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Original Article

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

Palaniselvam Kuppusamy[a,†], Kyung Dong Lee[b,†], Chae Eun Song[c], Soundharrajan Ilavenil[a], Srisesharam Srigopalram[a], Mariadhas Valan Arasu[d], Ki Choon Choi[a,*]

[a] Grassland and Forage Division, National Institute of Animal Science, Rural Development Administration, Cheonan, Republic of Korea
[b] Department of Oriental Medicine Materials, Dongsin University, Naju, Republic of Korea
[c] Lifelong Education Center, Chonnam National University, Kwangju, Republic of Korea
[d] Department of Botany and Microbiology, Addiriyah Chair for Environmental Studies, College of Science, King Saud University, Riyadh, Saudi Arabia

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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

* Corresponding author.
E-mail: choiwh@korea.kr (K.C. Choi).
† These authors have contributed equally to this work.

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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).

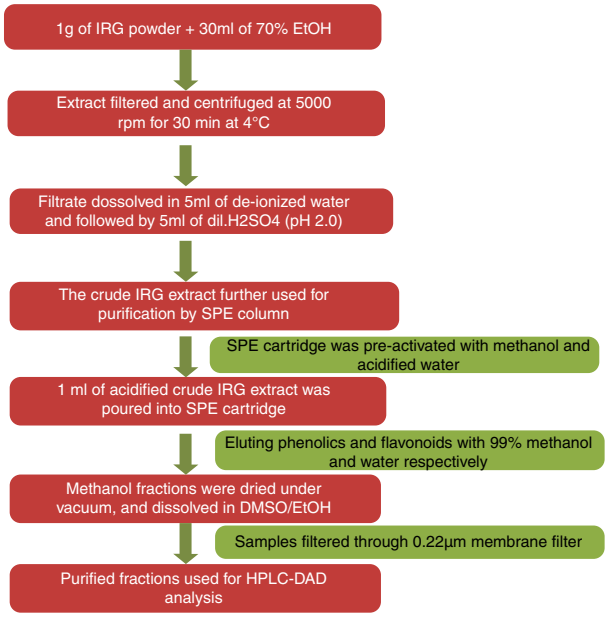

Fig. 1. Flowchart illustrating the extraction and separation of phenolics acids and flavonoids in *Lolium multiflorum* sample.

Total phenolic content (TPC) analysis for Lolium multiflorum *extract*

Total phenolic content in *L. multiflorum* extract was determined using the Folin Ciocalteu (FC) method described previously by Shirazi et al. (2014) with slight modifications. *L. multiflorum* silage extract was prepared in aliquots at concentration of 1 mg/ml. A calibration curve was prepared using gallic acid (1–0.05 mg/ml). *L. multiflorum* extract or gallic acid (1.5 ml) was added to 0.5 ml of FC reagent (3-fold diluted with distilled water), vortexed well, and allowed to stand at room temperature for 5 min. Sodium carbonate (1 ml, 7.5% w/v) was added to the mixture and kept at room temperature for 60 min. The absorbance of the mixture was then measured at wavelength of 760 nm. TPC was expressed as mg gallic acid equivalents (GAE)/g sample.

Total flavonoid content (TFC) analysis for Lolium multiflorum *extract*

Total flavonoid content (TFC) of *L. multiflorum* extract was studied using the aluminum chloride colorimetry method described by Chavan et al. (2013) with minor modifications. Briefly, *L. multiflorum* extract was prepared at different concentration (1, 0.5, 0.1, 0.05 mg/ml). A standard calibration curve was constructed using quercetin in different concentrations (0.05–1 mg/ml). *L. multiflorum* extract or quercetin (2 ml) was mixed with 500 μl of 10% aluminum chloride solution and 500 μl of 0.1 mM sodium nitrate solution. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at wavelength of 430 nm using a UV-VIS spectrometer. The amount of flavonoids was expressed as milligram quercetin equivalent per gram *L. multiflorum* extract (mg QCE/g).

