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In situ degradability of soyabean meal treated with *Acacia saligna* and *Atriplex halimus* extracts in sheep

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ABSTRACT

The effects of *Acacia saligna* (AC) and *Atriplex halimus* (AT) extracts were evaluated on ruminal soyabean meal (SBM) degradability using the nylon bag technique. Samples of SBM were treated with 0, 4, or 8 g of AC or AT extracts per 100 g SBM. Bags were incubated in two cannulated sheep for 2, 4, 6, 8, 12, 24, and 48 h. The chemical constituents (CCs) of extracts was determined using GC-MS. Rate and potential degradability of dry matter (DM) were decreased (P=0.015) to a greater extent than N degradability (P=0.145) with AC and AT doses. DM and N degradation were decreased (P<0.05) by 15% and 29%; 24% and 47% with AC, and 21% and 29%; 23% and 37% with AT at 4% and 8% for DM and N, respectively. The data suggest the possibility of using these extracts as feed additives to reduce ruminal degradability of SBM in ruminant diets.

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KEY WORD: plant extracts, soyabean meal, degradability, *Acacia, Atriplex*, sheep

INTRODUCTION

Manipulation of the ruminal microbial ecosystem to enhance digestibility of fibrous feed, reduce protein degradability and urinary N excretion, and lower methane emission are some of the most important goals for nutritional research. Plant extracts with high concentrations of secondary metabolites are good candidates for enhancing nutrient utilization (Patra et al., 2006; Patra and Saxena, 2010). Exploited as naturally safe feed additives, chemical plant constituents may inhibit enteric methanogenesis and reduce ammonia release in the rumen. This latter feature has been explained as due to inhibition of microbial urease, formation of complexes with feed proteins (Makkar, 1993), adsorption of free ammonia (Wang et al., 1998), and/or inhibition of proteases decreasing ruminal protein degradability (Kumar and Singh, 1984).

Whether plant extracts are beneficial or detrimental to ruminants depends on if the undegraded amino acids are subsequently available for utilization in the small intestine. This is associated, however, with decreased urine N excretion and lower ammonia disposal from the excreta (Wang et al., 1998). The effectiveness of plants or plant extracts on rumen fermentation depends on the source, type, and content of active compounds (Patra and Saxena, 2010). With low levels of secondary compounds, they exhibit some potential to improve rumen fermentation. The aim of the present work was to evaluate the potential effects of plant extracts rich in the chemical constituents of *Acacia saligna* and *Atriplex halimus* at different doses on ruminal degradation of a highly ruminally degradable protein (i.e., soyabean meals).

MATERIAL AND METHODS

Preparation of extracts and soyabean meal treatments

Leaf samples of *Acacia saligna* (AC) and *Atriplex halimus* (AT) were collected randomly from several young and mature trees during the summer and dried at 40°C for 72 h in a forced air oven and ground to pass a 1 mm sieve. Samples were immediately extracted at 1 g leaf/8 ml of solvent mixture. The mixture of solvents contained 10 ml methanol, 10 ml ethanol and 80 ml of distilled water. Plant materials were individually soaked and incubated in solvent in the laboratory at 25°C to 30°C for 48 to 72 h in closed black 500 ml bottles. After incubation, the bottles were heated at 39°C for 1 h, and then immediately filtered and the filtrates (extract) were collected. The extract was evaporated to obtain the crystallized extract (powder) to be used further in the treatment of soyabean meal and assay for secondary compounds.

Samples of 100 g soyabean meal (SBM; DM/kg 919 g; crude protein/kg DM 524 g and ash/kg DM 72 g) were ground using a hammer-mill fitted with a 2 mm screen and sprayed with 100 ml distilled water containing 0, 4, or 8 g of AC or AT extracts. Samples were dried at 35-40°C for 24 h and ground again through a 2 mm screen to remove lumps.

