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Original Article

Quantification of major phenolic and flavonoid markers in forage crop *Lolium multiflorum* using HPLC-DAD

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ABSTRACT

The objective of this study was to perform preliminary screening of phytochemical compounds and quantification of major phenolics and flavonoid markers in Italian ryegrass extract using HPLC-DAD. Previously, LC–MS analysis has identified different phenolic acids, including caffeic acid, ferulic acid, *p*-coumaric acid, chlorogenic acid, dihydroxy benzoic acid, propyl gallate, catechin, and six flavonoids including rutin hydroxide, luteolin, kaemferol, vitexin, narcissoside, and myricetin from Italian ryegrass extract. In the present study, Italian ryegrass silage powder was extracted with ethanol: water for 20 min at 90 °C. The extract targeted optimum yield of phenolic acids and flavonoids. Crude phenolic acid and flavonoids were then purified by solid phase extraction method. Purified fractions were then injected into HPLC with a diode-array detector. Quantified concentrations of isolated phenolic acids and flavonoids ranged from 125 to 220 μg/g dry weight. Limits of detection and limits of quantification for all standards (unknown compounds) ranged from 0.38 to 1.71 and 0.48 to 5.19 μg/g dry weight, respectively. Obtained values were compared with previous literatures, indicating that our HPLC-DAD quantification method showed more sensitivity. This method showed better speed, accuracy, and effectiveness compared to previous reports. Furthermore, this study could be very useful for developing phenolic acids and flavonoids from compositions in Italian ryegrass silage feed for pharmaceutical applications and ruminant animals in livestock industries.

Introduction

Lolium multiflorum Lam., Poaceae, commonly known as Italian ryegrass, has been widely used as a feed resource for ruminants. *L. multiflorum* is an excellent wildlife feed that provides high nutritional value in silage for domestic animals (Campagnoli and Dell'Orto, 2013). *L. multiflorum* grass also has various environmental benefits. It can increase nitrogen fixation from the atmosphere and control soil erosion by withstanding winter damage and high salinity conditions. It is widely grown in many regions such as Europe, America, and Asia. In South Korea, *L. multiflorum* is mainly cultivated for silage purposes and as feed for domestic and live stock animals and is one of common forage crops. It accounts for more than 60% of feed sources in livestock industries. Wild type *L. multiflorum* can be grown in different climatic conditions, particularly in cold temperatures and various types of soils and moisture environment (Choi et al., 2011; Buono et al., 2011). *L. multiflorum* based feed (silage) is less expensive compare to other commercial feed. It can also be more easily cultivated than other forage crops. Thus, it is widely used in different forms of feed such as hay, silage, haylage, baleage, and green chop.

Italian ryegrass, *L. multiflorum*, produces different stimulatory chemicals within the plant that could enhance feed intake in lactating cows (Baldinger et al., 2012). It derived bioactive compounds such as phenolics, flavonoids, alkaloids, sugars, proteins, fatty acids, terpenoids, and organic acids may also enhance feed intake and growth of ruminants with human health benefit (Dohi et al., 1997; Robbins, 2003). We have previously reported that *L. multiflorum* ethanolic extract contains phenolic acids, flavonoids, anthocyanins, and volatile oil that may act as potent antimicrobial substances

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against pathogenic bacteria (Valan Arasu et al., 2014; Choi et al., 2017). These metabolites can also act as more potent antioxidant compounds than other commercial drugs. Thus, *L. multiflorum* extract could have potential as a natural drug to treat infectious diseases.

Phytochemicals are derived from plants that require passable quality and quantity in herbal medicines to control various epidemic diseases (Fan et al., 2006; Ghasemzadeh and Ghasemzadeh, 2011). In particular, a large group of phenolic acids and flavonoids have been found in many plant species. They possess different biochemical functions such as chemical stability, color, flavor balance, bitterness, and micro-biologic control in plants. Besides, polyphenols are important compositions in plants with different biochemical functions for instant ripening, variety, and the ability to withstand atmospheric stress conditions and growth production (Pereira et al., 2010). The chemical structure of phenolic acid may contain one or more hydroxyl groups in the phenyl ring. Plant phenolic acids have a variety of subclasses such as benzoic acid (C6-C1) and cinnamic acid (C6-C3). Conversely, flavonoids belong to a cluster of polyphenols. They are grouped into four main classes according to the position of the aromatic ring in benzopyran moiety. Plants derived flavonoids are classified into different subclasses including flavonol, flavone, flavanone, flavanol, isoflavone, and anthocyanin (De Rijke et al., 2006). Chemical structures of flavonoids include a C15 (C6-C3-C6) skeleton joined to a phenyl-benzopyran ring. Plant synthesized flavonoids also possess anticancer, antioxidant, and anti-inflammatory activities in *in vitro* and *in vivo* studies. Both phenolic acids and flavonoids from natural sources are beneficial for human health by scavenging free radicals, chelating trace metals, and inhibiting bacteria growth, viral translation, and their enzymatic functions (Naczk and Shahidi, 2004). It is essential to learn amounts and varieties of plant derived metabolites due to their medicinal and pharmaceutical values.

