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Content of phenolic, extractable and bound condensed tannins and their effect on *in vitro* gas production from browse leaves

C.D.K. Rubanza[1,4], M.N Shem[2], R. Otsyina[3], S.S Bakengesa[3], T. Ichinohe[4] and T. Fujihara[4,5]

[1]United Graduate School of Agricultural Science, Tottori University
Tottori 680-8553, Japan
[2]Department of Animal Science and Production, Sokoine University of Agriculture
P.O Box 3004, Morogoro, Tanzania
[3]International Centre for Research in Agroforestry (ICRAF)
P.O Box 797, Shinyanga, Tanzania
[4]Laboratory of Animal Science, Shimane University
Matsue, Shimane 690-8504, Japan

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ABSTRACT

Two studies were conducted to screen leaves of four browse species (*Dichrostachys cinerea*, *Flagea villosa*, *Harrisonia abyssinica* and *Piliostigma thorningii*): 1. to quantify levels of total extractable phenolics (TEP), extractable tannins (TET), total condensed tannins (CT), extractable and bound CT fractions and proanthocyanidins (PAs) using chemical assays; 2. to investigate effect of tannin anti-nutritive activity on *in vitro* gas production assessed by polyethylene glycol (PEG) tannin bioassay. Crude protein (CP) varied ($P<0.05$) between fodder species from 109 (*P. thorningii*) to 160 g/kg DM (*D. cinerea*). The fodders had detectable TEP that varied from 112 (*P. thorningii*) to 234 mg/g DM (*F. villosa*). TET varied ($P<0.05$) between species from 95 (*P. thorningii*) to 220 mg/g DM (*F. villosa*). The content of CT varied ($P<0.05$) from 53.2 (*F. villosa*) to 98.3 mg/g DM in *P. thorningii*. High proportion of CT was bound to protein (40-51.4%) compared to soluble (19.5-33.1 %) and fibre-bound (22.7-27.3%) CT fractions. Characterization of PAs in leaves revealed presence of flavan-3-ol and flavan-3,4-diols flavonoids. The increase in GP due to PEG supplementation varied ($P<0.05$) from 44.4 ml/g OM (14.5%) to 132.3 ml/g OM (69.4%) at 16 h, and from 34.9 ml/g OM (10.3%) to 132.2 ml/g OM (57.2%) at 24 h, in *H. abyssinica* and *D. cinerea*, respectively. Variable responses on gas production between species' leaves due to PEG supplementation demonstrate adverse effects of tannins on depressed feed digestibility. Improved gas production and digestibility were due to the ability of PEG to bind and complex tannins, and recover feed nutrients bound by tannins.

[5] Corresponding author: e-mail: fujihara@life.shimane-u.ac.jp

Therefore, utilization of these browse species' leaves as protein supplements to ruminants could be optimized through reduction of levels of phenolic and tannin anti-nutritional factors (ANFs).

KEY WORDS: browse, phenolics, tannins, *in vitro* gas production, PEG, bioassay

INTRODUCTION

Browse tree foliages could be used as source of protein in the tropical region for improved ruminant nutrition and production. Livestock production in arid and semi-arid regions of the world is limited by inadequate feed nitrogen (N) supply from low protein content of basal feeds (hay, straw and stover) (Leng, 1990). In north-western Tanzania during dry seasons ruminants are grazed on standing hay forages that have too low crude protein (CP) contents to meet the normal protein requirement of 80 g/kg DM of CP needed for optimal rumen microbial function (Annison and Bryden, 1998). Browse fodder from semi-arid regions of Tanzania indicate high CP values (100-200 g/kg DM) (Topps, 1997; Rubanza et al., 2003). However, utilization of browse legume fodder as protein supplements to ruminants fed on low-quality roughages could be limited by their contents of phenolic and tannin feed anti-nutritional factors (ANFs) due to their effects on reduced feed digestibility and nutrient utilization (Mangan, 1988; Makkar, 1989).

Tannins are phenolic plant secondary compounds that possess enough hydroxyl groups to form complexes with proteins and carbohydrates (Mangan, 1988; Makkar, 1989). Tannins are classified into hydrolysable tannins (HT) that represent the polyesters of gallic acids (gallotannins) and hexahydroxydiphenic acids (ellagitannins) (Haslam, 1998), and condensed tannins (CT) or proanthocyanidins (PA), which refer to complex of oligomers and polymers of flavonoid units (Haslam, 1998; Schofield et al., 2001). There is scanty information from the literature on nutritive potential and content of tannin in tropical browse fodder. Similarly, levels of proanthocyanidins (PA), and proportions of soluble CT fraction and CT bound to dietary protein and fibre have rarely been reported. Also, little is known on forages' proanthocyanidins composition, tannin structure and tannin structure-biological activity relationship in ruminants (Waterman, 2000).

Protein analogies such as polyethylene glycol (PEG) could alleviate adverse effects of tannins on feed digestibility due to their ability to neutralize tannins' anti-nutritive activity (Makkar et al., 1995). PEG contains sufficient oxygen molecules capable of forming strong hydrogen bonds with the phenolic and hydroxyl groups in tannins; and to precipitate tannin from solutions (Silanikove et al., 2001a). This property of binding tannins using tannin binding agents has been used for assessment of adverse effects of tannins on nutrients availability *in vitro* (Makkar et al., 1995; Getachew et al., 2000), *in sacco* (Silanikove et al., 1996) and *in vivo*

(Silanikove et al., 2001b). Despite wide application of PEG in nutritional studies, there is little information from the literature on the use of PEG to neutralize tannin in different tropical forages. Available data are limited to those of Makkar et al. (1995) and Makkar and Becker (1996) on *Acacia bateri*, *A. saligna* and *Dichrostachys cinerea*; Getachew et al. (2000) on *Dichrostachys* spp.; and Rubanza et al. (2003) on leaves and pods of *Acacia nilotica*, *A. polyacantha* and *A. tortilis*.

A study was therefore, conducted to screen four browse fodder species' leaves from *D. cinerea*, *F. villosa*, *H. abyssinica* and *P. thorningii* indigenous to Tanzania, in order to: 1. quantify levels of phenolic and tannin ANFs; 2. determine soluble, protein-bound and fibre-bound CT fractions; 3. elucidate proanthocyanidins' structure and composition, and 4. assess adverse effects of tannins' anti-nutritive activity on *in vitro* gas production using PEG (MW 6000) tannin bioassay.

