Total phenolics and phenolic acids content in low (*Chrysopogon gryllus*) and mediocre quality (*Festuca vallesiaca*) forage grasses of Deliblato Sands meadow-pasture communities in Serbia

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ABSTRACT: *Chrysopogon gryllus* and *Festuca vallesiaca* are components of a number of meadow-pasture communities in Serbia. We performed the analyses of phenolics that influence quality and digestibility of grasses to a great extent. Total phenolics were measured spectrophotometrically and phenolic acids by HPLC analysis. The aboveground parts of *C. gryllus* contained 10.6 mg/g whereas *F. vallesiaca* of 21.6 mg/g total phenolics. Bound phenolics dominated over free ones in both species. The content of both free and bound *p*-coumaric, ferulic, *p*-hydroxybenzoic and syringic acid was higher in *C. gryllus* (6.34 mg/g) than in *F. vallesiaca* (3.96 mg/g). Derivatives of cinnamic acid prevailed in both species compared to the benzoic acid derivatives. Low quality of *C. gryllus* is connected with its high tissue phenolic acids and mediocre quality of *F. vallesiaca* with the high content of total phenolics that act unfavourably on digestibility of such grasses.

Keywords: digestibility; feed quality; secondary plant products; cinnamic and benzoic acid derivatives

Chrysopogon gryllus (L.) Trin. inhabits the Mediterranean region and towards the east to Asia Minor, the Caucasus, Syria, Iran, East India and Australia whereas *Festuca vallesiaca* Schlech. is distributed in southern and eastern parts of Europe, Asia and Asia Minor. *C. gryllus* grows on warm, dry, illuminated, sandy grassy slopes and hills as well as on dry pasture land. These two species are the components of a number of meadow-pasture communities of hill-mountain regions in Serbia. In Deliblato Sands they form two of the most widespread steppe communities *Festuceto-potentilletum arenariae* Stjep.-Ves. and *Chrysopogonetum Pannonicum* Stjep.-Ves. (Stjepanović-Veselićić, 1979). *C. gryllus* and *F. vallesiaca* account for 60% and 20% of such communities, respectively.

Although these two species represent grasses of low (*C. gryllus*) and mediocre quality (*F. vallesiaca*) with regard to their use as fodder, they are of great economic importance because of the large area they cover. The acceptability of a plant as food for herbivores is determined by a combination of feeding incentives and deterrents. Several phenolic acids as the constituents of cell wall lignin were detected in different parts of forage plants (Theodoru *et al.*, 1987; Kamisaka *et al.*, 1990; Provan, 1994; Ben-Hammouda *et al.*, 1995). Plant phenolics influence both the quality and digestibility of grasses to a great extent. Buchsbaum *et al.* (1984) claimed that the content of phenolic compounds was the most significant constituent influencing feeding selection, but the nutrient content had low or no effect on feeding selection.

The aim of this study was to establish the relation between the quality of *C. gryllus* and *F. vallesiaca* as fodder plants and their total contents of tissue phenolics and cinnamic (*p*-coumaric and ferulic acid) and benzoic acid derivatives (*p*-hydroxybenzoic, vanillic and syringic acid).

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MATERIAL AND METHODS

The Deliblato Sands are a vast, 35 km long and about 20 km wide region of Southern Banat extending between the Danube and the Carpathian Mountains. In part 331/1 of the Rošijana locality, an experimental area of 80 × 60 m in the plant community *Festuceto-potentilletum arenariae* was selected. The habitat lies on a moderate slope of a dune exposed to the northeast. The entire community has a characteristic appearance because of short and well developed tufts of the dominant grass *Festuca vallesiaca* while the other meadow-steppe species such as *Chrysopogon gryllus*, *Andropogon ischaemum*, *Carex humilis*, *Poa bulbosa*, *Potentilla arenaria*, *Euphorbia seguieriana*, *Thymus glabrescens*, *Asperula cynanchica*, *Festuca sulcata*, *Achillea millefolium ssp. collina* and *Teucrium chamaedrys* are present in relatively low numbers.

The experimental area (70 × 60 m) in the *Chrysopogonetum pannonicum* community is situated in part 335 (beginning of Lipar), on the right side of the road leading to Deliblato. In addition to *Chrysopogon gryllus* as a dominant species in this community, several meadow-steppe species such as *Potentilla arenaria*, *Thymus glabrescens*, *Carex humilis*, *Festuca sulcata* var. *Wagneri*, *Andropogon ishaemum*, *Stipa capillata* and *Poa bulbosa* were also present, but in a low number of individual plants per unit area.

