

The Effects of Mimosa Tannin on the Protein Precipitation in *Gliricidia* (*Gliricidia sepium*) and Protein Solubility in the Rumen of Cattle (*Bos indicus*)

(Pengaruh Mimosa Tanin terhadap Pengikatan Protein pada Daun *Gliricidia* (*Gliricidia sepium*) dan Tingkat Kelarutan Protein pada Rumen Sapi (*Bos indicus*))

Satrijo Widi Purbojo

Fakultas Peternakan Universitas Jenderal Soedirman

Abstrak

Suatu studi dilakukan untuk menentukan apakah penambahan mimosa tannin pada daun *Gliricidia* yang ditumbuk dapat merangsang pembentukan ikatan tannin dan protein sehingga dapat melindungi degradasi protein oleh bakteri rumen. Daun *Gliricidia* yang telah ditumbuk, diberi perlakuan penambahan 0, 3, 6, 9, 12 dan 15% mimosa tannin, kemudian dibiarkan selama 0, 3, 6, 12 dan 24 jam. Selanjutnya, bahan tersebut diekstrak menggunakan 70% acetone dan kandungan protein yang terikat diukur dengan metoda *radial diffusion assay*. Percobaan pencernaan protein *in situ* menggunakan 4 ekor sapi berfistula, dilakukan untuk membandingkan derajat proteksi protein pada tumbukan daun *Gliricidia* dan *Leucaena* yang diberi perlakuan 0, 3 dan 6% mimosa tannin dan dibiarkan selama 1 jam. Hasil penelitian menunjukkan bahwa tumbukan daun *Gliricidia* yang diberi perlakuan mimosa tannin lebih dari 3% mampu menghasilkan pengikatan protein. Kadar pengikatan protein tertinggi tercatat setelah 6 jam waktu inkubasi dan cenderung menurun setelah itu. Percobaan pencernaan protein *in situ* menunjukkan bahwa tumbukan daun *Gliricidia* dengan perlakuan 6% mimosa tannin mempunyai pencernaan protein terendah di dalam rumen yakni 13.31%, dibandingkan dengan 22.38 dan 26.60% pada tumbukan daun *Gliricidia* dengan perlakuan 0 dan 3% mimosa tannin. Demikian pula nilai efektif degradasi terendah pada $k=0.02$ adalah tumbukan daun *Gliricidia* dengan perlakuan 6% mimosa tannin. Hasil penelitian ini menyarankan bahwa kandungan protein pada daun *Gliricidia* dapat diikat dengan 6% mimosa tannin dan selanjutnya dapat melindungi kandungan protein dari fermentasi mikroba rumen.

Kata kunci : Mimosa tannin, protein precipitation complex, radial diffusion assay.

Introduction

In the tropics, low protein nutrition of ruminants is a primary concern as it directly affects the rate of fermentation of highly fibrous roughage as well as the protein nutrition of the host animal. The function of the rumen to degrade low quality roughage can be enhanced by supplementation of the diet with readily soluble urea or more traditionally by feeding leguminous fodder leaves to meet the nitrogen requirement of the microorganisms. The protein requirement of the host animal, on other hand, is dependent on the amount of undegraded dietary protein (UDP) leaving the rumen into the small intestine as source of amino acids for absorption. The amount of UDP

flowing into the small intestine is inversely related to its solubility in the rumen. It is, therefore, beneficial to protect some of the proteins from microbial degradation. Tannin, a naturally occurring compound in trees and shrub, is known to form complex with protein, which renders it undegradable in the rumen, but enzymatically digestible in the small intestines (Barry and McNabb, 2000). Although a decrease in feed intake and protein digestibility has been reported in animals fed with high concentration (>5%) of condensed tannin (CT), an increase in the quantity of dietary protein flowing into the small intestine has been observed (Navas-Camacho, *et al.*, 1993). Hence, fodder trees and shrubs with moderate amount of CT are potential sources of rumen by-pass proteins. Hence, this study was conducted to

determine whether the addition of mimosa tannin source in macerated *Gliricidia* could effectively protect its protein from rumen degradation.