MATERIAL AND METHODS

Study area

This study was conducted in Shinyanga region located at 1100-1300 m above sea level in north-western Tanzania (2-3^{o}S; 31-31.5^{o}E). Average annual rainfall ranges from 600-800 mm that rains from November through mid April. Dry season starts from June to October. Minimum and maximum temperatures vary from 15 to 18.3^{o}C and 27.6 to 30.2^{o}C, respectively. Common vegetation includes short grasses and scattered shrubs and trees.

Forage sample collection and processing

Browse leaves (soft twigs less than 5 mm diameter) samples were hand plucked from 8 to 10 trees selected at random in each of four sub-plots (70 × 70 m) in four rangelands in five administrative districts (Bariadi, Kahama, Meatu, Shinyanga Urban and Shinyanga Rural) in the study area. The samples were harvested at an advanced maturity stage in late rainy season between mid April and mid May in 2002. The samples were pooled for each individual tree species, and dried at 50^{o}C in a forced air oven for 48 h to constant weight. Foliage leaf samples for chemical analysis were ground to pass through a 2.00 mm-sieve and sub-sampled into 12 bulk samples (three samples for each species) for further laboratory analyses and nutritional studies. Foliage leaf samples for phenolic and tannin assays and for *in vitro* gas production PEG tannin bioassay were further ground to pass through 1.0 mm-sieve. Chemical compositions including phenolics and tannin assays were

determined in duplicates. *In vitro* gas production and tannin PEG bioassay were conducted in triplicates.

Chemical analyses

Chemical composition: dry matter (DM), organic matter (OM), ash and crude protein (CP) (N X 6.25) (Kjeldahl technique) were analysed according to the AOAC (1990) standard procedures. Fibre components: neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) were estimated using detergent solvents as described by Van Soest et al. (1991).

Phenolics and tannin assays

Extraction and determination of total phenolics and tannins. Extraction of phenolics and tannins from leaf samples was carried out using the method of Makkar (2000). The TEP and TET were assayed according to the method of Jolkumen-Tiito (1985) using Folin-Ciocalteu's reagent (Sigma-Aldrich Chemie, Steinhein, Germany) and tannic acid standard (Sigma-Aldrich Chemie, Steinhein, Germany) as described by Makkar (2000). TET was estimated gravimetrically as a difference of phenolics remaining from total phenolics after binding tannins with polyvinyl polypyrrolidone (PVPP; Sigma- Aldrich Chemie, Steinhein, Germany) (Makkar et al., 1993). The contents of TEP and TET were expressed as tannic acid equivalent.

Soluble and bound condensed tannins assay

Extraction and determination of soluble and bound condensed tannin fractions. The soluble or extractable, protein-bound and fibre-bound CT fractions were estimated using the modified butanol/HCl technique (Porter et al., 1986) by extracting the samples with 1 % aqueous sodium deodocyl sulphate (SDS) and 5% 2-β-mercaptoethanol solution as described by Jackson et al. (1996) and Stewart et al. (2000). A sample of approximately 0.1 g DM (1.0 mm) was extracted (in eppendorff tubes) three times with a mixture of 4 ml of acetone/water (7:3 v/v), containing 1 g/l ascorbic acid, and 2 ml of dichloromethane. The tubes were vortexed, then centrifuged at 3 000 rpm for 15 min while transferring the upper (aqueous) layer to a 10 ml volumetric flask, and the lower (dichloromethane and acetone) was discarded. The combined aqueous fractions containing soluble or extractable CT were made up to 10 ml with distilled water.

Extraction and determination of protein-bound condensed tannins. The solid residues following extraction of free-bound CT were dried for few minutes

in a stream of nitrogen at room temperature to drive off any residual volatile solvents. A-3 ml of 10 g/l aqueous sodium deodocyl sulphate (SDS) containing 50 g/l 2-β-mercaptoethanol (SDS) solution was added to the residues and heated at 95°C for 45 min as described by Stewart et al. (2000). The mixture was centrifuged for 15 min at 3 000 rpm and the supernatant solution transferred to a 10 ml volumetric flask and repeated for three times. The combined supernatant solution was made to 10 ml with SDS solution. Contents of CT in these fractions were assayed by butanol/HC/Fe^{3+} (Porter et al., 1986). A mixture of aqueous extract (0.25 ml) and SDS-solution (0.5 ml) was added to 6 ml of butanol/HCl (95:5 v/v); vortexed and heated at 95°C on a metal heat block for 1 h, with further vortexing after 30 min. Absorbance of the red anthocyanidin products (condensed tannins) was measured at 550 nm.

Extraction and determination of fibre-bound condensed tannins. Fibre-bound CTs were estimated using same procedure as for the protein-bound, except that 6 ml of butanol/HCl and 0.5 ml of SDS-solution were added directly to the solid residue during the extraction process as described by Stewart et al. (2000). The mixture was heated at 100°C on a metal heat block for 1 h, cooled, centrifuged at 3 000 rpm for 15 min before the absorbance of the supernatant was measured at 550 nm. Absorbance values for extractable, protein-bound and fibre-bound CT fractions for each of four browse species were compared with the respective species' blank values. Blank samples constituted plant extracts that were extracted in a similar procedure with butanol/H_2O (95:5 v/v) replacing the butanol/HCl as described by Jackson et al. (1996) and Stewart et al. (2000). Absorbance values for soluble, protein-bound and fibre-bound CT fractions were converted to condensed tannin concentrations by including external tannin standard purified from *Acacia nilotica* at known concentrations in each of the three runs. The concentration of CT in the browse samples were converted to mg *A. nilotica* tannin equivalent/g DM from *A. nilotica* tannin regression equation:

$$Y = 0.0636 + 112.83X \ (R^2 = 0.99)$$

where *Y* is absorbance at 550 nm, and *X* is CT concentration expressed as *A. nilotica* tannin. Purification of tannin from *A. nilotica* leaves for the standard was achieved by back extraction with diethyl ether to remove low molecular weight phenolics and pigments as detailed by Terrill et al. (1992).