Plants were collected on June 18, 2002, during flowering. The aboveground parts of *C. gryllus* and *F. vallesiaca* were uniformly collected on transect from five plots of 10 × 10 m (5 × 500 g) from both experimental areas. The material was air-dried, milled and screened through a 0.5 mm-mesh sieve before the analyses of phenolic compounds.

Both phenolic acids and total phenolic compounds were extracted from 5 × 2.0 g aliquots of dry plant material for both species with 80% (v/v) boiling aqueous methanol solution (3 × 30 ml; 4 h) followed by ethyl acetate (3 × 30 ml; 4 h) in glass flasks of 100 ml volume. The extraction was done in Soxlet apparatus under refluxing conditions. After filtration pooled methanol and ethyl acetate extracts were evaporated with a rotary evaporator under N_2, the residue was dissolved in 10 ml of distilled water adjusted to pH 2.0 with 2N HCl and phenolics were transferred to ethyl acetate (3 × 30 ml). The ethyl acetate was evaporated to dryness and the residue was dissolved in 4.0 ml of 80% (v/v) methanol solution. With this procedure free phenolics (high soluble fraction) were prepared. Bound phenolics (fraction of phenolics either ester or ether linked to the cell wall polysaccharides, hemicelluloses or polymerized into lignin) were prepared by boiling the insoluble residue that remained after the first procedure in 10 ml of 2N HCl for 60 min (acid hydrolysis) and transferring to ethyl acetate (3 × 30 ml). The ethyl acetate was evaporated to dryness using a rotary evaporator and the residue was dissolved in 4.0 ml of 80% (v/v) methanol solution. Both samples of free and bound phenolics were used for immediate HPLC analysis or stored at –20°C until use.

A 0.005 ml aliquot of the sample solution in methanol was taken and 7 ml of distilled water plus 0.1 ml Folin-Ciocalteu's phenol reagent was added, and after 3 min 0.2 ml of 20% Na_2CO_3 was added. After boiling at 90°C (exactly 5 min) samples were cooled at room temperature and diluted with H_2O to the volume of 10 ml. As a blank distilled water and reagents only were used. The absorbance of total phenolics (free and bound) was measured at 660 nm by a spectrophotometric method (a Shimadzu UV 160 spectrophotometer) according to Feldman and Hanks (1968), with a sensitivity of 0.05 μg/g dry weight. A standard curve was constructed with different concentrations of ferulic acid (Serva, Germany). The concentrations of ferulic acid have to be in the range of 0.33–80 μg/ml.

Phenolic acids were detected between 210 and 360 nm using a Hewlett Packard diode array detector (HP 1 100 HPLC system). The separation was achieved with a Nucleosil 100-5 C_{18} column; 5 μm; 4.0 × 250 mm (Agilent Technologies, U.S.A.). The following step-gradient of acetonitrile in water was used: 15% acetonitrile (5 min), 30% acetonitrile (20 min), 40% acetonitrile (25 min), 60% acetonitrile (30 min), 60% acetonitrile (35 min) and 100% acetonitrile (45 min, isocratic). In order to avoid the tailing of phenolic acids, 0.05% o-phosphoric acid was added to the solvents. The flow rate was 1 ml/min and the injection volume 5 μl. Phenolic acids were identified by comparison of the retention times and absorption maximum of known peaks obtained with analytically pure standards. For this purpose, *p*-hydroxybenzoic and syringic acids (Acros organics, USA), ferulic, vanillic and *p*-coumaric acid (Serva, Germany) were used. Units of phenolic acids were expressed in μg per gram dry weight.

Statistical evaluation of the differences in total content of phenolics and the composition of phe-

nolic acids in *C. gryllus* and *F. vallesiaca* was performed with ANOVA tests. A standard deviation did not exceed 15% of the mean values of phenolics and phenolic acid content.

RESULTS

The content of total phenolic compounds in *F. vallesiaca* and *C. gryllus* was quite different; the aboveground parts of *F. vallesiaca* contained 6.9 times more free phenolics and 1.3 times more bound phenolics than *C. gryllus*. As seen from Figure 1, the content of bound forms of phenolic compounds in the tissues of both species was much higher than that of free ones (1.3 times and 6.9 times in *F. vallesiaca* and *C. gryllus*, respectively).

HPLC analyses of both plant species revealed five phenolic acids. Two of these phenolic acids are cinnamic acid derivatives (*p*-coumaric and ferulic) and three are benzoic acid derivatives (*p*-hydroxybenzoic, vanillic and syringic acid) (Figures 2–5). *C. gryllus* contained significantly higher amounts of free *p*-coumaric, ferulic, *p*-hydroxybenzoic and syringic acid than *F. vallesiaca*. Both species contained the highest amounts of ferulic and *p*-coumaric acid while the content of *p*-hydroxybenzoic acid was the lowest (Figure 2).