HPLC instrumentation and separation conditions

Chromatography study was performed on an HPLC-DAD (Agilent Technologies 1100 series, CA, USA). Briefly, 10 μl of sample was injected using an HPLC system equipped with G1315A auto sampler and then separated on an Agilent Zorbax SB-C18 column (5 μm particle size, 4.6 × 150 mm, CA, USA) maintained at 38 °C. A binary gradient solvent system of (A) formic acid in distilled water (95:5; v/v) and (B) acetonitrile in distilled water (98:2; v/v) was used as shown in Table 1. Peak purity and absorbance were automatically detected by a DAD (UV–Vis) detector at 240 nm. Purified fractions of phenolic acids and flavonoids were identified by matching retention time and spectra of standards with unknown peaks. External standards were used for the identification and quantification of compounds. Results are expressed as μg/g of dry weight (DW).

Quantification of Lolium multiflorum *phenolic acids and flavonoids*

Quantification of phenolic acids and flavonoids was performed by using different internal standards such as catechin and kaempferol. Stock solutions of phenolic acids and flavonoids standards were prepared at 1 mg/ml after dissolving in DMSO and ethanol, respectively. For quantification of phenolic acids, four mass concentrations (1, 0.5, 0.25 and 0.125 mg/ml) were prepared separately for caffeic acid, ferulic acid, *p*-coumaric acid, chlorogenic acid, dihydroxy benzoic acid, propyl gallate, and catechin. They were then mixed to make a final concentration of 1, 0.5, 0.25, or 0.125 mg/ml. Likewise, four concentrations (1, 0.5, 0.25, and 0.125 mg/ml) of flavonoid standards such as rutin hydroxide, luteolin, kaemferol, vitexin, narcissoside, and myricetin were prepared separately. They were then mixed to obtain a final concentration of 1, 0.5, 0.25, or 0.125 mg/ml for all standards. Then 10 μl of the sample was analyzed in the HPLC-DAD system.

Concentrations of phenolic acids and flavonoids were calculated by preparing a calibration curve of mass concentration *vs.* peak area. Slope of the regression line and values of correlation coefficient (R^2) for each standard curve were obtained by using MS Excel 2007 software (Inbaraj et al., 2010). Regression equations for standards and purified fractions of phenolic acids and flavonoids were calculated. Purified fractions of phenolic acids and flavonoids in *L. multiflorum* were quantified using the following equation:

$$\text{Amount of phenolic acids/flavonoids } (\mu g/g) = \frac{\text{Amount}_{is} \times \text{Amount}_{sc} \times \text{IRF}_{sc}}{\text{Area}_{is}}$$

where internal response factor (IRF) is

$$\text{IRF} = \frac{\text{Area}_{is} \times \text{Amount}_{sc}}{\text{Amount}_{is} \times \text{Area}_{sc}}$$

and is = internal standard; sc = separated phenolic acids and flavonoids from *L. multiflorum*.

Quality control

Limits of detection (LOD) and limits of quantification (LOQ) were calculated for each sample in triplicates. Calibration curves were obtained consecutively by plotting concentration against peak area. The mean of the slope (S) and standard deviation of intercept (σ) were calculated from the standard curve of three replicates. LOD and LOQ were calculated with the following equations:

$$\text{LOD} = 3.3 \times (\sigma/S) \quad (1)$$

$$\text{LOQ} = 7 \times (\sigma/S) \quad (2)$$

The accuracy was determined through recovery (%) by preparing two different concentrations (1 and 10 mg/ml) of reference standard with 10 mg of *L. multiflorum* sample. The percentage of recovery of each standard was calculated based on the ratio of the standard concentration after and before HPLC (changed amount minus original amount) (Q2B CH, 1996).

Statistical analysis

All analyses were carried out in triplicates. Data are expressed as mean ± standard deviation (SD). Slopes and intercepts of calibration graphs were calculated by linear regression. Correlation coefficient (R^2) values were obtained from MS Office Excel 2007 software.

Results and discussion

Determination of TPC and TFC in Lolium multiflorum *silage extract*

Total phenolic content of *L. multiflorum* extract was measured using the FC method. TPC value in *L. multiflorum* extract was 21.54 ± 0.36 mg GAE/g (Table 2). This result was similar to TPC in extract of *Ceropegia* species and a variety of edible common herb

Table 1
HPLC-DAD gradient solvent system for phenolic acids and flavonoids separation.