Characterization of condensed tannin flavonoids

The condensed tannins in leaves were assayed into proanthocyaninds or leucoanthocyanidin flavonoid composition by high performance liquid chroma-

tography (HPLC) based on techniques described by Hedqvist et al. (2000) and Stewart et al. (2000). A 1.00 g DM (1.00 mm) sample was extracted for 30 min in an ultrasonic water bath with 4.0 ml aqueous acetone (7:3 v/v) containing 1 g/l ascorbic acid; then centrifuged at 3 000 rpm (15 min). A-0.5 ml supernatant sample was then vortexed with 0.25 ml of dichrolomethane and centrifuged again. A 50 µl aliquot sample of the aqueous upper layer was combined with 3.0 ml of butanol/HCl (95:5, v/v) and heated at 95°C for 1 h. Butanol/HCl was evaporated to dryness under nitrogen stream with the tubes kept at 50°C. The residue was re-dissolved in 0.5 ml of methanol/HCl (99:1, v/v), and filtered through a 0.02 polytetrafluoroethylene membrane, followed by injection of 10.0 µl aliquot into Inertsil ODS-80A (C 18) column, 150 × 4.6 mm (Shimadzu Co., Kyoto, Japan). Water/acetic acid (96:4, v/v; solvent A) and methanol (solvent B) were used for gradient elution at 2 ml/min. The gradient profile was 5-40% B (0-5 min); 40-50% B (6-10 min), 50-100% B (11-15 min) and 100-5% B (16-20 min). The absorbance at 525 nm was recorded using a LC-10AT HPLC system (Shimadzu Co., Kyoto, Japan) fitted with a CR-6A data processor (Shimadzu Co., Kyoto, Japan) and a SPD-10A variable wavelength detector (Shimadzu Co., Kyoto, Japan). The peaks were identified using cyanidin chloride, delphinidin chloride and pelargonidin chloride (Extrasynthese Co., Genay, France), which had retention times of 7.48, 10.24 and 12.64 min, respectively.

Effect of polyethyethylene glycol supplementation on in vitro gas production

Animals and management. Rumen fluid for *in vitro* tannin bioassay was obtained from three healthy mature Japanese Corriedale female sheep fitted with permanent rumen cannulae (70 mm). The fistulated animals were kept on standard daily ration of 800 g timothy hay and 200 g concentrates divided into two equal meals fed at 8.00 and 16.00 h daily. The animals were supplemented with minerals and had free access to water throughout the experimental period. Rumen fluid was obtained from the three sheep early in the morning before feeding, and filtered through three layers of cheese-cloth.

Preparation of mineral buffer medium. Preparation of an *in vitro* mineral buffer media for PEG tannin bioassay was conducted as described by Makkar et al. (1995), and as detailed by Makkar (2000). Composition of the rumen mineral buffer medium for PEG tannin bioassay was prepared according to Tilley and Terry (1963). Reduced buffer medium composition, were, g per litre: $NaHCO_3$, 35.0; NH_4HCO_3, 4.00; Na_2HPO_4, 5.7; KH_2PO_4, 6.2; NaCl, 2.22; $MgSO_4 \cdot 7H_2O$, 0.6; Na_2S, 0.52; $CaCl_2 \cdot H_2O$, 13.2; $MnCl_2 \cdot 4H_2O$, 10.00; $CoCl_2 \cdot 6H_2O$, 1.00 and sodium resazurin, 0.01 and, 60 ml freshly prepared reduction solution containing 580 mg $Na_2S \cdot 9H_2O$ and 3.7 ml 1M NaOH. Rumen fluid was mixed with an anaerobic mineral buffer solution (1:2 v/v) as described by Makkar et al. (1995).

The mixture was kept stirred under CO_2 flushing at 39°C using a magnetic stirrer fitted with a hot plate.

Polyethylene glycol (PEG) tannin bioassay. Adverse effects of tannin ANFs on *in vitro* gas production, and thus digestibility, were assessed by incubation of approximately 500 mg DM test feed samples (in triplicates) with or without 1.0 g PEG molecular weight (MW, 6 000) (Wako Pure Chemicals Industries Ltd., Osaka, Japan). PEG (MW 6 000) was preferred due to its higher binding capacity to tannins at near neutral pH that approaches true biological value. The feed samples were incubated in 100 ml glass syringes based on Menke and Steingass (1988) standard procedure. A portion (40.0±0.5 ml) of the rumen fluid/buffer medium was transferred into graduated syringes (100 ml) and incubated into water bath at 39±0.1°C as described by Blümmel and Ørskov (1993). Vaseline oil was applied to the piston to ease movement and prevent escape of gas. The syringes were pre-warmed (39°C) for 1 h, before addition of 40±0.5 ml of rumen fluid/buffer mixture into each syringe, and incubated in a water bath maintained at 39±0.1°C. The syringes were gently shaken every hour during the first 8 h of incubation. Gas production readings (ml) were recorded after 2, 4, 6, 8, 12, 16 and 24 h for both PEG treated samples and blank samples.

Statistical analyses

Data on chemical compositions, phenolics, condensed tannins and CT fractions in forages were subjected to the analysis of variance (ANOVA) using the General Linear Model Procedure (GLMP) (SAS/Statview, 1999), and were analysed based on statistical model:

$$Y_{ij} = \mu_{ij} + S_i + e_i$$

where: Y_{ij} is the general observation on chemical composition, phenolics, condensed tannins, CT fractions and proanthocyanidins' composition; μ_{ij} is the general mean common for each parameter under investigation; S_i is the i^{th} effect of browse fodder species on the observed parameters; e_i is the standard error term.

Data on *in vitro* gas production were subjected to the analysis of variance (ANOVA) using the General Linear Model Procedure (GLMP) (SAS/ Statview, 1999), and were analysed based on statistical model:

$$Y_{ij} = \mu_{ij} + S_i + P_j + (SxP)_{ij} + e_i$$

where: Y_{ij} is the general observation on gas production, μ_{ij} is the general mean common for each observation; S_i is the i^{th} effect of browse fodder species on the observed parameters; P_j is the j^{th} effect of PEG supplementation on *in vitro* gas production; $(SxP)_{ij}$ refer to i^{th} and j^{th} interaction effects of fodder species and PEG supplementation on *in vitro* gas production, e_i is the standard error term.

