl at −18 °C during a period of 63 days was determined to be above 78%. On the other hand, the concentration of the frozen and thawed standard (10 times) and samples dropped below 74% and 88%, respectively.

1. Introduction

Anthocyanins are structurally glycosides of polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium or flavylium salts within the group of flavonoids (Kong et al., 2003; Pina et al., 2015). These natural phenolic pigments have individual characteristics imparted by the number of hydroxyl groups, the position and number of sugars attached to the molecule, and number of aliphatic or aromatic acids attached to sugars in the molecule (Kong et al., 2003). The sugars such as glucose, galactose, rhamnose and arabinose attach generally at the C_3 position as shown for cyanidin-3-*O*-glycoside (Fig. 1) (Takeoka and Dao, 2002).

Besides their primary role of attracting animals for pollination and seed dispersal in plants, anthocyanins are involved in the defense mechanism of plants against insect attack (Kong et al., 2003). They exert their antioxidant effect by preventing free radicals from causing damage that is believed to form the basis of many diseases such as cardiovascular disease, myocardial infarction, cognitive decline, neural dysfunction, liver disorders, colon cancer and gastric cancer (Cassidy et al., 2013; Kahkonen and Heinonen, 2003; Kamei et al., 1998; Kong et al., 2003; Wang et al., 1997). They have also anti-inflamatory, antimutagenic, antitumor activities and strong inhibition on critical enzymes like glutathione reductase, cyclooxygenase, xanthine oxidase, lipid peroxidases, and α-glucosidase (Cassidy et al., 2013; Elliott et al., 1992; Kahkonen and Heinonen, 2003; Seeram et al., 2001; Tall et al., 2004; Tsuda et al., 1996; Wang et al., 1997, 1999).

The most commonly known anthocyanidins are pelargonidin, cyanidin, peonidin, delfinidin, petunidin and malvidin (Clifford, 2000). Because the flavylium cation has high reactivity with nucleophilic reagents; chemical structures of anthocyanidins are unstable in shifting pH, oxygen, light and heat (Amr and Al-Tamimi, 2007; Aramwit et al., 2010; SatueGracia et al., 1997; Tonon et al., 2010). Anthocyanidins in their flavilium forms are quite stable at pH 1–3 after 60 days at 10 °C. Their extraction yields are also highest in these conditions. Therefore acidic modifiers, for instance hydrochloric acid, formic acid or trifluoroacetic acid, are usually used for extraction and storage to avoid degradation (Amr and Al-Tamimi, 2007; Bjoroy et al., 2009; Cabrita et al., 2000; Fan et al., 2008; Park et al., 2014). Anthocyanidins are mostly stable around

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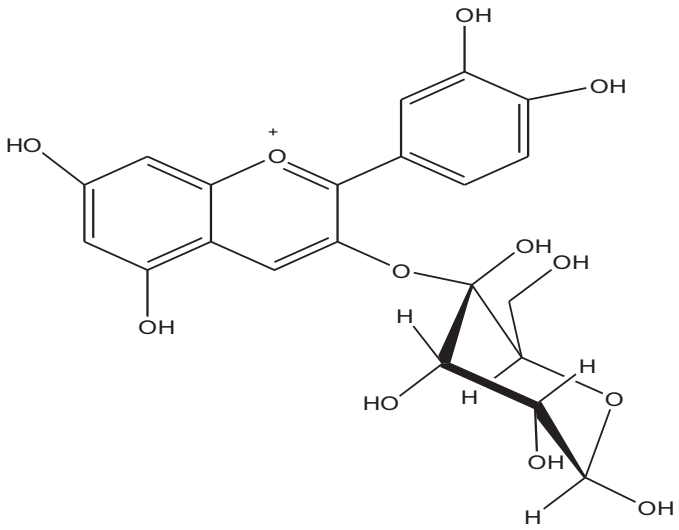

Fig. 1. Cyanidin-3-*O*-glycoside.

−20 °C (Gomez-Plaza et al., 2006; Withy et al., 1993). Almost all naturally occuring anthocyanins have *O*-linkages and are less stable than their *C*-linkage containing synthetic counterparts (Bjoroy et al., 2009).

Thymus praecox subsp. *caucasicus* var. *caucasicus, also* known as Anzer tea, is a kind of thyme in *Lamiaceae* family with highly aromatic pinkish-white to purple flowers (Diaz-Garcia et al., 2015). Family members are being used in pharmacology and perfume industry because of their influential essential oils and aromatic compounds (Orhan et al., 2009). Their essential oils possess antioxidant, antiaflatoxigenic, antimicrobial, antifungal and antibacterial activities (Bounatirou et al., 2007; Pinto et al., 2006; Razzaghi-Abyaneh et al., 2009; Rota et al., 2008; Youdim and Deans, 1999). Anzer tea is one of the herbs constituting Anzer honey and is used as a medicine by local people. Its antioxidant property has been described in recent studies (Boros et al., 2010; Orhan et al., 2009; Turumtay et al., 2014). In our previous study, phenolic acids (caffeic acid, syringic acid, ferulic acid, *p*-coumaric acid, protocatechuic acid, *p*-hydroxybenzoic acid, and vanillic acid) and flavonoids (catechin, epicatechin, kaempferol and quercetin) were identified and quantified in the Anzer tea extracts by HPLC-UV (Turumtay et al., 2014). The plants from the *Lamiaceae* family are rich for anthocyanins as well (Phippen and Simon, 1998; Saito and Harborne, 1992).

