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Intraspecific differences in ash, crude protein contents and protein precipitation activity of extractable tannins from Nepalese fodder trees

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Abstract *Leaves of different ages and from different positions on three* Artocarpus lakoocha *and three* Quercus semecarpifolia *trees were sampled in November, January and March 1990/91 and analysed for ash, crude protein and protein precipitation activity of extractable tannins. Much of the variation within species related to leaf age and position appeared to be random in nature. Protein contents generally fell and ash contents rose over the period, but the trend was generally not statistically significant (*P>0·05*). Some significant (*P<0·05*) tree-to-tree differences were found in these components. Variability in leaf protein precipitation activity was significant (*P<0·05*) within trees, between trees and between bimonthly samples. Significant (*P<0·05*) within-tree differences were found in fresh leaf samples of* Q. semecarpifolia *and* Ficus glaberrima, *but not* Castanopsis tribuloides. *Intraspecific differences in chemical composition may complicate the assessment of the nutritive value of tree leaf fodders.*

Keywords: tannins, protein precipitation, fodder trees, protein, ash.

Introduction

Ruminants are important to many farmers in Nepal as sources of food, income, traction and manure but lack of fodder can be a serious constraint to livestock production, particularly in the dry season (Panday 1982). Panday estimated that about 40% of the fodder available annually in Nepal comes from forest trees, but this figure obscures regional and seasonal variations. Fodder trees are particularly important in the dry season. The Lumle Agricultural Centre grows and distributes seedlings of fodder trees in response to requests from farmers increasingly looking to produce their own fodder as traditional forest resources come under pressure.

Data have been published on the concentrations of the major nutrients in fodder leaves used in Nepal (Panday 1982). However, little is known of the variability within species of the main nutrients such as crude protein and organic matter. Furthermore, many leaves contain

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antinutritional factors which may lower the actual nutritive values from those expected from the proximate analysis. Tannins, probably the most widespread of these factors, are chemically diverse but ill-defined (Mangan 1988). Tannins are known to occur in oilseeds, oak *(Quercus* sp.*)* trees, legumes and other fodder species (Kumar and Singh 1984; Leiner 1990). The digestibility of protein by rumen microorganisms is reduced by tannin binding (Mangan 1988), but in some circumstances this binding may be advantageous, increasing protein availability in the lower gut (Barry *et al.* 1986). Soluble phenolics may be absorbed by the animal and have direct toxic effects (Robbins *et al.* 1987).

Hagerman and Butler (1989) recommended measuring protein precipitation activity to estimate the biochemical activity of tannins. Hanley *et al.* (1992) found that protein precipitation assays were useful indicators of the inhibition by tannins of protein and dry matter digestibility in deer fed on seven tannin-containing forages and twigs from one browse species. This approach was used in this study.

The commonly used Nepalese fodder tree species *Artocarpus lakoocha* and *Quercus semecarpifolia* were investigated over a period of 4 months during the dry season to assess variability within and between trees. Data on this variability was extended by analysing samples from other *Q. semecarpifolia* trees and from four *Ficus* spp. Data on within-tree variation was extended by a repeat sampling of different *Q. semecarpifolia* trees and sampling of the tree species *Ficus glaberrima* and *Castanopsis tribuloides.* All of the samples were obtained from the region near the Lumle Centre.

Experimental

Sample preparation

Samples of *A. lakoocha* and *Q. semecarpifolia* were taken in November 1990, and January and March 1991. Samples of leaves of different ages and at a number of positions were taken from three trees of each species. Leaf samples contained some non-woody thin stem material as well as whole leaves including leaf veins. Within a single species leaves were stripped from the trees in a consistent manner. A list of the sample types is given in Table 1.