The aboveground parts of *C. gryllus* contained higher amounts of bound phenolic acids than *F. vallesiaca* with the exception of vanillic acid, for which no statistically significant difference was recorded (Figure 3). Out of the five phenolic acids, both species contained the highest amounts of bound ferulic and *p*-coumaric acid (1.2–3.4 mg/g dry weight). The content of other phenolic acids was significantly lower and the concentration of *p*-hydroxybenzoic and vanillic acid ranged from 0.17 to 395.17 μg/g. It was characteristic of both grasses that the amount of bound forms of dominant phenolic acids, i.e. ferulic and *p*-coumaric acid, was 12.7–29 times higher than that of their free forms.

When the percentages of individual free and bound phenolic acids were calculated in relation to the content of total free and bound phenolics (content of total free and bound phenolics = 100%), the proportions of each of the phenolic acids examined in total phenolic content were clearly distinct in comparison with the amounts of individual phenolic acids only. The tissues of *F. vallesiaca* and *C. gryllus* contained 1.98% and 18.09% of free cinnamic acids (ferulic and *p*-coumaric acid), respectively. The proportion of benzoic acid derivatives was significantly low (Figure 4). The tissues of *C. gryllus* contained twice the amount of bound cinnamic acids in comparison with *F. vallesiaca* (57.69% and 26.43%, respectively). Ferulic and *p*-coumaric acids were dominant phenolic compounds in the tissues of both grasses. The percentage of bound benzoic acid was significantly lower (Figure 5). Phenolic acids accounted for 89% of total phenolics in the tissues of *C. gryllus* and only for 33% in the tissues of *F. vallesiaca*. The obtained results demonstrate large differences in the content of total phenolics as well as in the composition

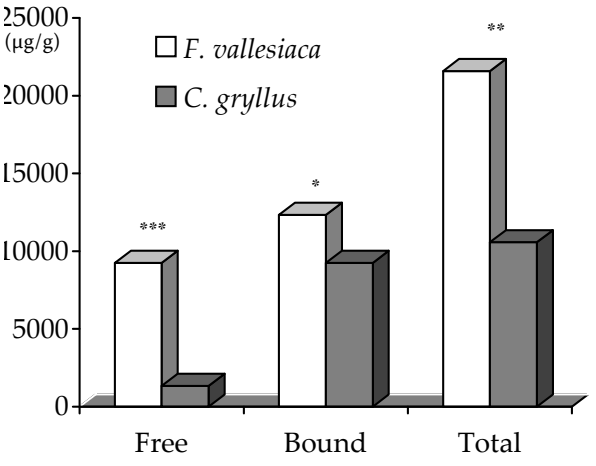

Figure 1. Total phenolics content (ANOVA; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $n = 5$)

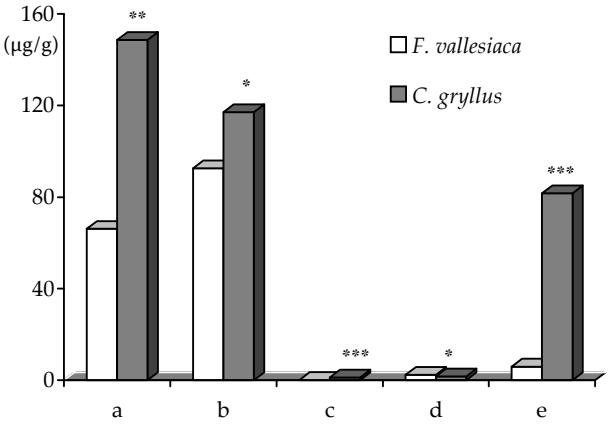

Figure 2. Content of free phenolic acids: (a) *p*-coumaric; (b) ferulic; (c) *p*–hydroxybenzoic; (d) vanillic; (e) syringic; (ANOVA, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $n = 5$)