Time/min	Solvent A (%)	Solvent B (%)
0–2	98	2
2–25	90	10
25–40	85	15
40–48	80	20
48–68	75	25

Table 2
Total phenolic content and total flavonoid content of *Lolium multiflorum* silage extract.

Phytochemical assays	*L. multiflorum* ethanolic-water crude extract
Total phenolic content (TPC) GAE/5 g	19.54 ± 0.36
Total flavonoid content (TFC) QUE/5 g	34.16 ± 0.54

species (Chavan et al., 2013; Shirazi et al., 2014). TFC in *L. multiflorum* extract was 34.16 ± 0.54 mg QUE/g (Table 2). Moreover, the amount of TPC/TFC fluctuated depending on the solvent system, species/cultivars, and various parts of the plant.

Quantification of phenolic acids and flavonoids using HPLC-DAD

HPLC analysis enables simultaneous separation and identification of a broad range of phenolics acids and flavonoids from a plant sample. We found that a binary solvent system in gradient mode could determine both phenolic acids and flavonoids from *L. multiflorum* extract. For example, a binary solvent system with formic acid and acetonitrile obtained good peak resolution and symmetry due to buffering in the solution. When 1% phosphoric acid instead of formic acid was used, it caused inability to detect many phenolic and flavonoids peaks in the sample due to its low pH and isoelectric point. Parameters for the gradient mobile phase are described in Section 2.3 and Table 1. Solvents play important roles in extraction efficiency and yield of extract. For instance, various combinations of ethanol and water delivered the highest extraction yield with lower concentration of ethanol extract. Ethanol and water also have less toxicity with easy availability for *in vivo* assays. Similarly, Numonov et al. (2015) have compared extraction yields with various compositions of aqueous ethanol. They found that 80% ethanol extraction delivered the highest extraction yield (3.76 mg/g) among tested solvents. Increased solvent polarity may affect quantification of highly polar phenolics groups such as rutin, quercetin, and apigenin in the extraction sample. However, 80% aqueous ethanol could be an effective solvent for extraction due to its functional groups. It can also lead to the maximum groups of bioactive metabolites in the crude extract (Seal, 2016).

Separations were accomplished by gradient elution with mobile phases of formic acid, phosphoric acid, methanol, water, and acetonitrile. Formic acid was used as a good volatile mobile phase due to its buffering capacity in pH range of 2–4 (Jin et al., 2008). Moreover, 0.1% formic acid was efficient for separating phenolic acids and flavonoids. A gradient elution system is mainly used to ensure efficient separation of compounds from a mixture. It also extends the lifetime of a column. Eluent compositions by modifying solvent ratio, elution time, and concentration of eluent may influence the separation of analyte. Therefore, the elution mode should be simple and robust (Pulkkinen et al., 2015). HPLC system using reverse phase C18 columns can produce promising results with most common eluents. Mobile phase of acetonitrile and acidified water (with acetic acid, 1% v/v; pH 3.0) instead of methanol and phosphoric acid can give high polarity and solubility. HPLC gradient solvents of acetonitrile with water could give better peak resolution and sharp and symmetrical peaks with minimal noise that help precise measurement of the peak area ratio.

Typical HPLC chromatograms of phenolic acid and flavonoid standards mixture recorded at 240 nm are shown in Fig. 2A and B *L. multiflorum* purified fraction sixteen contains numerous phenolic and flavonoid peaks (Fig. 3). Of thirteen major peaks shown in Fig. 2, seven peaks belonged to phenolic acids (caffeic acid, ferulic acid *p*-coumaric acid, chlorogenic acid, dihydroxy benzoic acid, propyl gallate, catechin) while six peaks belonged to flavonoids (rutin hydroxide, luteolin, kaemferol, vitexin, narcissoside, myricetin) by comparing their retention times with respective standards and literature data. Amounts of these seven phenolic acids and six flavonoids were quantified by constructing external calibration curves compared with authentic standards. SPE cartridge was used for purifying phenolic fractions identified by HPLC-DAD with corresponding internal standards. These purified fractions showed the presence of phenolic acid derivatives such as caffeic acid, dihydroxy benzoic acid, and *p*-coumaric acid. Thus, phenolic and flavonoid derivatives were present in *L. multiflorum* extract. They were identified by comparing retention time with previously published literature.