Ruminal degradation

Rumen degradation was measured by *in situ* methods. Nylon bags (12.0 × 8.5 cm) made of filter cloth with an approximate pore size of 45 μm diameter was used. Five grams (DM) of each SBM were incubated in the rumen of each of the two ruminally cannulated adult sheep for 2, 4, 6, 8, 12, 24, and 48 h. Two bags for each of the five treated meals were introduced in two different days into the rumen of each sheep before the morning feeding, and remained in the rumen for the intended incubation time. At the end of each incubation time, the bags were removed and washed under cold tap water until the rinsing water was clear. They were then dried in a forced air oven at 40°C and weighed. Duplicate bags containing samples of the treatments were washed and dried as described for incubated samples to estimate zero-time disappearances.

Chemical analysis and secondary constituents assays

Treated meals and residues from the nylon bags at each incubation time were analysed for dry matter (AOAC, 1990). Crude protein was determined by a Kjeldahl procedure (AOAC, 1990).

One gram of the powdered individual extract was dissolved in 10 ml of the same solvent used in the extraction and was fractionated by funnel separation with a double volume of ethyl acetate (99.7/100, (Sigma®-Aldrich) to determine total phenolics (TP) by drying and quantifying the TP layer in the funnel. After TP separation, a double volume of n-butanol (99.9/100, (Sigma®-Aldrich), was added to fractionate the saponins (SP; Makkar et al., 1998). For the alkaloid (AK) extract, dried samples were first extracted with ethanol and then dissolved in diluted hydrochloric acid. This solution was filtered and extracted with petroleum ether to remove fat (Arambewela and Ranatunge, 1991). The remaining solution was considered to be the aqueous fraction (AF) which contained other secondary metabolites including lectins, polypeptides, and starch (Cowan, 1999).

Samples from AC and AT extracts were dissolved in an equal amount of methyl alcohol to give a concentration of 100 ppm. The dissolved extracts were injected into a GC/MS (Hewlett Packard HP 5890 series II with HP 5890 Mass Spectrometer GC/MS, Germany) to assay the chemical constituents of each plant extract. The area under each peak was considered the concentration of each chemical constituent.

Calculations and statistical analysis

Ruminal disappearance (dg) data were used to determine nutrient degradation parameters using the equation from Ørskov and McDonald (1979):

$$DIS = a + b\,(1 - e^{-c(t - L)})$$

where: *DIS* - the fraction that disappeared from the bag after *t* hours, *a* - the fraction that immediately disappears from the bag (intercept), *b* - the insoluble fraction that is potentially degraded over time, *c* - the fractional rate of degradation of fraction *b,* and *L* - the lag time (h). The equations were fitted to *in situ* degradation profiles using the NLIN procedure of the SAS package (SAS, 1989).

Extent of degradation (*dg*) was estimated according to the equation described by Ørskov and McDonald (1979): $dg = a+(bc)/(ck_p)$, by using the parameters *a*, *b* and *c* and a ruminal passage fractional rate (k_p) value of 0.06/h.

Data relating to DM and N disappearances were subjected to one-way analysis of variance. The effects of experimental treatments were separately tested for each incubation time with the animal effect as a block. Comparisons among soyabean meals for the rumen degradation parameters and the *dg* were also performed using one-way analysis of variance with the animal effect as a block. All data were analysed using the Statistical Analysis Systems (SAS, 1989).

RESULTS

Atriplex halimus extract (AT) had a higher concentration of potentially antimicrobial compounds than *Acacia saligna* extract (AC). Secondary compounds, i.e., TP, SP, and AF (lectins, polypeptides and starch), were higher in the AT extract than in the AC extract, whereas the AK contents were lower in the AT extract (Table 1).

Table 1. Secondary metabolites of *Acacia saligna* and *Atriplex halimus* extracts, g/kg DM

Metabolite	*A. saligna*	*A. halimus*
Total phenolics	61.2	112.9
Saponins	23.5	123.8
Alkaloids	3.2	2.3
Aqueous fraction[1]	68.3	475.1

[1] lectins, polypeptides and starch, according to Cowan (1999)

Numerous chemical constituents (CCs) were detected by GC-MS in the two extracts In the AC extract, 16 CCs were found, whereas only 8 were seen in the AT extract (Table 2). All of the CCs could be grouped in such categories as

Table 2. Detection of chemical components in *Acacia saligna* and *Atriplex halimus* extracts using the GC-MS