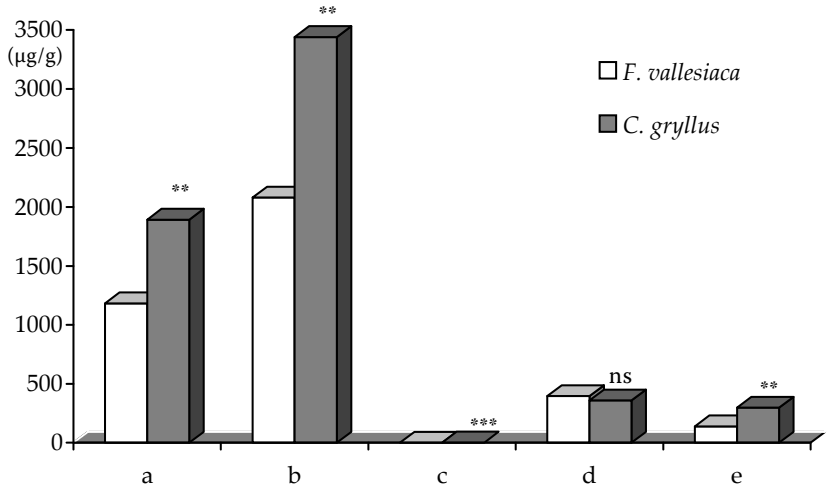

Figure 3. Content of bound phenolic acids: (a) *p*-coumaric; (b) ferulic; (c) *p*-hydroxybenzoic; (d) vanillic; (e) syringic; (ANOVA, ns – not significant; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $n = 5$)

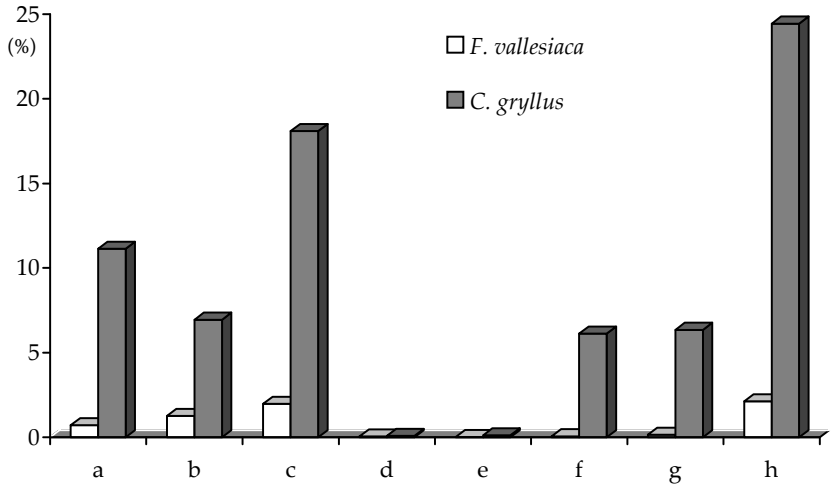

Figure 4. Percentage of free cinnamic and benzoic acid derivatives: (a) *p*-coumaric; (b) ferulic; (c) total cinnamic; (d) *p*-hydroxybenzoic; (e) vanillic; (f) syringic; (g) total benzoic; (h) total benzoic + cinnamic. The percentages of individual free phenolic acids were calculated in relation to the content of total free phenolics (content of total free phenolics = 100%)

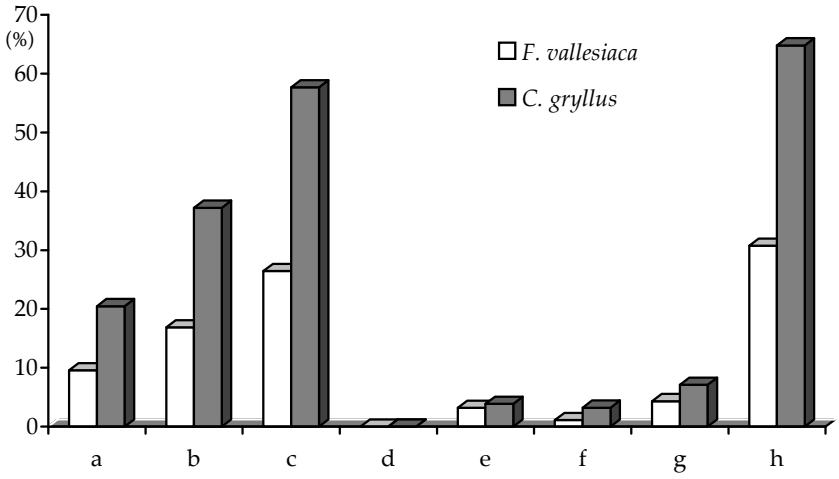

Figure 5. Percentage of bound cinnamic and benzoic acid derivatives: (a) *p*-coumaric; (b) ferulic; (c) total cinnamic; (d) *p*-hydroxybenzoic; (e) vanillic; (f) syringic; (g) total benzoic; (h) total benzoic + cinnamic. The percentages of individual bound phenolic acids were calculated in relation to the content of total bound phenolics (content of total bound phenolics = 100%)

of phenolic acids in the tissues of these two grass species. So *C. gryllus* is characterized by a higher content of phenolic acids whereas *F. vallesiaca* by a higher amount of total phenolics.