HPLC-DAD quantification of phenolic compounds (phenolic acids and *C*-glycosylflavonoids) from *Cecropia* species has been achieved in a single run with a relatively long run time (40 min) (Costa et al., 2011). Only *C*-glycosyl-flavonoids showed better separation of all peaks compared to a short time run (Costa et al., 2011). On the other hand, Vagiri et al. (2012) have reported RP-HPLC-DAD chromatographic separations of different organic acids and polyphenol compounds in wine sample without sample pretreatment. DAD detection is the most common method to quantify several plant derivatives such as aromatic acid (hydroxycinnamic acid), flavonoids, and anthocyanins with good resolution and separation of compounds in black currant sample. HPLC-DAD method has been optimized for simultaneous quantification of phenolic acids and flavonoids in fruit sample.

HPLC-DAD identification and quantification may also accurately determine each compound based on peak area and concentration of plant derivatives. Phenolics and flavonoids at concentrations of 104 mg/100 ml and 13 mg/100 ml, respectively, in plant extract with a mixture of individual peaks at 280 nm have been reported (Badalica-Petrescu et al., 2014). Gomez-Alonso et al. (2007) have obtained HPLC chromatograms of fruit extract at different wavelengths (280, 320, 360, and 520 nm). The chromatogram corresponded to excitation at 280 nm and emission at 320 nm. Although non-flavonol compounds including pigments were absorbed at this wave length, from red wines and red grape samples.

Among thirteen phenolic acids and flavonoids identified in *L. multiflorum*, *p*-coumaric acid was present with the largest mass fraction (219.69 μg/g) in phenolic acids, followed by ferulic acid (207.05 μg/g), chlorogenic acid (199.75 μg/g), dihydroxy benzoic acid (187.5 μg/g), propyl gallate (181.73 μg/g), caffeic acid (126.8 μg/g), and catechin (131.52 μg/g), while myricetin (198.05 μg/g) had the largest mass fraction among flavonoids, followed by rutin (192.7 μg/g), luteolin (180.5 μg/g), vitexin (172.1 μg/g), kaempferol (160.7 μg/g), and narcissoside (122.8 μg/g) (Table 3). The remaining unknown peaks were not characterized. Their identities require further study. HPLC-DAD/UV instruments use wavelength switching for simultaneous detection of phenolic and flavonoids compounds. They provide more accuracy with fewer noise peaks than using constant absorbance at 280 nm. Phenolic group of metabolites can provide response to UV range with different absorption intensities for respective structural characteristic and functional groups (Plazonić et al., 2009). Therefore, selection of suitable detection wavelengths for each analyte should be critically considered. A single one is inadequate for simultaneous determination of different compounds in plant extracts. Phenolic and flavonoid metabolites exhibit more than one absorption peaks in their DAD spectra except some phenolic compounds. It seems that wavelength range of 230–280 nm is mostly used for simultaneous determination of different phenolic compounds from plant materials (Zhang et al., 2013).

Previously, we have reported that *L. multiflorum* extract is rich in phenolic acids, flavonoids, proteins, and their derivatives (Choi et al., 2017). They might exert adipogenesis activity *in vivo* with antioxidant and antiseptic properties (Choi et al., 2017). On the