To our knowledge, the anthocyanin composition of the Anzer Tea has not been reported so far. In this study, the anthocyanin content of flowers of Anzer tea was identified by HPLC-UV. Stability of cyanidin in the methanol with 1% HCl was also investigated according to time and temperature.

2. Materials and methods

2.1. Chemicals and solvents

Analytical grade delphinidin chloride, cyanidin chloride, apigeninidin chloride, pelargonidin chloride and malvinidin chloride purchased from Sigma–Aldrich (St. Louis, MO, USA). HPLC grade acetonitrile, methanol, formic acid and hydrochloric acid were from Merck (Darmstadt, Germany).

2.2. Sample preparation

The Anzer tea was collected from the Anzer Plateau (2300 m) in Ikizdere (Rize, Turkey) in July 2012. The specimens were dried at room temperature and identification was performed in September 2012 by Professor Salih Terzioğlu from the Department of Faculty of Forestry, Karadeniz Technical University in Trabzon, Turkey. The purple flower part of the plant was separated and ground in a blender (Waring Commercial, CT, USA). Moisture of dried flower samples was determined as 9.86% by incubating at 100 °C for 3 h in oven.

Since a number of studies reported that the stability of anthocyanins is best at pH 1 (Bąkowska et al., 2003; Cabrita et al., 2000; Ma et al., 2012), extraction was performed by two methods (A & B) with 1% hydrochloric acid (HCl) containing solutions as detailed below.

Extraction A: 1 g of plant sample mixed with 20 mL of the solution consisting of 80% methanol, 19% water, 1% HCl over night at room temperature in the dark and filtered. The extract was separated into 2 parts (A1 and A2). Solvents of both parts were evaporated by a rotary evaporator (Heidolph, Laborota 4000, Schwabach, Germany). A1 was resuspended in 5 mL of 3% formic acid in water while A2 was dissolved in 6 mL of 1% HCl in methanol and they were filtered prior to HPLC-UV analysis.

Extraction B: 0.25 g sample and 5 mL of 1% HCl in methanol were mixed for 2 h on a shaker at 45 °C in the dark and the extract was filtered. 300 μL of extract, 900 μL of methanol, 285 μL of water and 15 μL of HCl were mixed (80% methanol, 19% water and 1% HCl) and filtered prior to HPLC-UV analysis (B1). 2 mL of this extract was subjected to acidic hydrolysis with 4 mL of 2N HCl solution for 2 h at 100 °C. 300 μL of hydrolyzed extract, 900 μL of methanol, 285 μL of water and 15 μL of HCl were mixed and filtered prior to HPLC-UV analysis (B2).

2.3. Determination of anthocyanidins in Anzer tea extracts by HPLC-UV

HPLC-UV analyser was used with a UV–vis detector (Thermo Finnigan, San Jose, CA, USA) supplying double wavelength simultaneously. ChromQuest manager software was used with this instrument. Analyses were performed on a C18 reverse phase column (150 mm × 4.6 mm, 5 μm particle; ACE) using a gradient elution with minor modifications of the method described by (de Villiers et al., 2011).

The eluents were 5% aqueous formic acid solution (A) and 5% formic acid in acetonitrile (B). The elution gradient established was: 0–3 min 10% B; 3–8 min 10–16% B; 8–12 min 16–20% B; 12–15 min 20–25% B; 15–20 min 25–30% B; 20–30 min 30–10% B; 30–40 min 10% B. The sample running time was 40 min. The injection volume was 25 μL, the column temperature was 30 °C and the flow rate was 1.0 mL min^{-1}. Five anthocyanidin standards were analyzed at 520 nm.

Stock solutions of delphinidin chloride, cyanidin chloride, apigeninidin chloride, pelargonidin chloride and malvinidin chloride were prepared in methanol at 100 mg L^{-1} for the quantitative analysis. The stock solutions contained 1% HCl and 19% water for preservation of stability. Stock solutions were diluted 1:19:80 (v/v), respectively with HCl:H_2O:MetOH to the concentrations of 0.5, 1, 3, 5, 10, 20 and 30 mg L^{-1} for external calibration curves.

Limit of detection (LOD) values were calculated according to the EPA method as an S/N level of 3 and limit of quantification (LOQ) was calculated as an S/N level of 10. 0.5 mg L^{-1} of five standards were prepared and the mixture was injected 7 times to verify the LOD and LOQ of each compound. The percentage relative standard deviation (RSD %) of peak areas and retention times were calculated to validate the reproducibility of the method.