Research Methods

Protein Precipitation

Treatments. Mature leaves of *Gliricidia* (*Gliricidia sepium*) and *Leucaena* (*Leucaena leucocephala*) were macerated using mortar and pestle and then thoroughly mixed with 0, 3, 6, 9, 12, and 15% of a commercial preparation of mimosa tannin on a dry-matter basis. The tannin treated samples were then allowed to stand for 0, 3, 6, 12 and 24 hours before tannin-precipitated proteins were determined using radial diffusion assay (Hagerman, 2002).

Tannin extraction. Tannin was extracted from 200 mg of each sample with 10 ml of 70% aqueous acetone in centrifuge tubes suspended in an ultrasonic water bath for 20 minutes at 4°C and centrifuged for 10 minutes at 3,000 RPM. The supernatant was decanted and the precipitate was twice treated with 5 ml of 70% aqueous acetone, subjected to ultrasonic treatment for 20 minutes at 4°C and centrifuged. The final precipitate was used for radial diffusion assay.

Radial diffusion assay. An agarose agar was prepared in petri dishes using the following chemicals: Agarose – Type I, low EEO, gel point 36°C (Sigma A-3350), Bovine Serum Albumin – Fraction V powder, 96-99 albumin (Sigma A-9647), 70% aqueous acetone and 0.05M buffer containing 60 µmg ascorbic acid with pH 5.0.

Using a well puncher with 4 mm diameter, 4 wells were punched in the solidified agarose agar in petri dishes. The plugs of agarose were removed from the petri dishes gently using gentle suction. Each well was then filled up with 20 µl of the tannin extract, covered and sealed with parafilm. These were then placed in an incubator with a temperature of 30°C for at least 96 hours to allow the formation of the rings. Thereafter, the diameter of the ring was measured using a caliper and transformed into mm².

Protein Solubility in the Rumen

Treatments. Macerated mature leaves of *Gliricidia* and *Leucaena* were mixed with mimosa tannin at the rate of 0%, 3% and 6% on a DM basis and allowed to stand for one hour before oven drying at 60°C for 48 hours. The dried samples were ground in a hammer mill and stored for digestibility trial.

Animals. Four grade cattle (*Bos indicus*) with an average of 350 kg live weight and fitted with permanent rumen fistula, were used in this study. The animals were placed in individual digestion stalls and maintained in good condition prior to and throughout the duration of the study. Animals were fed with a ration of 80% forage and 20% concentrate. The amount of feed offered was calculated at 2.5% of live weight on a dry-matter basis. The forages were composed of combination of 80% Napier grass (*Pennisetum purpureum*) and 20% *Gliricidia*. The concentrate mixture consisted of 60% wheat pollard, 36% copra meal, 2% urea, 1% salt and 1% dicalcium phosphate. Feeding was done twice daily at 08.00 and 14.00 hours. The animals were provided with clean water at all times.

In situ digestibility trial. Approximately 5 g of each sample was put in nylon bags measuring 10 x 20 cm (White Polyester Monofilament) with pore size 53 µm (± 10) and free of nitrogen. Two bags per sample were used per incubation period. Zero time disappearance value (0 hour) was obtained by washing the empty nylon bag with distilled water. On day one of incubation, all the were inserted in the ventral sac of rumen immediately before the morning feed. The samples were incubated at 0, 6, 12, 24, 48 and 72 hours. After each incubation period, the nylon bags were removed from the rumen and washed with distilled water to remove the rumen fluid. These were then dried at 60°C for 48 hrs.

The *in sacco* digestibility data was estimated by the equation $D = a + b(1 - e^{-ct})$, where D = degradation of protein (%) after t hr, a = readily soluble fraction or solubility (the intercept with the y-axis = zero time intercept), b = slowly soluble fraction (insoluble potential), c = degradation rate

constant (h^{-1}) and t = the incubation time (h) (Ørskov and McDonald, 1979). Effective degradability was estimated using equation of Madsen and Hvelplund (1994). Four fraction outflow rates of (k) 0.02, 0.04, 0.06, and 0.08 (h^{-1}) were assumed as proposed by ARC (1984). From parameters a , b , c of above non-linear equation, effective degradability was calculated as follows: $K = a + (b \times c) / (k + c)$.