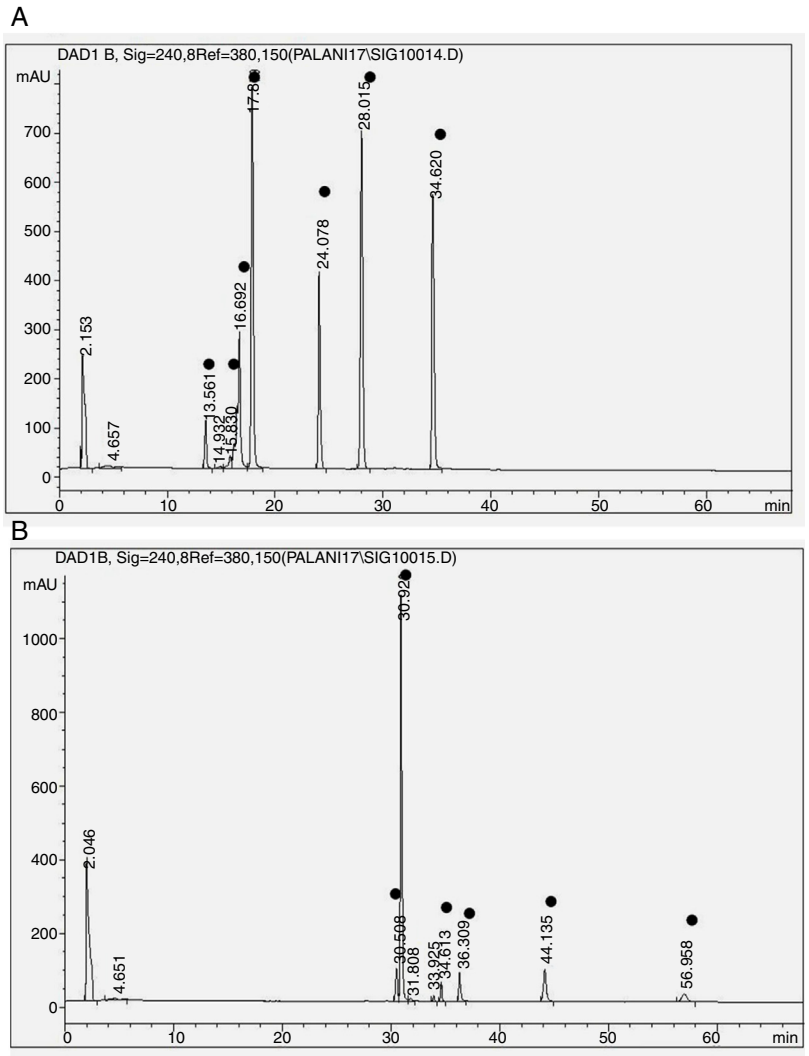

Fig. 2. HPLC chromatograms of internal standards. (A) Chromatogram of phenolic acids standards (*from left to right, caffeic acid, ferulic acid, *p*-coumaric acid, chlorogenic acid, dihydroxy benzoic acid, prophyl gallate, catechin). (B) Chromatograms of flavonoids standards (*from left to right, rutin hydroxide, luteolin, kaemferol, vitexin, narcissoside, myricetin).

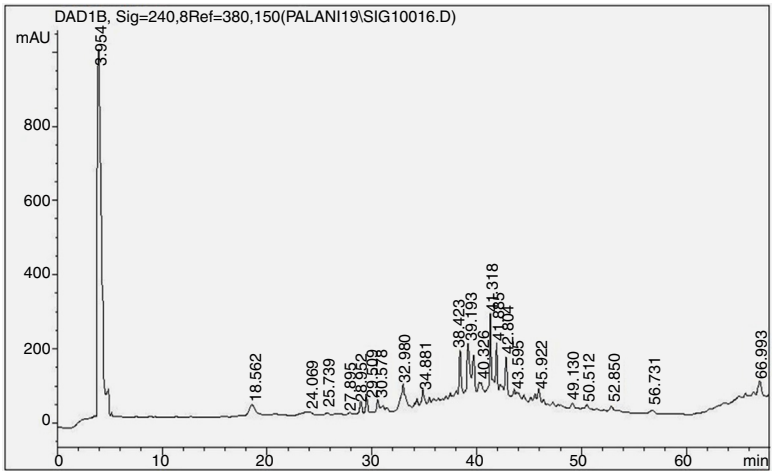

Fig. 3. HPLC chromatogram of simultaneous separation of phenolic acids and flavonoids in the extract of *Lolium multiflorum.*

Table 3
Identification data (retention time, recovery, LOD, and LOQ) of phenolic acids and flavonoids in the extract of *Lolium multiflorum*.