Chemical constituents	Area, %[1]	Retention time, min	Classification
A. saligna extract			
2-phenyl-3-oxetanone	3.90	2.37	Ketone
1-methylpyrazole	0.20	9.06	Azole
1-propanamine, 3-(10,11-dihydro-5H-dibenzo (a,d)cyclohepten-5-ylidene)-N-methyl	0.44	10.09	Alkaloids
2-decyloxyethanol	0.13	12.45	Ether
N-undecane	0.35	14.62	Liquid alkane hydrocarbon
2-propyldecan-1-ol	0.13	16.67	Volatile compound
Humuladienone	0.25	17.04	Ketone
Oxirane, 2-methyl-3-propyl 2-(2-hydroxyl-3-methoxypropyl)-4,6-dibenzoylresorcinol	0.09	18.65	Phenolics
1,3,5-triazine-2,4-diamine	0.22	19.35	Alkaloid
Carboxymethylamine hemihydrochloride	0.09	19.55	Carboxylic compound
Dimethylphosphinic azide	0.11	19.92	Alkaloid (Amide)
Cyclopentanamine	1.58	20.52	Alicyclic hydrocarbon
Pentanoic acid	1.46	20.63	Straight fatty acid
1-benzyl-4-methyl-2-azatidinone	0.15	21.09	Heterocyclic ketone
2-methyleneamino-propyl	0.12	21.18	Alkaloids
3-methyl-2,1-benzisoxazole	0.10	21.40	Azole
A. halimus extract			
Benzenecarbothioic acid	8.16	2.30	Ester acid salt
Benzamide	1.80	2.46	Amide
2-decyloxyethanol	0.13	12.44	Ether
Octadecane	0.30	14.60	Higher chain alkane
Imidazole-2,4,5-d3	0.09	16.69	Azole
2, 2-dideuterio-4-phenyl-butanenitrile	0.14	19.35	Cyano compound
benzene, azidomethyl	0.07	19.55	Azide
4-methoxy-1,4-dihydro-2h-pyrrole	0.24	20.63	Alkaloid

[1] concentration based on the total areas of the identified peaks in the chromatogram of the GC-MS

phenolics, alkaloids, toxic amino acids, and fatty acids. The relative concentration of each CC was considered as the % area under the peak of the spectrum analysis. The CCs present in the largest amounts in the AC extract were 2-phenyl-3-oxetanone, cyclopentanamine, and pentanoic acid (3.9%, 1.58%, and 1.46%, respectively), while in the AT extract, benzenecarbothioic acid and benzamide (8.2% and 1.8%, respectively). In the AC extract, oxirane, 2-methyl-3-propyl,2-(2-hydroxyl-3-methoxypropyl)-4,6-dibenzoylrecorcinol, carboxymethylamine hemihydrochloride, and 3-methyl-2,1-benzisoxazole were found in the smallest amounts. Benzene, azidomethyl, and imidazole-2, 4, 5-d3 appeared to be present in the smallest amounts in the AT extract.

Both doses (i.e., 4% and 8%) of plant extracts had a negative effect on both DM and N disappearance from treated SBM compared with control at all the incubation times. This effect was dependent on the incubation time and dose of

Table 3. Ruminal dry matter disappearance at different hours of incubations and degradability of soyabean meal treated with different doses of *Acacia saligna* (AC) and *Atriplex halimus* (AT) extracts

	Control	AC4	AC8	AT4	AT8	SEM[1]	P-values for contrasts, linear effect		
							AC	AT	AC vs AT
Disappearance (D)[2]									
D02	0.584	0.377	0.355	0.356	0.349	0.0363	0.027	0.033	0.73
D04	0.668	0.481	0.382	0.457	0.372	0.0180	0.003	0.001	0.38
D12	0.816	0.748	0.578	0.624	0.580	0.0225	0.007	0.002	0.042
D24	0.911	0.840	0.733	0.778	0.704	0.0333	0.020	0.025	0.24
D48	0.956	0.881	0.793	0.873	0.821	0.0230	0.012	0.020	0.67
Degradability									
a[2]	0.420	0.366	0.340	0.356	0.327	0.0159	0.047	0.043	0.50
b[2]	0.514	0.521	0.463	0.526	0.508	0.0235	0.31	0.088	0.33
$a+b$[2]	0.934	0.887	0.803	0.882	0.835	0.0206	0.032	0.012	0.53
c (per h)	0.147	0.115	0.094	0.079	0.070	0.0078	0.018	0.029	0.012
L (h)	0.00	2.19	4.60	1.80	2.71	0.579	0.019	0.005	0.10
dg[3]	0.784	0.666	0.554	0.623	0.560	0.0136	0.001	0.001	0.23