The ages of the leaves (young, intermediate and old) were relative rather than strictly defined. Young leaves did not include very immature developing leaves. *A. lakoocha* trees 1 and 3 were located in a rising terrace near each other, tree 2 on a terrace of cultivated land. All three *Q. semecarpifolia* trees were located near each other in a south-facing sector of a

Table 1. Samples from different types of fodder trees and from different positions on the trees

Type and location of sample	Sample code
Young leaves	Y
Intermediate age leaves	I
Old leaves, north facing	ON
Old leaves, south facing	OS
Old leaves, top of tree	OT
Old leaves, bottom of tree	OB

forest. Additionally, pooled samples were taken from a further five *Q. semecarpifolia* trees in April 1991.

All the samples were dried at 60°C to constant weight (dry matter being recorded), ground and sent to the UK for analysis. Further fresh leaf samples were taken in November 1991, extracted and analysed for tannins in Nepal. For both procedures about 1 kg of fresh leaf was taken for each category of sample. The sample was thoroughly mixed (bulked sample) and a 100 g sub-sample taken for drying or homogenizing for fresh extractions. Samples comparing different trees consisted of 500 g fresh leaf collected from the lower parts of the trees and pooled.

Analyses of dry matter, ash and crude protein

Samples were analysed for dry matter, ash and crude protein by the methods described in the Feeding Stuffs (Sampling and Analysis) Regulations (1982).

Preparation of extracts for tannin analysis

For dried samples 500 (±10)mg was weighed out accurately in duplicate into 10 ml glass beakers. To this was added 5 ml of 70% v/v aqueous acetone (analytical grade, BDH Chemicals) and the mixture was homogenized and mixed for 3 min using an Ultraturrex (10 mm probe, 13 500 rpm; Janke and Kunkel). The mixtures were then transferred to a centrifuge tube and spun at 2000*g* in a bench centrifuge for 10 min.

Fresh leaf samples

From the bulked sample 100 g of material was removed and ground using a hand grinder. To 5 g ground material 20 ml of 80% v/v aqueous acetone was added. The mixture was thoroughly stirred with a glass rod and left covered for 10 min at ambient temperature. It was then centrifuged in the same way as the extracted dried samples.

Tannin analysis

The protein precipitation activity (PPA) was measured using the radial diffusion method of Hagerman (1987), except that 1·0 g/l haemoglobin (Bovine blood, Sigma Chemicals) in agarose was used. A Hamilton syringe was used to apply 15 µl of the supernatant to wells in two agarose plates, and the plates were sealed, incubated and assayed. PPA values were calculated as the activity of the test extract (in cm^2) per gram dry weight of sample.

Statistical analysis

Multifactor Analysis of Variance was used to assess the extent of the differences between trees, positions within trees (for old leaves only), sample times and leaf ages. The analysis was used as a basis for a simplified presentation of means and provided estimates of standard errors for comparison of means. The analysis was performed separately for ash, crude protein and PPA. The extent of analytical variation for PPA was also studied. The analytical variation was proportional to the mean level of PPA. This relationship was used to derive an

equation for computing a pooled estimate of standard error for the PPA data for analyses of dried and fresh leaf samples.

Results

Ash and crude protein contents

Table 2 shows the average ash and crude protein contents of samples collected in November and the pooled standard errors of the averages. Averages are given by age of sample and position of sample for both species. The contents of young and top of the tree samples of *A. lakoocha* were significantly lower ($P<0.05$) than other age or position related samples, but otherwise there were no significant differences in ash content between leaves of different ages or between old leaves from different positions. Much of the variation appeared to be random in character. As it was unlikely that the variations observed would make much difference to the nutritional quality of the leaves, only the pooled sample from each tree was analysed for ash content for January and March samples: Table 3.

The ash content increased over the sampling period for *A. lakoocha* and *Q. semecarpifolia*, but this was generally not significant ($P>0.05$). Five additional *Q. semecarpifolia* trees sampled in April 1991 were analysed, giving values of 22·5, 23·5, 27·7, 23·3 and 25·7 g/kg, indicating that between-tree differences in ash contents were unlikely to greatly affect the nutritive value of the leaves.