Peak No.	Compounds	λ (nm)	RT (min)	Standard curve (regression equation)[a]	R^2	Recovery (%)	LOD (μg/ml)	LOQ (μg/ml)
Phenolic acids								
1	Caffeic acid	260	15.4	$y = 276.61x - 24.87$	0.99	95.178	1.71	5.19
2	Ferulic acid	260	27.5	$y = 39.499x - 7.6522$	0.99	90.079	0.39	1.20
3	*p*-Coumaric acid	260	23.3	$y = 190.98x - 14.957$	0.99	93.984	1.02	3.10
4	Chlorogenic acid	260	16.5	$y = 950.53x - 162.09$	0.99	90.527	0.21	0.65
5	Dihydroxy benzoic acid	260	13.3	$y = 47.005x - 8.087$	0.99	91.023	0.44	1.36
6	Prophyl gallate	260	34.1	$y = 245.77x - 13.565$	0.99	96.874	1.60	4.86
7	Catechin	260	16.3	$y = 26.845x - 4.087$	0.99	90.628	0.67	2.05
Flavonoids								
8	Rutin hydroxide	260	30.7	$y = 162.41x - 46.783$	0.99	92.139	1.33	4.04
9	Luteolin	260	44.1	$y = 332.94x - 24.652$	0.99	94.418	1.15	3.48
10	Kaemferol	260	56.9	$y = 195.98x - 9.6522$	0.99	90.255	0.15	0.48
11	Vitexin	260	30.4	$y = 244.51x - 19.13$	0.99	94.454	0.38	1.16
12	Narcissoside	260	34.5	$y = 368.06x - 53.261$	0.99	90.632	0.75	2.28
13	Myricetin	260	36.2	$y = 104.17x - 0.7826$	0.99	99.987	1.06	3.21

[a] Peak area *vs.* concentration (amount of extract).

other hand, *L. multiflorum* forage crop has been consistently used as a nutritional feed for live stock ruminants and domestic animals. It may increase meat and milk production in ruminants due to its high nutritional value (NRC, 1996). *Lolium multiflorum* also contains various phenolic compounds that may decrease live stock animal diseases. Mocan et al. (2014) have found that hydroxycinnamic acids derivatives such as caffeic acid, ferulic acid, and *p*-coumaric acid possess higher antioxidant activities than commercial drugs. On the other hand, *L. barbarum* fruit contains flavonoid compounds such as kaemferol, quercetin, and their derivatives that may possess overall biological activity such as antibacterial, anticancer and anti aging.

Validation of HPLC method

A standard curve was constructed for each internal standard by plotting the concentration of the standard (mg/ml) against the peak area at specific wavelength of 240 nm. Slopes and intercepts of calibration curves, recovery percentage, and limits of detection and quantification are shown in Table 2. All internal standards and samples gave almost linear calibration curves through zero point. From Eq. (1), the limit of quantification calculated for phenolic acids and flavonoid compounds was the highest for caffeic acid (5.19 μg/g) but the lowest for chlorogenic acid (0.65 μg/g) (Table 2). LOD results are comparable or lower than those found in reported studies. From Eq. (2), quantitation limits of caffeic acid, chlorogenic acid, p-coumaric acid, propyl gallate, rutin, and kaempferol were determined to be 5.19, 0.65, 3.10, 4.86, 4.04, and 0.48 μg/g, respectively. On the other hand, recovery percentages of caffeic acid, chlorogenic acid, *p*-coumaric acid, rutin, and kaempferol-3-*O*-rutinoside were calculated to be more than 90%. Average recovery of less than 100% might be due to high temperature used for the extraction (90 °C) to speed up extraction of phenolic compounds within a short time.