RESULTS

Chemical composition

Chemical composition varied significantly (P<0.001) between fodder species (Table 1). The forages had (P<0.05) high organic matter (OM) contents that ranged from 906 (*D. cinerea*) to 940 g/kg DM in *P. thorningii*. There was no (P>0.05) difference in OM content between *D. cinerea* and *H. abyssinica* (906 vs 913 g/kg DM). The forages had (P<0.05) variable crude protein (CP) content that ranged from 109 to 160 g/kg DM in *P. thorningii* and *D. cinerea*, respectively (Table 1). There was no (P>0.05) difference in CP content between *D. cinerea* and *F. villosa*, which was also not (P>0.05) from *H. abyssinica*. The forages had (P<0.001) variable contents of neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) with *P. thorningii* containing (P<0.05) notably highest fibre values (Table 1). The NDF content varied (P<0.05) between fodder species from 306 to 559 g/kg DM in *H. abyssinica* and *P. thorningii*, respectively. There was no (P>0.05) difference in NDF content between *H. abyssinica* and *F. villosa* (306 vs 317 g/kg DM, respectively). The forages had (P<0.05) variable ADF that ranged from 214-417 g/kg DM in *F. villosa* and *P. thorningii*, respectively. The ADL varied (P<0.05) from 73 to 183 g/kg DM in *F. villosa* and *P. thorningii*, respectively. The results show that D. cinerea contained higher (P<0.05) fibre contents than both *F. villosa* and *H. abyssinica* (Table 1).

TABLE 1

Chemical composition of selected browse tree species' leaves harvested from fodder banks in north-western Tanzania, g/kg DM

Fodder species	Dry matter, %	Organic matter	Crude protein	NDF	ADF	ADL
D. cinerea	90.8[a]	906[a]	160[a]	450[a]	267[a]	124[a]
F. villosa	88.4[b]	923[b]	154[ab]	317[b]	214[b]	73[b]
H. abyssinica	90.4[a]	913[a]	147[b]	306[b]	221[c]	94[c]
P. thorningii	90.3[a]	952[c]	109[c]	559[c]	417[d]	183[d]
Mean	90.0	924	143	408	280	119
SEM	2.90	0.7	2.8	9.6	1.1	1.4
Significance of effect species	*	***	***	***	***	***

[a,b,c,d] different letters in the same column indicate significant differences (P<0.05)
*** P<0.001

Phenolics and tannins compositions

Total extractable phenolics and tannins. The selected browse forages had significantly (P<0.05) variable levels of TEP, TET and CT among fodder species (Table 2). *Flagea villosa* had (P<0.05) highest TEP content (234 mg/g DM) compared to *P. thorningii* that had (P<0.05) lowest total phenolics (112 mg/g DM). There was no difference (P>0.05) in TEP content between *D. cinerea* and *P. thorningii* (114 vs 112 mg/g DM, respectively). *Flagea villosa* had (P<0.05) higher TET content (220 mg/g DM) than *H. abyssinica* (139 mg/g DM), while *P. thorningii* and *D. cinerea* had the least TET contents of 95 and 96 mg/g DM, respectively. The content of CT varied (P<0.05) from 53.2 to 98.3 mg/g DM in *F. villosa* and *P. thorningii*, respectively. There was no (P>0.05) difference in CT between *F. villosa* and *H. abyssinica*.

Soluble and bound-condensed tannin fractions. The forages had variable (P<0.05) CT fractions (Table 2) with *D. cinerea* having higher (P<0.05) soluble CT fraction of 22.9 mg/g DM compared to *H. abyssinica* that had the least soluble CT fraction (14.5 mg/g DM). *Piliostigma thorningii* had high (P<0.05) protein-bound and fibre-bound CT contents of 50.5 and 28.6 mg/g DM, respectively, compared to *F. villosa* and *D. cinerea,* which contained (P<0.05) lowest contents (22.2 and 13.4 mg/g DM, respectively). The forages had most of their CT in the protein-

TABLE 2

Content of total extractable phenolics (TEP), total extractable tannins (TET) and total condensed tannins (CT), and CT fractions (free, protein-bound and fibre-bound CT) in selected fodder species' leaves native to north-western Tanzania rangelands, mg/kg DM

Fodder species	TEP[1]	TET[1]	CT[2]	Free CT		Protein-bound CT		Fibre-bound CT	
				mg/kg DM	% of total	mg/kg DM	% of total	mg/kg DM	% of total CT
D. cinerea	114[a]	96[a]	75.4[a]	22.9[a]	30.4	35.4[a]	46.9	17.1[a]	22.7
F. villosa	234[b]	220[b]	53.2[b]	17.6[b]	33.1	22.2[b]	41.7	13.4[b]	25.1
H. abyssinica	156[c]	139[c]	54.3[b]	14.5[c]	26.7	25.0[c]	40.0	14.8[b]	27.3
P. thorningii	112[a]	95[a]	98.3[c]	19.2[b]	19.5	50.5[d]	51.4	28.6[c]	29.1
Mean	154	138	70.3	18.6		33.2		18.5	
SEM	5.3	6.3	1.61	1.58		1.72		1.44	
Significance of effect									
species	***	***	***	***		***		***	

[a,b,c,d] different letters in the same column indicate significant differences (P<0.05)

*** P<0.001

[1] total extractable phenolics (TEP) and total extractable tannins (TET) were expressed as mg/g tannic acid equivalent

[2] total condensed tannins (TCT), protein-bound CT and fibre-bound CT were expressed as mg/g *Acacia nilotica* CT tannin equivalent

bound fraction that constituted 40.0-51.4% of the total CT in *H. abyssinica* and *P. thorningii*, respectively. Soluble CT fraction constituted 19.5-33.1% of the total CT in *P. thorningii* and *F. villosa*, respectively. The browse species had relatively high fibre-bound CT fraction that constituted 22.7-29.1% of the total CT in *D. cinerea* and *P. thorningii*, respectively.

Characterization of condensed tannins (CT) in selected browse leaves

Flavonoid composition in *D. cinerea*, *F. villosa*, *H. abyssinica* and *P. thorningii* leaves is shown in Table 3. Browse leaves had detectable and variable ($P<0.01$) proanthocyanidins (PAs) or leucoanthocyanidins among fodder species. *Dichrostachys cinerea* contained highest ($P<0.05$) delphinidins, cyanidins and pelargonidins flavonoid contents of 1.63, 1.42 and 4.39 mg/g DM, respectively, compared to the other fodder species. *Piliostigma thorningii* had significantly ($P<0.05$) the lowest contents of delphinidins, cyanidins and pelargonidins (0.19, 0.31 and 0.01 mg/g DM), respectively. There was no difference ($P>0.05$) in delphinidins and cyanidins between *F. villosa* and *H. abyssinica*. Similarly, there was only slight ($P>0.05$) difference in pelargonidin flavonoids content among *F. villosa* (0.004 mg/g DM), *H. abyssinica* (0.02 mg/g DM) and *P. thorningii* (0.01 mg/g DM). *Dichrostachys cinerea* showed higher delphinidin/ cyanidin (D:C) ratio of 1.14 than *F. villosa*, *H. abyssinica* and *P. thorningii*, that had relatively lower D:C ratios of 0.86, 0.62 and 0.61, respectively.