Table 2. Ash and crude protein contents of leaves of different ages and from different positions in trees

	Ash content (g/kg dry matter)		Crude protein content (g/kg dry matter)	
Sample code	*A. lakoocha*	*Q. semecarpifolia*	*A. lakoocha*	*Q. semecarpifolia*
*Different ages**				
Y	139	27	126	144
I	156	27	127	142
O†	165	28	124	141
Pooled SE	5·8	3·1	2·7	3·2
*Different positions**				
ON	168	26	130	139
OS	167	31	124	141
OT	155	26	118	139
OB	168	28	126	144
Pooled SE	5·8	3·1	2·7	3·2
*All samples**				
Average	159	28	125	142
Pooled SE	3·2	1·0	2·9	2·3
Range	121–176	21–34	90–147	96–160

**Average of three trees, November samples only for ash contents; November, January and March samples for crude protein.*
†Average of all 'old' samples (ON, OS, OT, OB).

Table 3. Ash and crude protein contents of leaves of different trees at different times of sampling

	A. lakoocha				*Q. semecarpifolia*			
	Tree 1	Tree 2	Tree 3	Average*	Tree 1	Tree 2	Tree 3	Average*
Ash contents (g/kg dry matter)†								
November 1990	154	158	165	159	29	31	22	27
January 1991	ND	ND	ND	163	ND	ND	ND	25
March 1991	ND	ND	ND	184	ND	ND	ND	30
Pooled SE	3·3	3·3	3·3	3·3	1·8	1·8	1·8	1·8
Crude protein contents (g/kg dry matter)†								
November 1990	122	138	137	109	133	149	143	147
January 1991	117	130	137	132	140	153	146	150
March 1991	119	104	120	118	134	140	136	143
Pooled SE	5·7	5·7	5·7	5·7	4·4	4·4	4·4	4·4
All months								
Average	118	126	132	120	136	148	141	147
Pooled SE	2·7	2·7	2·7	2·7	3·2	3·2	3·2	3·2

ND = not determined.
*Data for pooled samples averaged for trees 1, 2 and 3.
†Monthly averages by tree for sample types Y, I, ON, OS, OB.

Table 2 also gives crude protein content data averaged by sample age and position; Table 3 gives similar data averaged by tree and month. Additionally, in Table 3 average data from the pooled samples are given for each sampling time. No significant age-related differences were observed, but significant ($P<0·05$) positional differences were seen in *A. lakoocha* but not *Q. semecarpifolia*. Significant ($P<0·05$) falls in protein contents from January to March were observed for *A. lakoocha* for the pooled samples from trees 2 and 3, and *Q. semecarpifolia* tree 2, but not in other trees. Overall, the within-tree variations in crude protein content appeared to be largely random in nature. The five additional *Q. semecarpifolia* trees sampled in April gave values of 123, 120, 109, 112 and 126 g/kg crude protein, significantly ($P<0·05$) lower than the average crude protein values obtained for the three test trees. This confirmed that significant ($P<0·05$) tree-to-tree differences in leaf crude protein content do occur, as indicated in the main study.

PPA of tannins

The standard error of measurements followed the following relationships:

$$\text{for dried samples, SE} = 0·02 \times \text{mean activity}^{1·05}$$

$$\text{for fresh samples, SE} = 4·87 + (0·048 \times \text{mean activity})$$

where mean activity was the average activity of a single extract measured in two axes on two agarose plates. This was used as a measure of analytical error.

Data on the PPA of the *A. lakoocha* and *Q. semecarpifolia* samples of different ages and from different positions are given in Table 4. Young *A. lakoocha* leaves had significantly higher PPAs than intermediate and old leaves, but no statistically significant differences were observed between *Q. semecarpifolia* leaves of different ages. For *A. lakoocha*, north-facing leaves had a significantly ($P<0.05$) higher PPA than south-facing leaves; conversely for *Q. semecarpifolia* the south-facing leaves were significantly ($P<0.05$) higher. In both cases the trend was not consistent for all three trees studied (data not shown). Significantly higher ($P<0.05$) activities were observed at the top of the tree than at the bottom for *A. lakoocha*, but the leaves at the bottom of *Q. semecarpifolia* trees had a significantly ($P<0.05$) higher PPA than those at the top, although the trend was not consistent for all three trees at all three sampling times (data not shown).