Chemical analysis. The dry matter and crude protein content of the samples were measured following the AOAC (1993) procedure.

Statistical analysis. All data were analyzed using the Analysis of Variance in a Randomized Completely Block Design using (SAS, 1996). To examine the difference within parameters, the Duncan test was used, as described by Gomez and Gomez (1984).

Results and Discussions

Protein Precipitation of Macerated Gliricidia with Mimosa Tannin

Table 1 presents the size of the rings formed from macerated Gliricidia samples treated with different levels of mimosa tannin at different times of incubation with the use of radial diffusion assay. The treatment of macerated Gliricidia with mimosa tannin at levels of 0 and 3% did not show any formation of the ring from the agar wells. This

implies that serum albumin in the agar was not precipitate with the amount of tannin used. It is therefore possible that the protein present in Gliricidia precipitated all of the 3% tannin added and none was left to precipitate the albumin in the agar. However, at levels equal to or more than 6% of mimosa tannin, there was tannin-albumin binding as shown by the rings form in the agar. Thus, the highest protein precipitation was recorded with the highest amount of mimosa tannin mixed with Gliricidia. The effect of incubation period on the amount of protein precipitated was curvilinear, peaking after 6 hours of incubation and then started to decrease. Nonetheless, it was evident that protein precipitation was almost spontaneous as shown by the ring formation at 0 hour in treatments with more than 6% tannin.

Although radial diffusion assay may be less useful for tannin quantification, it is useful for estimating the protein-binding ability of forages (Reed, 1995). The radial diffusion assay involves formation of tannin – bovine serum albumin complexes with no interference from other proteins and therefore suitable for routine analysis of samples (Hagerman, 1987).

Hence, results from this study would indicate that the optimum mimosa tannin level needed to precipitate the protein present in Gliricidia was then diffused in the agar and formed complex with serum albumin.

Table 1. Radial Diffusion Assay (at mm²) for macerated Gliricidia treated at different levels of tannin and different times of incubation

Level of Tannin (%)	Incubation Periods (hr)					Means
	0	3	6	12	24	
0	nd	nd	nd	nd	nd	nd
3	nd	nd	nd	nd	nd	nd
6	536.58	345.62	397.13	478.30	333.00	418.13 ^c
9	493.26	493.26	564.15	687.47	596.93	567.01 ^b
12	670.16	671.21	884.14	766.16	766.16	759.02 ^a
15	832.76	884.14	799.46	719.20	549.45	773.48 ^a
Means	593.78 ^b	598.76 ^b	661.22 ^a	648.01 ^{ab}	529.25 ^c	

Legend: Row and column with different superscript are significantly different ($P < 0.05$). nd = non detected.

Table 2. Mean crude protein digestibility, degradation parameters and effective degradability of macerated *Gliricidia* and *Leucaena* leaves treated with tannin

	Leucaena	Gliricidia	Gli+3% tannin	Gli+6% tannin
Mean CP digestibility, %	38.35 ^a	22.38 ^c	26.60 ^b	13.31 ^d
Degradation parameters				
a	9.83 ^{ab}	2.77 ^b	10.35 ^a	4.31 ^{ab}
b	56.95 ^a	47.71 ^{ab}	40.02 ^b	22.36 ^c
a+b	66.78 ^a	50.48 ^b	50.36 ^b	26.66 ^c
c	0.028	0.023	0.023	0.030
D	25.01 ^a	14.50 ^{bc}	18.11 ^{ab}	9.85 ^c
Effective Degradability, %				
k=0.02	40.56 ^a	27.66 ^b	27.15 ^b	15.54 ^b
k=0.04	31.48 ^a	19.89 ^{bc}	21.93 ^b	12.16 ^c
k=0.06	26.61 ^a	15.87 ^{bc}	19.21 ^{ab}	10.40 ^c
k=0.08	23.55 ^a	13.40 ^{bc}	17.54 ^{ab}	9.30 ^c

Legend: Row means with different superscript are significantly different ($P < 0.05$).