TABLE 3

Condensed tannins' flavonoid compositions in selected fodder species' leaves native to north-western Tanzania rangelands, mg/kg DM

Fodder species	Delphidins	Cyanidins	Pelargonidins	Delphinins/ cyanidins ratio
D. cinerea	1.630[a]	1.424[a]	4.392[a]	1.14
F. villosa	0.461[b]	0.537[b]	0.004[b]	0.86
H. abyssinica	0.384[b]	0.619[b]	0.017[b]	0.62
P. thorningii	0.19[c]	0.311[c]	0.009[b]	0.61
Mean	1.510	1.324	0.705	0.81
SEM	0.160	0.056	0.137	n.a.
Significance of efect fodder species	**	**	**	

[a, b, c] different letters in the same column indicate significant differences ($P<0.05$)

** $P<0.01$

n.a. = not applicable

Effect of polyethylene glycol treatment on in vitro gas production

Effect of incubation of browse leaf samples with polyethylene glycol (PEG) on *in vitro* gas production (GP) is shown in Figure 1. Incubation of the fodder species *in vitro* with PEG resulted in increase ($P<0.05$) in gas production with variable responses among fodder species. D*ichrostachys cinerea* showed the highest response on the increase in gas production of 132.3 ml/g OM (69.4%) to PEG treatment at 16 h incubation interval compared to *H. abyssinica*, which showed the lowest increase of 44.4 ml/g OM (14.5%). The increase in gas production due to PEG treatment at 24 h incubation interval varied from 34.9 ml/g OM (10.3%) to 132.2 ml/g OM (57.2 %) in *H. abyssinica* and *D. cinerea*, respectively. The response on the increase in gas production from *D. cinerea* due to PEG supplementation was slightly higher than *P. thorningii*. *Flagea villosa* showed the relatively lower responses than *D. cinerea* and *P. thorningii*.

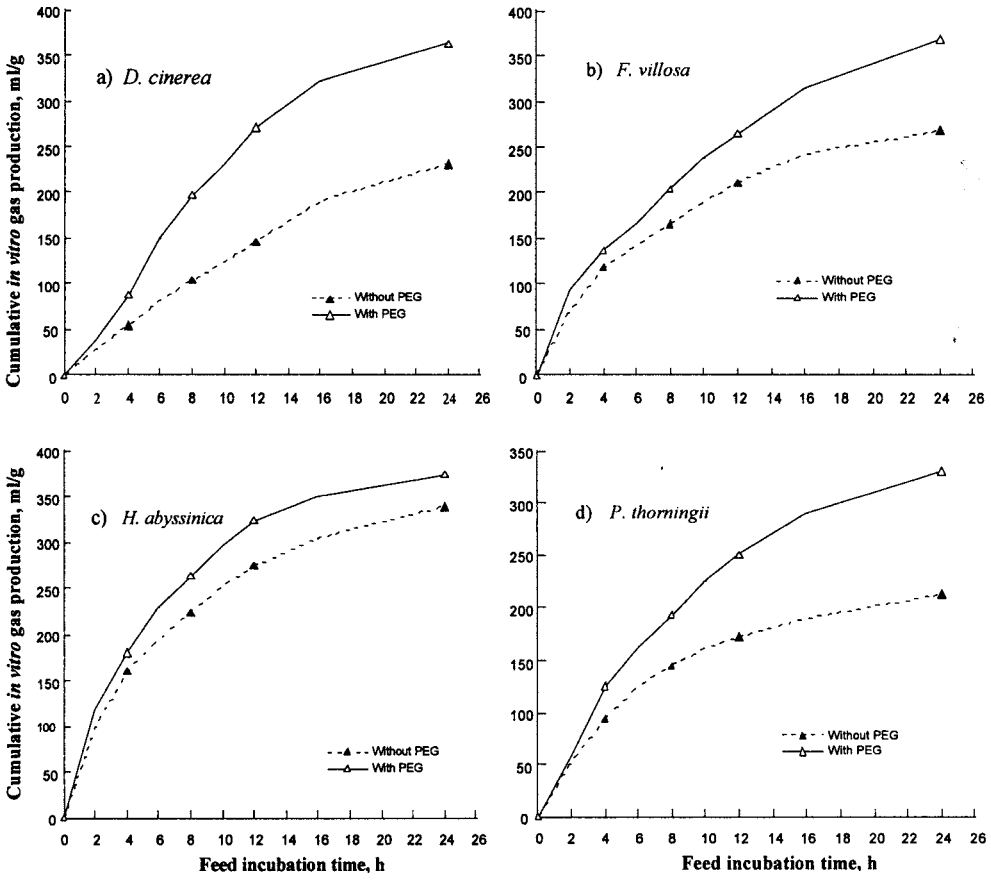

Figure 1. Effect of polyethylene glycol (PEG) treatment on cumulative *in vitro* gas production (ml/g OM) from selected browse tree leaves at different incubation intervals, h

DISCUSSION

The current findings on CP were higher than the previously reported CP content of standing hay forages (30 g/kg DM) (Rubanza, 1999) in semi-arid western Tanzania in the dry season. Following high CP contents, browse leaves could be used as ruminants' feed supplements for N in the tropics especially during dry seasons. Variations in chemical compositions between fodder species could be partly explained by genotypic differences in factors that control synthesis and accumulation of nutrients in plant tissues; and partly by forages' stage of growth and plant proportion that was harvested for analysis. Minson (1990) reported decrease in cell contents (CP, sugars, vitamins, neutral detergent soluble contents) and digestibility with forage maturity at the expense of increased fibre content and lignification. Similarly, Topps (1997) reported a decrease in CP and increase in fibre fractions in *Acacia* spp. due to advanced leaf maturity. Despite browse fodder nutritive superiority, high levels of phenolic and tannin ANFs could limit utilization of N from browse legume foliages.

Higher levels of tannin (>50 mg/g DM) have been associated with deleterious effects in ruminant nutrition through lowered feed palatability, intake, and digestibility (Makkar, 1989; Aerts et al., 1999).