Table 5 presents data on PPA of tannins from different trees for samples collected at different times. For *A. lakoocha*, differences between trees were generally not consistent with time although in individual trees significant ($P<0.05$) differences were observed. The data for *Q. semecarpifolia* were far more consistent, with all three specimens showing significant ($P<0.05$) falls in PPA over the sampling period, particularly between January and March. For both species the tree 2 specimens had significantly ($P<0.05$) lower average (of all sample types at all sampling times) PPAs than trees 1 and 3. The five additional *Q. semecarpifolia* trees sampled in April 1991 had PPAs of 767, 666, 752, 808 and 604 cm^2/g, further illustrating the existence of tree-to-tree differences. With both *A. lakoocha* and *Q. semecarpifolia* there was generally more variation between trees than between positions on the same tree. The between-tree differences and changes with time showed the same trends for both the pooled samples and average data of samples from different positions and ages.

Table 4. PPA of tannins of leaves from trees of different ages and from different positions

	Tannin PPA (cm^2/g dry matter)	
Sample code	*A. lakoocha*	*Q. semecarpifolia*
*Different ages**		
Y	256	850
	222	856
O†	229	850
Pooled SE	10·2	28·3
*Different positions**		
ON	249	747
OS	214	823
OT	264	881
OB	187	949
Pooled SE	10·2	28·3
All samples		
Average	239	829
Pooled SE	16·7	57·1
Range	92–392	381–1478

*Average of three trees, November, January and March samplings.
†Average of all 'old' samples (ON, OS, OT, OB).

Data obtained from fresh leaf samples are given in Tables 6 (different leaf positions) and 7 (different trees). Significant ($P<0.05$) positionally related differences were found in *Q. semecarpifolia* and in *F. glaberrima*, but no significant ($P>0.05$) differences were observed in *C. tribuloides*. Leaves at the bottom of *Q. semecarpifolia* trees had significantly ($P<0.05$) higher PPAs than those at the top (also seen in dried samples; Table 4) but the opposite trend was seen in *F. glaberrima*. Significant ($P<0.05$) tree-to-tree differences were also observed in the four *Ficus* species.

Discussion and conclusions

Crude protein and ash analyses

Some significant leaf age and positional differences in crude protein and ash contents were observed in *A. lakoocha* but such variations did not appear to be systematic and much of the variability appeared to be random in nature. No significant differences were noted in the *Q. semecarpifolia* samples. The observed variations in ash content were unlikely to make much difference to the nutritive value, but the variations in protein content would affect the nutritive value of the leaves, particularly if they were being fed with crop residues low in protein. This study indicates that leaves of different ages should be taken from the different positions to obtain representative samples.

Panday (1982) quoted crude protein and ash levels of 156·7 g/kg and 100·5 g/kg, respectively, for a sample of *A. lakoocha* collected in June when the leaves were new. The relatively high protein and low ash contents in June are probably indicative of the differences between newly grown leaves and the more mature ones we analysed. For *Q. semecarpifolia*,

Table 5. PPA of tannins* of leaves from different trees at different times of sampling

	A. lakoocha			*Q. semecarpifolia*		
	Tree 1	Tree 2	Tree 3	Tree 1	Tree 2	Tree 3
Monthly averages†						
November 1990	245	127	268	1189	729	1193
January 1991	323	172	208	995	706	1072
March 1991	189	256	300	650	476	649
Pooled SE	17·7	17·7	17·7	49·0	49·0	49·0
All months						
Average	252	185	259	945	637	971
Pooled SE	10·2	10·2	10·2	28·3	28·3	28·3
Pooled samples§						
November 1990	248	107	241	938	564	1278
January 1991	380	158	187	812	485	779
March 1991	167	197	225	617	452	591

*Expressed in cm^2/g dry matter.

†monthly averages by tree for all sample types given in Table 1.