Method linearity was calculated by plotting peak area ratio (A) *vs.* analyte concentration (C in mg/ml) to obtain calibration curves. For the limit of detection (LOD), signal to noise ratio of approximately three is normally considerable to calculate LOD range. For the limit of quantification, a typical signal–noise ratio of seven replicates is the lowest concentration of compounds that can be measured with accuracy and precision (Zhang et al., 2013). Pereira et al. (2010) have reported HPLC-DAD quantitative analysis of polyphenols in wine samples. The lowest LOD were in the range of 0.07–0.49 mg/l for cinnamic and gallic acids. Since these LOD are very low, it is realistic to conclude that this method can be used for quantification of phenolic acids. Similarly, Burin et al. (2011) have studied phenolic compounds in Brazilian wine sample quantified by HPLC-DAD and reported that there are variations in phenolic quantity of wine samples depending on geographical origins. Temperature and climate may affect the content of these compounds in the product. However, phenolic standards in a synthetic sample and red wine showed recovery percentage of 80–120%. This indicates that HPLC-DAA method is accurate and acceptable for quantification of phenolic compounds. LOQ values for phenolic acids and flavonoids were very small, ranging from 1.35 to 5.02 and 4.51–16.72 μg/ml, respectively (Table 3). RSD values for all retention times ranged from 0.45–1.67, indicating good stability. This confirms that the HPLC-DAD method is precise and accurate (Pereira et al., 2010).

Moreover, our validation method is better regarding separation of different phenolics and flavonoids molecules with a total run time of 68 min compared to other reports (Chen et al., 2012). The HPLC-DAD method with various gradient mobile phase conditions identified a total of nine phenolic acids and four flavonoids standard in two fractions with a total run time of 72 min. However, a poor resolution occurred when this was applied to cranberry juice. The validation analysis performed using the lowest concentration range gave a visible signal with LC instrumentation. Obtained values are between 0.3 mg/g for LOD and 0.1 mg/g for LOQ for phenols with an HPLC-DAD equipped amide-embedded phase (Restivo et al., 2014). LOD and LOQ for caffeic acid, cis-caftaric acid, 3,5-di-caffeoylquinic acid, chlorogenic acid, chicoric acid, luteolin-7-*O*-glucoside and quercetin all ranged from 50 to 300 ng/ml. Obtained recovery data correlated well with those reported in the literature, although they were lower than earlier reports.

Similarly, Kao et al. (2008) have used HPLC-DAD and identified one phenolic acid and eight flavonoids in wine within 17 min of run time. Pirisi et al. (2000) have used a ternary solvent system of acetic acid (1%), acetic acid (6%), and water-acetonitrile (65:30; v/v) and identified sixteen phenolic acids and flavonoids in plant extract with a total run time of 105 min. Thus, HPLC instrument, run time, solvent combination, and flow rate may play potentially affect the separation of compounds from complex mixture of metabolites. Further investigation needs to focus on pharmacokinetic effects of phenolic and flavonoid compounds, their half-life time, and degradation rate in ruminant juices.

Conclusion

In this study, we demonstrated the quantification of phenolic and flavonoid compounds in *L. multiflorum* extract using HPLC-DAD technique. An extract of *L. multiflorum* forage crop was obtained using 60% aqueous ethanol. Its purification was then carried out by using SPE cartridge. Purified phenolic and flavonoid fractions were separated by using C18 column and a gradient solvent sys-

tem of 0.1% formic acid and acetonitrile at flow rate of 0.8 ml/min and UV–Vis detection at 240 nm. A total of thirteen compounds, including seven phenolic acids and six flavonoids, in *L. multiflorum* extract were identified based on peak area of an unknown compared to standards peak values and published literature. Various unknown peaks were also noted in HPLC spectra. They might be other soluble phenolic acids and flavonoids. Their structural and functional studies need to be characterized by NMR and LC–MS/MS. Finally, we developed a rapid and efficient method for identification and quantification of phenolic acid and flavonoid compositions in forage crop *L. multiflorum*. These compounds might have potential applications in pharmaceutical, food, and livestock industries.

Author's contribution

KCC conceived and designed experiments. SI and SS collected and extracted *L. multiflorum* plant extract. PK performed separation of phenolic acids and flavonoids from *L. multiflorum* plant, developed the HPLC-DAD method, and conducted measurements. CES and MVA helped method development and performed data analysis. PK wrote the manuscript. All authors read, commented, and approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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