Waterman (2000) and Brooker *et al.* (1999) reported that in forage legumes with moderate levels of tannin (less than 4%) higher growth and milk yield of ruminants were obtained. However, where tannin levels exceeded 6% of the diet, deleterious effects were reported in terms of growth rates (Norton, 2000). Other researchers reported that while tannin has beneficial effects on ruminants, excessive intake of tannin may result in decrease in nutrient utilization, depressed palatability and voluntary feed intake, decreased rumen ammonia, reduced growth and even death of the animal (Hagerman *et al.*, 1992; Pell *et al.*, 2000; Kumar and D'Mello, 1995). Our results suggest that treatment of forage legumes with 6% mimosa tannin is the optimum and safe level to impart protection on forage proteins from rumen fermentation.

Crude Protein Degradation

Table 2 shows the data for the mean rumen crude protein digestibility, degradation parameters and effective degradability of macerated *Gliricidia* and *Leucaena* leaves treated with tannin after 72 hours of incubation in the rumen.

The leucaena sample had the significantly highest mean crude protein digestibility compared with the *Gliricidia* samples. Among *Gliricidia* samples, treatment with 6% mimosa tannin resulted in the lowest protein solubility (13.31%),

which suggests that there was protein-tannin complex formation. The protein digestibility of *Gliricidia* samples treated with 3% mimosa tannin was significantly higher than with *Gliricidia* treated with 0% mimosa tannin. This finding validated the results of the radial diffusion assay, which indicated that treatment of 3% mimosa tannin may or may not have been sufficient to bind all proteins contained in *Gliricidia*. Further, these results imply that 6% mimosa tannin treatment protected the feed protein from rumen microbial action.

The readily degradable protein fraction (a) was lowest in untreated *Gliricidia* compared with that treated with 3% mimosa tannin. The effect of mimosa tannin on protein can be seen on the degradability of the slowly degradable fraction (b) between *Gliricidia* treated with 3 and 6% mimosa tannin. The addition of 6% mimosa tannin in macerated *Gliricidia* significantly decreased the solubility of its protein as shown in the lower fraction of the potentially soluble fraction (a+b) compared with all the other treatments. Similarly, 6% mimosa tannin in *Gliricidia* resulted in the lowest amount of protein degradation (D) due to the formation of tannin-protein complex. The rate constant for the degradation of the slowly degradable fraction of protein was not significantly affected by the treatment of mimosa tannin.

These results indicate that the addition of mimosa tannin in macerated *Gliricidia* tree leaves

at the level 6% has a positive effect in protecting crude protein degradability of feed samples. This is further shown by the lower effective protein degradability at different rumen outflow rates. Min *et al.* (2001) reported that the presence of tannins in the feed reduces protein degradability in the rumen. Further, effective degradability values would indicate that when the rate of protein degradation is slow relative to the rate of rumen outflow, feed protein escapes microbial degradation (Norton, 2000; Broderick, 1995).

The role of tannin on the protection of protein is well recognized, albeit there also exists negative effects on the performance of the animals as well as the digestibility of the other nutrients in the feed. These variations on the effects of tannin in ruminant feeding have been attributed partly to the wide differences in chemical structure and composition of tannins (Barahona *et al.*, 1977; Stern *et al.*, 1996; Odenyo *et al.*, 1999). In this study, it was demonstrated that mimosa tannin, which is used in leather tannery, can provide ample protection for the proteins in *Gliricidia* when added at 6% of dry matter.

Conclusions

Mimosa tannin supplementation at level 6% protects the crude protein digestibility of the feed in the rumen. Our results suggest that treatment of forage legumes with 6% mimosa tannin is the optimum and safe level to impart protection on forage proteins from rumen fermentation.