Adverse effects due to high levels of tannins on digestibility could be through inhibition of digestive enzymes and complex formation with dietary nutrients and toxic effects on rumen microorganism. Variation of phenolics and tannin concentration among browse fodder species could be due to factors which control synthesis and accumulation of polyphenolic compounds in plants, mainly plant genotype, environmental factors, genotype-environment interaction (Wong, 1973) and foliages' stage of growth.

The reported total condensed tannins (CT) contents were comparable to CT contents reported earlier in *Acacia* spp. and *Dichrostachys* spp. leave and pods. Rubanza et al. (2003) reported CT content (butanol/HCl) of 46, 51, 55 and 74 mg/g for *A. polyacantha*, *A. tortilis, A. nilotica* and *Dichrostachys* spp. leaves, respectively. In the current work, *F. villosa* had higher CT content than *Acacia* spp. leaves. The CT content in *D. cinerea* was comparable to *Dichrostachys* spp. reported previously (74 vs 75 mg/g DM) (Rubanza et al., 2003). Comparison of the presented results on the total condensed tannins and CT fractions is limited by lack of information from the literature on CT composition in tropical browse foliages. However, results presented in the current work on total CT contents were higher than values of CT fractions reported by Terrill et al. (1992) in grasses and herbaceous legumes, and even higher than those reported by Jackson et al. (1996) and Mupangwa et al. (2000). Mupangwa et al. (2000) reported lower total CT of 29.5, 16.9 and 12.4 mg/g DM in *Cassia rotundifolia*, *Lablab purpureum*

and *Macroptilium atropurpureum*, respectively, compared to the current findings of 53, 54, 75 and 98 mg/g DM in *F. villosa*, *H. abyssinica*, *D. cinerea* and *P. thorningii*, respectively. High levels of CT in browse species' leaves in this study could cause depressed N and dry DM digestibility (Aerts et al., 1999) through formation of tannin-protein complexes (Mangan, 1988).

The four browse species' leaves in the current study were lower (19.5-33.1% of total CT) in the soluble fraction than those reported by Jackson et al. (1996) for tropical legumes *Arachis pintoi*, *Centrosema latidens* and *Desmodium ovalifolium* (70-95% of total CT). Higher contents of CT fractions of browse species' leaves in this study than values reported by Terrill et al. (1992) in herbage forages were due to the fact that the latter forages contained negligible CT contents. Reported results on the distribution of CT fractions compare slightly with values of 12-44% of total CT (butanol/HCl) for soluble CT fraction in *C. rotundifolia*, *L. purpureum*, and *M. atropurpureum* (Mupangwa et al., 2000). High proportion of protein-bound CT fraction (40.0-51.4%) could be associated with depressed browse fodder digestibility. The fibre-bound CT fraction constituted a significant proportion (22.7-29.1%) of the total CT. High proportion of bound CT reduce rumen fibre digestion possibly due to a reduction caused by inactivation of hemicellulase and cellulase enzymes or inhibition of the attachment of the rumen microbes (Muhammed et al., 1994). The latter author reported inhibitory effect of tannins on microbial endoglucocanase activity and the attachment of fungi to cellulose *in vitro*, which result to reduced degradation of cellulose. Min et al. (2003) further noted reduced rumen proteolytic bacterial growth, and some specific bacterial populations measured *in vivo* due to CT anti-nutritive activity.

Variation in flavonoid classes between studied browse fodder species could be due to differences in genetic and biochemical processes that control synthesis and accumulation of flavonoids in plant tissues, environmental factors (light intensity, mineral deficiency and moisture stress) and interactions between the environment and genetic factors (Wong, 1973; Haslam, 1998; Aerts et al., 1999). Some important genetically controlled biochemical processes include overall flavonoid production, specific flavonoid synthesis and distribution of flavonoids in different plant tissues (Wong, 1973). Variable levels of pelargonidin, delphinidin and cyanidin flavonoids composition between foliage species could be associated to tannin reactivity and thus biological activity due to the chemical structure or stereochemistry of the polymerized proanthocyanidins. The detected proanthocyanidins in *D. cinerea*, *F. villosa*, *H. abyssinica* and *P. thorningii* browse fodder species could have different tannin anti-nutritive activity and thus variable biological activity due to their variable flavonoid structure (Haslam, 1998).

Effect of tannin on in vitro digestibility

Increase in gas production due to *in vitro* supplementation with PEG in browse leaves in this study shows the negative role of tannin on lowered fermentation and digestibility of the feed organic matter (OM). Therefore, condensed tannins in *D. cinerea*, *F. villosa*, *H. abyssinica* and *P. thorningii* leaves could limit digestibility of browse fodder and thus lower their nutritive potential. Variable responses on the increase in *in vitro* gas production shown by tannin-PEG bioassay among browse species in this study could be associated with depressed feed OM digestibility potential probably due to content of tannin and variable tannin anti-nutritive activity among browse leaves (Aerts et al., 1999). Tannins bind and complex feed proteins and carbohydrates (Mangan, 1988; Makkar, 1989), which result to lowered feed digestibility (Makkar et al., 1995).

Improved gas production and probably OM digestibility in tannin-rich browse could be due to high affinity of PEG to tannins (Makkar and Becker, 1996), and due to its ability to bind and inactivate tannins by forming tannin-PEG complexes (Makkar et al., 1995) and the release of feed nutrients, for example, protein, from tannin-protein complexes (Getachew et al., 2000). Improved *in vitro* gas production in the current study concur to findings reported elsewhere (Makkar et al., 1995; Makkar and Becker, 1996; Rubanza et al., 2003) in different browse fodder species. Makkar et al. (1995) and Makkar and Becker (1996) reported improved gas production due to PEG treatment in *Acacia barteri*, *A. saligna*, *Dichrostachys cinerea* leaves incubated with PEG.