[1] standard error of the mean

[2] g/g incubated

[3] dg, extent of degradation (g degraded/ g ingested; k_p = 0.06/h)

Table 4. Ruminal nitrogen disappearance at different hours of incubations and protein degradability of soyabean meal treated with different doses of *Acacia saligna* (AC) and *Atriplex halimus* (AT) extracts

	Control	AC4	AC8	AT4	AT8	SEM	P-values for contrasts, linear effect		
							AC	AT	AC vs AT
Disappearance (D)[2]									
D02	0.621	0.194	0.151	0.168	0.141	0.0197	0.001	0.001	0.40
D04	0.658	0.336	0.155	0.322	0.230	0.0411	0.003	0.008	0.49
D12	0.862	0.799	0.421	0.521	0.538	0.0243	0.003	0.004	0.021
D24	0.946	0.834	0.639	0.824	0.757	0.0365	0.014	0.008	0.20
D48	0.953	0.870	0.810	0.875	0.780	0.0286	0.070	0.001	0.68
Degradability									
a[2]	0.466	0.252	0.145	0.173	0.150	0.0130	0.001	0.001	0.035
b[2]	0.504	0.632	0.713	0.746	0.645	0.0324	0.038	0.004	0.50
$a+b$[2]	0.971	0.884	0.858	0.919	0.795	0.0334	0.16	0.001	0.69
c (per h)	0.167	0.146	0.061	0.076	0.113	0.0082	0.017	0.67	0.34
L (h)	0.00	3.70	4.05	1.85	2.92	0.265	0.001	0.007	0.002
dg[3]	0.789	0.610	0.426	0.543	0.503	0.0121	0.001	0.001	0.68

[1] standard error of the mean

[2] g/g incubated

[3] dg, extent of degradation (g degraded/ g ingested; k_p = 0.06/h)

plant extracts used to treat the meals. The lowest ($P<0.05$) disappearance of DM and N were observed for SBM treated with AC extract at 8%, while the greatest disappearance rates were for control SBM. The differences between treated SBM and control in *in situ* DM and N disappearance became noticeable after 12 h of incubation. Beyond this time, differences between treated and control SBM were similar to those observed at 12 h. The response to extract treatment was dose dependent, especially with AC extract-treated SBM (Tables 3 and 4).

Overall, ruminal degradability fractions of SBM were depressed ($P=0.015$) by the addition of AC and AT extracts. Degradability of DM (i.e., extent of degradation (*dg*) and fractional degradation) was depressed ($P=0.015$) more than N degradability ($P=0.145$) by all of the extract doses tested.

DISCUSSION

The protein supply to ruminants is largely microbial protein reaching the small intestine. Dietary protein that escapes ruminal microbial degradation can be utilized provided that it can be hydrolysed and absorbed post-ruminally (Noftsger and St-Pierre, 2003). Plants produce a huge variety of secondary metabolites and many of these substances with antimicrobial properties may prove particularly useful in modifying rumen fermentation (Wallace, 2004). A major aim is providing enough N supply from degraded feed proteins for the growth and maintenance of the ruminal microbial population. Plant secondary compounds can play an important role modifying ruminal fermentation *via* their interaction with ruminal microorganisms or with animal feed components. It seems that the secondary constituents of AC and AT extracts (Tables 1 and 2) might be detrimental to some important rumen microbial species.