Literature Cited

- AOAC. 1993. Official methods of analysis (16th Ed). AOAC, Washington, D.C.
- ARC. 1984. The nutrient requirement of ruminant livestock. Commonwealth Agriculture Bureau, Slough, England. U.K.
- Barahona, R., C.R. Lacsano, R. Cochran, J. Morril and E.C. Titgemeyer, 1997. Intake, digestion and nitrogen utilization by sheep fed tropical legumes with contrasting tannin concentration and astringency. *J. Anim. Sci.* 75(6): 1633-1640
- Barry, T.N. and W.C. McNabb. 2000. The effect of condensed tannins in temperate forages on animal nutrition and productivity. In: Brooker, J.D. (editor). Tannin in livestock and human nutrition. *ACIAR Proceeding* 92:30-35.
- Broderick, G.A., 1995. Desirable characteristics of forage legumes for improving protein utilization in ruminants. *J. Anim. Sci.* 73(9): 2760-2773
- Brooker, J.D., L. O'Donovan, I. Skene and G. Sellick. 1999. Mechanisms of tannin resistance and detoxification in the rumen. In: BELL, C.R., M. BRYLINSKY and P. JOHNSON-GREEN (eds). Microbial biosystems: New frontiers. Proceedings of the 8th International Symposium on Microbial Ecology. Atlantic Canada Society for Microbial Ecology, Halifax, Canada.
- Gomez, K.A. and A.A. Gomez. 1984. Statistical procedure for agricultural research. 2nd Ed. An International Rice Research Institute Book, Singapore.
- Hagerman, A.E. 1987. Radial diffusion method for determining tannins in plant extracts. *Journal of Chemical Ecology* 13: 437-449.
- Hagerman, A.E. 2002. Tannin handbook. Department Chemistry and Biochemistry, Miami University Oxford, U.S.A. (<http://www.muohio.edu> or e-mail: hagermae@muohio.edu).
- Hagerman, A.E., C.T. Robins, Y. Weerasuriya, T.C. Wilson and C. McArthur. 1992. Tannin chemistry in relation to digestion. *Journal of Range Management* 45:57-62.
- Kumar, R. and J.F.P. D'Mello. 1995. Antinutritional factors in forage legumes. In: D'Mello, J.F.P. and C. Davendra (eds). Tropical legumes in animal nutrition. CAB International, Wallingford, UK. p.95-133.
- Madsen J. and T. Hvelplund. 1994. Prediction of in situ protein degradability in the rumen. Results of a European ring test. *Livestock Production Science*. Volume 39, Issue 2, July 1994, p.201-212.
- Min B.R., G.T. Attwood, W. McNabb and T.N. Barry. 2001. Effect of condensed tannins on proteolytic bacterial populations in the rumen and on nitrogen flow to the abomasum of sheep. *J. Anim. Sci.* Vol.79, Suppl.1.
- Navas-Camacho A., M.A. Laredo, A. Cuesta, H. Anzola and J.C. Leon. 1993. Effect of supplementation with a tree legume forage on rumen function. *Livestock Research for Rural Development* Volume 5, Number 2, September 1993.

- Norton, B.W. 2000. The significance of tannins in tropical animal production. In: BROOKER, J.D. (editor). Tannin in livestock and human nutrition. *ACIAR Proceeding* 92:14-23.
- Odenyo, A.A., C.S. McSweeney, B. Palmer and P.O. Osuji, 1999. In vitro screening of rumen fluid samples from indigenous African ruminants provides for rumen fluid with superior capacities to digest tannin-rich fodders. *Aust. J. Agric. Res.* 45: 1147-1157
- Ørskov, E.R. and I. McDonald. 1979. The estimation of protein degradability in the rumen from incubation measurement weighed according to rate of passage. *J. Agric. Sci.* 92:499-503.
- Pell, A.N., T.K. Woolston, K.E. Nelson and P. Scofield. 2000. Tannins: biological activity and bacterial tolerance. In: Brooker, J.D. (editor). Tannin in livestock and human nutrition. *ACIAR Proceeding* 92:121-126.
- Reed, J.D. 1995. Nutritional toxicology of tannins and related polyphenols in forage legumes. *Journal of Animal Science* 73:1516-1528.
- SAS. 1996. SAS user's guide. Proprietary Software Release 6.12, TS020. SAS Institute Inc. Cary, NC., USA.
- Stern, J.L., A.E. Hagerman, P.D. Steinberg, P.K. Mason, 1996. Phlorotannin-protein interactions. *J. Chem. Ecol.* 22(10): 1877-1899
- Waterman, P.G. 2000. The tannins – An overview. In: Brooker, J.D. (editor). Tannin in livestock and human nutrition. *ACIAR Proceeding* 92:10-13