Higher increase in gas production due fermentation of *D. cinerea* with PEG than other species suggests different tannin anti-nutritive activity among browse species' leaves (Aerts et al., 1999). Results on variable improved gas production between browse species' leaves due to addition of PEG were consistent to previous findings reported earlier (Rubanza et al., 2003) in *Acacia nilotica*, *A. polyacantha*, *A. tortilis* and *Dichrostachys* spp. leaves and pods. Low digestibility of both *D. cinerea* and *P. thorningii* could also be attributed to their high proportions of free CT, protein-bound CT and fibre-bound CT fractions due to their effects on depressed protein and fibre digestibility (Muhammed et al., 1994), and probably due to their toxic effects on rumen microbes (Odenyo et al., 1997). Lower *in vitro* gas production potential and probably OM digestibility of *D. cinerea* compared to *F. villosa*, *H. abyssinica* and *P. thorningii* leaves could also be associated with its relatively higher delphinidin/cyanidin ratio than in *F. villosa*, *H. abyssinica* and *P. thorningii* (Table 3).

Variable *in vitro* gas production potential and probably their digestibility between these browse species could also be partly explained by type of fibre and extent of fibre lignifications (Fonseca et al., 1998). Low gas production

and probably low digestibility of D. *cinerea* and *P. thorningii* leaves could also be partly accounted for by their relatively higher lignin contents compared to *F. villosa* and *H. abyssinica*, which contained relatively lower lignin contents.

CONCLUSIONS

It was concluded that browse foliage leaves harvested from *D. cinerea*, *F. villosa*, *H. abyssinica* and *P. thorningii* could be used as N supplements to ruminants fed on low-quality roughages due to their high crude protein content. However, utilization of these browse fodder supplements to ruminants could be limited by their high contents of phenolics and tannins. Browse species' leaves in this study contained higher contents of phenolic and tannin ANFs than the lower beneficial level of tannin (50 mg/g DM) in ruminant diets. High proportion of total condensed tannins (CT) were bound to protein compared to soluble CT and fibre-bound CT fractions. The species' leaves contained detectable proanthocyanidins that constituted flavan-3-ols and flavan-3,4-diols that elucidate variable flavonoids' stereochemistry. Improved *in vitro* gas production due to fermentation of the browse species' leaves with PEG were mainly due to the deactivation and reversed tannin anti-nutritive activity by PEG binding tannins; and the release of feed nutrients bound by tannins.

Further studies are needed to investigate effect of PEG and PEG analogies supplementation on effects of tannins *in vivo* in ruminants fed on tannin-rich browse foliages, and effect of browse foliages supplementation on animal productivity.

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REFERENCES

Aerts R.J., Barry T.N., McNabb W.C., 1999. Polyphenols and agriculture: beneficial effects of proanthocyanidins in forages: a review. Agr. Ecosyst. Environ. 75, 1-12

Annison E.F., Bryden W.L., 1998. Perspectives on ruminant nutrition and metabolism. I. Metabolism in the rumen. Nutr. Res. Rev. 11, 173-198

AOAC, 1990. Association of Official Analytical Chemists, Official Methods of Analysis. 15th Edition. Arlington, VA

Blümmel M., Ørskov E.R., 1993. Comparison of *in vitro* gas production and nylon bag degradability of roughage in predicting feed intake in cattle. Anim. Feed Sci. Tech. 40, 109-119

Fonseca A.J.M., Dias-da-Silva A.A., Ørskov E.R., 1998. *In sacco* degradation characteristics as predictors of digestibility and voluntary intake of roughages by mature ewes. Anim. Feed Sci. Tech. 72, 205-219

Getachew G., Makkar H.P.S., Becker K., 2000. Effect of polyethylene glycol on *in vitro* degradability of nitrogen and microbial protein synthesis from tannin-rich browse and herbaceous legumes. Brit. J. Nutr. 84, 73-83

Haslam E. (Editor), 1998, Practical Polyphenolics from Structure to Molecular Recognition and Physiological Action. Cambridge University Press (UK)

Hedqvist H., Mueller-Harvey I., Reed J.D., Kruger C.G., Murphy M., 2000. Characterisation of tannins and *in vitro* protein digestibility of several *Lotus corniculatus* varieties. Anim. Feed Sci. Tech. 87, 41-56

Jackson F.S., Barry T.N., Lascano C., Palmer B., 1996. The extractable and bound condensed tannins of leaves from tropical tree, shrub and forage legumes. J. Sci. Food Agr. 71, 103-110

Jolkumen- Tiito R., 1985. Phenolics constituents in the leaves of northern willows: Methods for the analysis of certain phenolics. J. Agr. Food Chem. 33, 213-217

Leng R.A., 1990. Factors affecting the utilisation of 'poor- quality' forage by ruminants particularly under tropical conditions. Nutr. Res. Rev. 3, 277-303

Makkar H.P.S. (Editor), 2000. Quantification of tannins in tree foliage. A Laboratory Manual for the FAO/ IAEA Co-ordinated Research Project on 'Use of Nuclear and Related Techniques to Develop Simple Tannin Assays for Predicting and Improving the Safety and Efficiency of Feeding Ruminants on Tanniniferous Tree Foliage'. Joint FAO/ IAEA of Nuclear Techniques in Food and Agriculture. Animal Production and Health Sub-programme, FAO/ IAEA Working Document, IAEA Vienna (Austria), pp. 40

Makkar H.P.S., 1989. Protein precipitation methods for quantification of tannins: A review. J. Agr. Food Chem. 97, 1197-1202

Makkar H.P.S., Becker K., 1996. A bioassay for polyphenols (tannins). Polyphenols communications 96, July 15-18, Bordeaux (France)

Makkar H.P.S., Blümmel M., Becker K., 1995. Formation of complexes between polyvinyl pyrrolidones or polyethylene glycols and tannins and their implication in gas production and true digestibility in *in vitro* techniques. Brit. J. Nutr. 73, 897-913

Makkar H.P.S., Blümmel M., Borrowy N.K., Becker K., 1993. Gravimetric determination of tannins and their correlations with chemical and protein precipitation methods. J. Sci. Food Agr. 6, 161-165.

Mangan J., 1988. Nutritional effects of tannins in animal feeds. Nutr. Res. Rev. 1, 209-231

Menke K.H., Steingass H., 1988. Estimation of the energetic feed value obtained from chemical analysis and *in vitro* gas production using rumen fluid. Anim. Res. Develop. 28, 7-55

Min B.R., Barry T.N., Attwood G.T., McNabb W.C., 2003. The effect of condensed tannin on nutrition and health of ruminants fed on fresh temperate forages: a review. Anim. Feed Sci. Tech. 106, 3-19

Minson D.J. (Editor), 1990. Forage in Ruminants Nutrition. Academic Press Inc., San Diego, CA

Muhammed S., Stewart C.S., Acamovic T., 1994. Effects of tannic acid on cellulose degradation, adhesion and enzymatic activity of rumen microorganisms. Proc. Soc. Nutr. Physiol. 3, 174

Mupangwa J.F., Acamovic T., Topps J.H., Ngongoni N.T., Hamudikuwanda H., 2000. Content of soluble and bound condensed tannins of three tropical herbaceous forage legumes. Anim. Feed Sci. Tech. 83, 139-144

Odenyo A.A., Osuji P.O., Karanfil O., 1997. Effect of multipurpose tree (MPT) supplements on ruminal ciliate protozoa. Anim. Feed. Sci. Tech. 67, 169-180.