§SE (analytical error) = $0.02 \times \text{mean activity}^{1.05}$ for the pooled samples.

Panday (1982) gave crude protein and ash contents of 73·0 g/kg and 33·0 g/kg, respectively; a lower protein but similar ash content to those reported in this study. These differences are presumably due to tree-to-tree variation and seasonal factors. Our data show that single values can be misleading.

PPA of tannins

Significant ($P<0·05$) variations, but few consistent trends, were observed in the data. The most notable sources of variation were between species and between trees of the same species. Monthly differences were also significant ($P<0·05$).

Sample preparation can affect extractable tannin levels; for example, Hagerman (1988) reported that drying (lyophilization or drying at 40°C) could increase or decrease tannin extractability. We observed variability in both dried and fresh leaf samples so it did not appear to be an artefact of sample preparation.

There are indications in the literature that, within a single tree, differences in tannin levels may be expected with position and age of the leaf. Waterman *et al.* (1984) studied variations between leaves in *Barteria fistulosa*: total phenols between 13·6 and 106·9 g/kg (tannic acid standard) and condensed tannins between 14·9 and 294·8 g/kg, (quebracho tannin standard) were observed in leaves from different positions. Exposure to strong sunlight was associated with high tannin levels. Variations in sunlight could account for the increased PPAs in leaves from top of *A. lakoocha*, but with *Q. semecarpifolia* exhibiting the opposite trend no firm conclusion can be drawn.

Table 6. Fresh leaf samples: within-tree differences in the PPA of tannins

	PPA of tannins (cm^2/g dry matter)				
	Bottom of tree	Middle of tree	Top of tree	Sunny position	Shady position
Ficus glaberrima	339	345	396	449	454
Castanopsis tribuloides	234	235	243	238	211
Quercus semecarpifolia	655	631	506	764	922

SE = 4·87 + (0·048 × mean activity).

Table 7. Fresh leaf samples: between-tree differences in the PPA of tannins

	PPA of tannins (cm^2/g dry matter)			
	Tree 1	Tree 2	Tree 3	Tree 4
Ficus roxburgii	232	191	108	275
Ficus semicordata	212	285	271	403
Ficus nerrifolia	211	229	195	315
Ficus glaberrima	555	412	477	ND

ND = not determined.
SE = 4·87 + (0·048 × mean activity).

Makkar *et al.* (1988, 1991) looked at samples of young and mature leaves from single trees of several oaks (*Quercus* sp.). The protein precipitation activity of 4 day old leaves was four times that of 1 year old leaves for *Quercus serrata*. Less extreme differences were observed in the other species studied, but young leaves had generally high tannin contents and activities. Makkar *et al.* (1991) demonstrated that the greatest changes occur during the very early stages of leaf development; these stages were not monitored in our study, hence the absence of significant age-related differences in *Q. semecarpifolia* (although young leaves had a significantly higher PPA than intermediate and old leaves of *A. lakoocha*).

Significant tree-to-tree differences were found. Such variations in tannin levels may be expected, owing to genetic and environmental factors. Barry and Forss (1983) found that soil conditions greatly affected the condensed tannin content of *Lotus pedunculatus*, reporting levels of 20–30 g/kg and 80–110 g/kg for plants grown on fertile and poor soils, respectively.

The composition of phenolic compounds (such as tannins and their metabolic precursors) in sorghum are very dependent on the environment in which they are grown (Mueller-Harvey and Dhanoa 1991).

We conclude that the PPAs of leaf extracts can vary significantly between trees of the same species, between leaves from single trees and with time. Variations in crude protein and ash contents appear to be generally random, rather than systematic in nature. Nevertheless, some statistically significant positional, tree-to-tree and time-related differences were observed. These variations were presumably related to the genetic properties of the trees and the immediate environment of the leaves. To evaluate the nutritive value of trees, multiple samples taken from different trees and at different times should be analysed to obtain a measure of variability within species. Representative samples should be collected from leaves of different ages from different positions.

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