DISCUSSION

In spite of the poor or mediocre quality for use by herbivores both grasses are of quite great eco-

nomic importance because they take up relatively large areas in Serbia. Such quality can be ascribed to a relatively high content of total phenolics in the tissues of *F. vallesiaca* and of phenolic acids in the tissues of *C. gryllus*. Ferulic and *p*-coumaric acids are the main phenolic acids present in the cell wall of monocotyledons and especially of Gramineae, as reported by Hartley *et al*. (1990). Theodoru *et al*. (1987) showed that cell walls of barley stems contain *p*-coumaric (6.79 mg/g), ferulic (3.38 mg/g) and *p*-hydroxybenzoic acid (0.10 mg/g). It has also been shown that nineteen species of dry-season tropical grasses contain high amounts of *p*-coumaric, ferulic and caffeic acid. Most of the grasses examined so far have been shown to contain about 10 mg of phenolic acids per g tissue. These acids are incorporated into the cell wall lignin as demonstrated by Lowry *et al*. (1993). In contrast to these data, the grasses examined in the present study contained somewhat lower amounts of the five phenolic acids, the content of which ranged between 3.96 and 6.34 mg/g. Ferulic and *p*-coumaric acid represent dominant compounds. Recently, Séne *et al*. (2001) identified eight phenolic acids and three associated aldehydes, *p*-hydroxybenzoic, *p*-coumaric and ferulic acid being the most abundant (3.2 mg/g) in the aboveground parts of *Sorghum bicolor*. Our results demonstrated a low content of *p*-hydroxybenzoic acid in the tissues of both examined grass species.

Phenolic constituents (lignins and phenolic acids) and carbohydrates are assembled in a tight architecture, which differs according to the plant species. Ferulic and diferulic acid are esterified with polysaccharides of the cell walls in *Avena sativa* L. Three cell wall fractions of Italian ryegrass and orchard grass were reported by several authors to contain ester- and ether-linked *p*-coumaric and ferulic acid (Kamisaka *et al*., 1990; Besle *et al*., 1994; Kondo *et al*., 1994).

Digestibility of grass cell walls depends on the content, structure and organization of lignin within the cell walls, as pointed out by Besle *et al*. (1994). Kondo *et al*. (1994) demonstrated that the removal or degradation of bound phenolic acids by sheep digestion occurred more extensively in the lignin fraction solubilized from the grass cell walls than in the insoluble lignin fraction that remained in the grass cell walls. The contents of ferulic and *p*-coumaric acids were inversely correlated with degradability. Goto *et al*. (1994) reported that the bmr3 maize variant had a higher rumen degradability of leaf blade, leaf sheath and stem and lower contents of cellulose, lignin, ferulic and *p*-coumaric acids esterified and/or etherified to the cell walls than the normal maize phenotype. In connection with these data and taking into account the results of the present study, *C. gryllus* could be classified as a firm and poorly digestible grass owing to high amounts of bound phenolic acids (6.34 mg/g dry weight), and *F. vallesiaca* as the grass of somewhat higher quality.

The results of free and bound phenolics content in F. *vallesiaca* and *C. gryllus* summarized in the present study and the data from the available literature concerning the role phenolics play in the diet of herbivores confirmed that both grasses had low or medium-high nutritional values. In spite of the low or mediocre quality both grasses are already of great importance in hill-mountain regions of Serbia because they take up large areas. Taking into consideration that drought becomes an urgent problem, *F. vallesiaca* as a xerophyte (adapted to the conditions of extreme drought) and *C. gryllus* as a subxerophyte (adapted to extremely dry and also to mesophilic phytocoenoses) will play an important role in future management of pasture areas.

CONCLUSIONS

Aboveground parts of *F. vallesiaca* contain 6.9 times more total free and 1.3 times more bound phenolics than *C. gryllus*. Bound phenolics prevailed in the tissues of both examined species in relation to free ones. *C. gryllus* contained 1.6 times more bound cinnamic acid derivatives than *F. vallesiaca*. The proportion of benzoic acid derivatives in the total content of phenolic compounds was significantly lower in both grasses. Also, ferulic and *p*-coumaric acids were dominant phenolics in the examined grasses. Low quality of *C. gryllus* is connected with its high tissue phenolic acids and mediocre quality of *F. vallesiaca* with high total content of phenolics that act unfavourably on digestibility of such grasses. The proposed scheme and methods in this paper might be useful for studies of the relationship between grass phenolics content and fodder quality.

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