Porter L.J., Hrstich L.N., Chan B.G., 1986. The conversion of proanthocyanidins and prodelphinidins to cyanidins and delphinidin. Phytochemistry 25, 223-230

Rubanza C.D.K., 1999. Effect of Leucaena leaf meal supplementation on growth performance of Zebu steers grazing on standing hay traditionally conserved forages (*Ngitiri*). MSc. Dissertation, Sokoine University of Agriculture, Tanzania

Rubanza C.D.K., Shem M.N., Otsyina E.R., Ichinohe I., Fujihara T., 2003. Content of phenolics and tannins in leaves and pods of some *Acacia* and *Dichrostachys* species and effects on *in vitro* digestibility. J. Anim. Feed Sci. 12, 645-663

SAS/ Statview, 1999. Using Statview. Statistical Analytical System (SAS) Inc. 3rd Edition. SAS Inc.

Schofield P., Mbugua D.M., Pell A.N., 2001. Analysis of condensed tannins: a review. Anim. Feed Sci. Tech. 91, 21-40

Silanikove N., Gilboa N., Nitsan Z., 2001b. Effect of polyethylene glycol on rumen volume and retention time of liquid and particulate matter along the digestive tract in goats fed tannin-rich carob leaves (*Ceratonia siligua*). Small Ruminant Res. 40, 95-99

Silanikove N., Pereovolotsky A., Provenza F.D., 2001a. Use of tannin- binding chemicals to assay for tannins and their negative postingestive effects in ruminants. Anim. Feed Sci. Tech. 91, 69-81

Silanikove N., Shinder D., Gilboa N., Eyal M., Nitsan Z., 1996. Binding of poly(ethylene glycol) to samples of forage plants as an assay of tannins and their negative effects on ruminal degradation. J. Agr. Food Chem. 44, 3230-3234

Stewart J., Mould F., Mueller-Harvey I., 2000. The effect of drying treatment on fodder quality and tannin content of two provenances of *Calliandra calothyrsus* Meissner. J. Sci. Food Agr. 80, 1461-1468

Terrill T.H., Rowan A.M., Douglas G.B., Barry T.N., 1992. Determination of extractable and bound condensed tannin concentrations in forage plants, protein concentrate meals and cereal grains. J. Sci. Food Agr. 58, 321-329

Tilley J.M.A., Terry R.A., 1963. A two stage technique for the *in vitro* digestion of forage crops. J. Brit. Grassl. Soc. 18, 104-111

Topps J.H., 1997. Nutritive value of indigenous browse in Africa in relation to the needs of wild ungulates. Anim. Feed Sci. Tech. 69, 143-154

Van Soest P.J., Robertson J.B., Lewis B.A., 1991. Methods for dietary fiber, neutral detergent fiber and non-starch carbohydrates in relation to animal nutrition. J. Dairy Sci. 74, 3583-3597

Waterman P.G., 2000. The tannins - An overview. In: J.D Brooker (Editor). Proceedings of an International Workshop: Tannins in Livestock and Human Nutrition, Adelaide (Australia)

Wong E., 1973. Plant phenolics. In: G.W. Buttler, R.W. Bailey (Editors). Chemistry and Biochemistry of Herbage. Vol. 1. Academic Press, London and New York, pp. 265-322

STRESZCZENIE

Zawartość fenoli ekstrakcyjnych i związków tanin w liściach krzewów strączkowych oraz ich wpływ na produkcję gazu *in vitro*

Przeprowadzono dwa doświadczenia celem zbadania właściwości liści czterech gatunków krzewów strączkowych *(Dichrostachys cinerea, Flagea villosa, Harrisonia abyssynica i Piliostigma thorningii)* pod względem: 1. zawartości ogólnych ekstrakcyjnych związków fenolowych (TEP), ekstrakcyjnych tanin (TET), ogólnych polimerów tanin (CT) oraz ekstrakcyjnych i związanych CT i proantocjanidyn (PAs), stosując metody chemiczne; 2. wpływu anty-żywieniowej aktywności tanin, oznaczając produkcję gazów w warunkach *in vitro* pod działaniem dodatku polietylenoglikolu (PEG). Zawartość białka ogólnego (CP) była zmienna (P<0,05) między gatunkami i wahała się od 109 *(P. thorningii)* do 160 g/kg s.m. *(D. cinerea)*. Zawartość TEP wynosiła od 112 *(P. thorningii)* do 234 mg/g s.m. (*F. villosa)*, a zawartość CT zmieniała się (P<0,05) między gatunkami od 53.2 (*F. villosa)* do 98,3 mg/g s.m. w *P. thorningi*. Znaczna ilość CT była połączona z białkiem (40-51%) w porównaniu z ilością tanin rozpuszczalnych (19,5-33,1%) i związanych z włóknem (22,7-27,3%) frakcji CT. W liściach stwierdzono obecność flavan-3-olowych i flavan-3,4-diolowych flawonoidów. Wzrost GP (P<0,05) w inkubatach liści z dodatkiem PEG wahał się od 44,4 ml/g substancji organicznej (OM) liści (14,5%) do 132,3 ml/g OM (69,4%) po 16 godzinnej inkubacji, a od 34,9 ml/g OM (19,3%) do 132,2 ml/g OM (57,2%) po 24 godzinnej inkubacji *H. abyssinica* i *D. cinerea,* odpowiednio. Ta zmienność GP pomiędzy gatunkami liści spowodowana dodatkiem PEG wskazuje na depresyjne oddziaływanie tanin na strawność paszy. Polepszenie strawności było wynikiem związania tanin przez PEG i odzyskanie składników odżywczych związanych z taninami. Tak więc, wykorzystanie badanych gatunków liści jako źródła białka dla przeżuwaczy może być zwiększone przez obniżenie poziomu antyżywieniowych związków fenolowych i tanin.