HPLC-DAD is a rapid and precise technique for quantification of individual compounds. HPLC is a commonly used analytical technique for separation and identification of biological substances in a mixture solution. HPLC system combines with high-resolution DAD/UV absorption detection can lead to easy automation with modest sample requirements. HPLC column is important to allow efficiencies in separation and greater resolution. An HPLC column with small particle size and length may increase efficiency in the separation method (Khoddami et al., 2013; Magwaza et al., 2016). Commonly, reversed phase C18 columns and a binary solvent system including water and a polar organic solvent (acetonitrile or methanol) and DAD for detecting the absorption in UV–Vis regions are used. These conditions may change according to chemical nature and structural forms of different phenolic acids and flavonoid compounds (Burin et al., 2011). Some phenolic compounds isolated from wine show typical absorbance peak at 260–280 nm. This range of absorption wavelength is suitable for detection of a large number of phenolic acids and flavonoids compounds by HPLC technique because of its versatility and accuracy (De la Torre-Carbot et al., 2005; Francisco and Resurreccion, 2009).

In the present study, we aimed to separate, identify, and quantify major phenolic acids and flavonoids in *L. multiflorum* extract using HPLC-DAD. Extraction and separation of phenolic acids and flavonoids in Italian ryegrass were optimized by using different solvent ratios, temperatures, and time to achieve high yield and single compounds. The HPLC-DAD method for quantification of these secondary metabolites from *L. multiflorum* extract was also validated. The validation of chromatographic identification of phenolic acids and flavonoids from plants may contribute to the standardization of crude extracts and drug development process.

Materials and methods

Chemicals used

HPLC grade solvents acetonitrile, water, formic acid (Merck, South Korea) were used as mobile phase. They were filtered through 0.45 μm membrane filter (Millipore, USA) and degassed prior to use. Ethanol, methanol, DMSO, and water used for extraction were purchased from Sigma Aldrich Corporation (USA). Phenolic acids standards (including caffeic acid, ferulic acid, *p*-coumaric acid, chlorogenic acid, dihydroxy benzoic acid, propyl gallate, and catechin) and flavonoids standards (including rutin hydroxide, luteolin, kaemferol, vitexin, narcissoside, and myricetin) at purity of 99.9% HPLC grade were purchased from Sigma Chemical Co. (USA). All glassware and plastic accessories were sterilized and used for extraction and separation experiments.

Plant material

The aboveground of healthy plant *Lolium multiflorum* Lam., Poaceae, for livestock was procured from National Institute of Animal Science (NIAS), Seonghwan, South Korea at late blooming stage of June 2016. It was taxonomically identified by plant taxonomist at NIAS, Jeonju campus. Plant voucher specimen for the sample was prepared and stored in Grassland and Forage Division for future reference. Its plant voucher specimen number was KCC FPVN002. Collected plant was transported in several plastic bags, cleaned well, and sterilized at a hot air-oven at 60 °C. Dried plant material was powdered using a mechanical grinder. Powdered sample was sealed under vacuum and stored at room temperature for further experimental studies.

Extraction and separation procedure

Phenolic acid and flavonoids were extracted from *L. multiflorum* according to the method described by Kao et al. (2008) with slight modifications of physio-chemical parameters. *L. multiflorum* phenolic acids and flavonoids extraction, isolation, and separation procedure are shown in flow chart (Fig. 1).

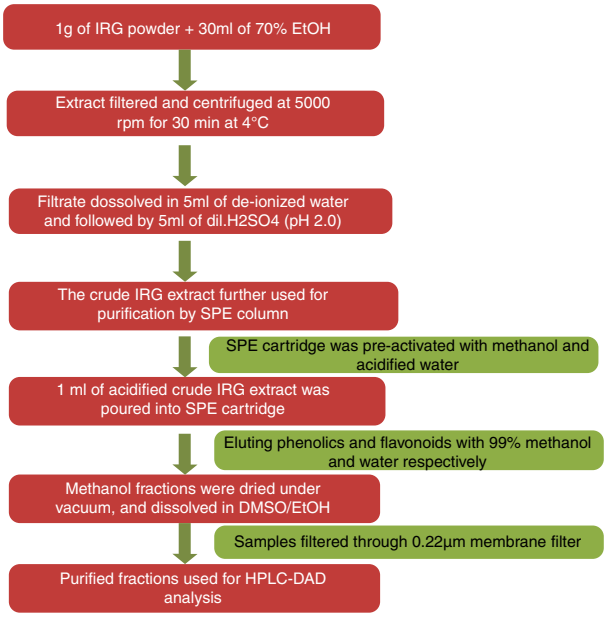

Fig. 1. Flowchart illustrating the extraction and separation of phenolics acids and flavonoids in *Lolium multiflorum* sample.

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