From tables 3 and 4 it can be seen that treatment of SBM either with AC or AT extracts at different doses markedly reduced rumen degradation kinetics of DM and N. The reduction was generally more pronounced with the AC extract, as it contains a greater concentration of secondary constituents (Table 2). Similar results were reported earlier by Hervas et al. (2000) using SBM treated with tannic acid or quebracho, with these treatments resulting in decreased NH_3-N concentrations in the rumen of sheep. Many phenolic compounds that could affect rumen microorganisms were detected in AC and AT extracts (Table 2), which could decrease SBM degradability. Microorganisms present in sheep rumen liquor can extensively degrade decarboxylate 4-hydroxybenzoic acid and demethoxylate 3-methoxy-4-hydroxybenzoic acid (Chesson et al., 1982). Decarboxylation of 4-hydroxycinnamic acids, however, is insignificant, as side chains are rapidly hydrogenated to give 3-(4-hydroxyphenyl) propionic (phloretic) acid and its

derivatives (Chesson et al., 1982). Martin (1978) suggested some further metabolic processes by rumen microbes giving rise to the appearance of 3-phenylpropionic acid in rumen liquor. On the other hand, ruminal microorganisms might be able to degrade the secondary constituents of extracts such as alkaloids, saponins, and phenolic compounds (Wachenheim et al., 1992). Ingested saponins from *Tribulus terrestris* were hydrolysed in the rumen (Miles et al., 1994). *Butyrivibrio* and *Bacteroides* strains have been isolated from the rumen; they are capable of degrading lucerne saponins (Gutierrez et al., 1959). Rothrock et al. (1955) have reported that the saponins from *Dioscorea tubers* can be cleaved into their component parts, diosgenin and the sugar moiety, by ruminal *Aspergillus terreus*. Some microorganisms that may be responsible for degradation of gallate, pyrogallol, phloroglucinol, and quercetin, have been isolated recently from the rumen, among them *Syntrophococcus sucromutans*, which demethoxylates various phenolic compounds (Krumholz and Bryant, 1986). Ferulic and p-coumaric acids in forage cell walls may act as cross-linking agents between polysaccharide and lignin components (Scalbert et al., 1985). Esterification of these phenolic acids to polysaccharides reduces microbial degradation rate of the polysaccharides (Jung and Sahlu, 1986). Differences between control and treated SBM disappeared, however, with increasing time of incubation; this may indicate that ruminal microorganisms are capable of degrading the secondary constituents of plant extracts.

In situ DM and N degradabilities of SBM were generally reduced ($P<0.05$) when treated with AC extract. The effect of treatment on N degradation could be due to decreased proteolysis, degradation of peptides, and deamination of amino acids in the rumen, as suggested by Newbold et al. (1990). The decreased degradation of N fractions could suggest that AC tannins can bind SBM protein making it less soluble and more resistant to rapid degradation in the rumen. The formation of undegradable complexes (Waghorn et al., 1994) between condensed tannins and protein and/or carbohydrates may have reduced the amount of substrate available for fermentation. The escape of these undegradable complexes from the rumen into the abomasum (Wiegand, 1996) could also decrease the N concentration in the rumen fluid, which may impair microbial growth and activity. This may partly explain the general low degradation of DM of SBM treated with either AC or AT extracts. This reduction can be attributed to the slower rate of degradation (parameter c) observed in all treatment. Likewise, the treatment clearly reduces the potential extent (a+b) of degradability, contrary to the results reported by Makkar et al. (1995).

It is likely that the use of high doses of plant extracts with antimicrobial activity would decrease microbial activity and diet fermentability (Evans and Martin, 2000). The effect of plant extracts on degradation parameters indicates

that the doses used were toxic to ruminal microbes. The effect of the treatment with plant extracts on degradation of DM and N was more pronounced during the 48 h of incubation. These results provide evidence of the dynamics of N utilization by ruminal microbes and suggest that these additives had a short-term effect on ruminal microbial fermentation (Tables 3 and 4).

CONCLUSIONS

Ruminal degradability of soyabean meal (SBM) was decreased by the addition of *A. saligna* and *A. halimus* extracts. Dry matter and nitrogen degradability was lower at both doses of *A. saligna* than with *A. halimus* extracts. This data suggests that the evaluated extracts of *A. saligna* and *A. halimus* have the potential to reduce the ruminal degradability of SBM in ruminant diets and may increase the post rumen by-pass of amino acids to the small intestine.

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