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Tannins determined by various methods as predictors of methane production reduction potential of plants by an *in vitro* rumen fermentation system

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ABSTRACT

Relationships between chemical constituents, including values obtained with tannin assays (*i.e.*, total phenols, total tannins, condensed tannins and tannin activity using a tannin bioassay) for plant materials ($n = 17$), and methane production parameters at 24 h of incubation in the *in vitro* Hohenheim gas method were established. The methane production reduction potential (MRP) was calculated by assuming net methane concentration for the control hay as 100%. The MRP of *Bergenia crassifolia* leaves and roots, and *Peltiphyllum peltatum* leaves, was >40%. Amongst the chemical constituents, neutral detergent fibre had a high correlation ($r = 0.86$) with methane concentration. There was negative relationship between total phenol, total tannins or tannin activity and methane concentration. However, a positive relationship existed between these tannin assays and the MRP, with r-values ranging from 0.54 to 0.79 ($P<0.05$). A very weak relationship ($r = 0.09$) occurred between condensed tannins and MRP. Similar results to those with MRP were obtained with the percent increase in methane on addition of polyethylene glycol. The highest correlations, 0.79 and 0.92 ($P<0.001$), were between tannin activity determined using the tannin bioassay and the MRP, or the percent increase in methane on addition of polyethylene glycol, respectively, suggesting that this tannin assay could be used to identify plants possessing antimethanogenic properties. Leaves of

Abbreviations: ADF, acid detergent fibre; AGPs, antibiotic growth promoters; CP, crude protein; CT, condensed tannins; MRP, methane reduction potential; NDF, neutral detergent fibre; PEG, polyethylene glycol; TP, total phenols; TT, total tannins.

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Rheum undulatum, *Vaccinium vitis-idaea*, *B. crassifolia*, *Rhus typhina* and *P. peltatum*, and roots of *B. crassifolia* have considerable potential (*i.e.*, >25%) to decrease enteric methane production from ruminants.

1. Introduction

Methane production from ruminants contributes to total global methane production, which is an important contributor to global warming (Hartung and Monteny, 2000; Lassey, 2007). Enteric methane contributes 30–40% of total methane production from agricultural sources (Moss et al., 2000). Many attempts, such as concentrate supplementation (Lovett et al., 2005), use of probiotics and prebiotics (Mwenya et al., 2004; Takahashi et al., 2005), lipid supplementation (Van Nevel and Demeyer, 1996; Ungerfeld et al., 2005), and addition of plant extracts (Sliwinski et al., 2002; Patra et al., 2006; Goel et al., 2008) have been made to decrease enteric methane production.

Antibiotic growth promoters (AGPs), such as monensin and lasalocid, also decrease methane production (Fuller and Johnson, 1981). However, AGPs have been banned in Europe since 2006, and many countries outside the European Union are also considering a ban. As a result, scientists have intensified efforts to exploit plants, plant extracts and natural plant compounds as potential natural alternatives to AGPs for enhancement of livestock productivity while minimizing environment impacts (Makkar et al., 2007), including decreased methane production (Soliva et al., 2008). There is a need to develop or identify in vitro methods to screen large numbers of plants in a short time and with limited resources. We evaluated relationships between tannin values from several tannin assays and chemical composition parameters with in vitro rumen methane production, with the aim at identifying analytes that could be used to identify plants possessing antimethanogenic properties.

2. Materials and methods

2.1. Experimental plants and chemical composition determination

All samples except *Salix alba*, *Rhus typhina* and *Peltiphyllum peltatum* were collected from Mongolia (Table 1). The plants collected from Mongolia are used locally in the region of production as medicinal plants. *S. alba* and *R. typhina* were collected from the Botanical Garden of the University of Hohenheim in Stuttgart (Germany) and *P. peltatum* was provided by Dr. John Wallace of the Rowett Research Institute in Aberdeen (UK). Air-dried samples were ground to pass a 1-mm sieve. The ground samples were analysed for dry matter (DM) by drying at 100 °C for 16 h. Crude protein (CP, #988.05) and ether extract (EE, #988.05) were determined using AOAC (1990), while neutral detergent fibre (NDF) and acid detergent fibre (ADF) were analysed by Van Soest et al. (1991). NDF analysis was without addition of amylase, and both NDF and ADF are expressed inclusive of residual ash.

2.2. Tannin assays

For the tannin assays, samples were ground to fine powder in a Ballmill MM 200 (Retsch GmbH, Haan, Germany) at 50 Hz for 2 min. Samples (0.2 g) were extracted in 10 ml aqueous acetone (acetone:water, 7:3) twice for 20 min in an ultrasonic waterbath (105W). The extracted samples were centrifuged (6000 × *g*, 10 min, 4 °C), and the supernatants were combined and used for tannin analysis on the same day.

Tannin assays were according to Makkar (2003a). Total phenols and total tannins in the extract were determined by a modification of the Folin-Ciocalteu method using polyvinylpolypyrrolidone (PVPP) to separate tannin phenols from non-tannin phenols, and condensed tannins were determined by the butanol–HCl–iron method. Both total phenols and total tannins were expressed as tannic acid equivalent and condensed tannins as leucocyanidin equivalent.

Polyethylene glycol (PEG), which has a high affinity for tannins and makes them inert by binding to them, is used to identify specific effects of tannins. Tannin bioassay was based on incubation of

Table 1
Chemical composition, total phenols (TP), total tannins (TT) and condensed tannins (CT) and percent gas increase on PEG addition (tannin bioassay) of plant materials.

Plant	Crude protein	Ether extract	NDF[a]	ADF[b] (g/kg DM)	TP[c]	TT[c]	CT[d]	Tannin bioassay (% increase in gas[e])
Artemisia frigida[f]	90.3	14.1	560.7	426.8	21.9	4.0	0.8	0
Tanacetum vulgare	151.8	34.5	462.3	416.0	37.5	5.7	0.4	0
Iris lactea	103.0	14.7	495.1	436.3	38.1	28.9	14.3	0
Rhenum undulatum	105.2	6.0	227.7	168.5	76.6	55.7	7.1	92.7
Thymus gobicus	103.6	23.3	540.5	443.0	35.9	11.6	0.2	33.4
Seratula centauroides	114.9	35.4	625.8	509.4	58.3	46.0	0.5	7.2
Stellera chamaejasme	133.9	27.7	391.3	312.2	43.8	15.4	0.3	0.4
Taraxacum officinale	245.9	26.2	317.7	270.6	22.7	7.1	0.3	11.0
Delphinum elatum	136.4	22.4	515.4	387.1	22.2	8.5	0.7	4.1
Artemisia frigida[g]	156.4	19.4	548.1	431.0	19.7	5.1	0.3	4.3
Vaccinium vitis idea	63.2	33.3	476.1	354.1	243.3	149.2	174.5	96.3
Salsola laricifolia	94.8	11.7	574.3	380.3	65.3	32.0	28.0	17.7
Bergenia crassifolia	63.1	23.9	260.9	190.4	320.2	178.4	14.1	169.8
Bergenia crassifolia[h]	32.7	7.7	226.0	197.0	309.3	165.0	33.1	204.7
Salix alba	169.0	17.0	322.0	185.0	56.5	35.5	14.5	0.7
Rhus typhina	140.0	56.0	220.0	174.0	221.6	209.3	0.8	45.7
Peltiphyllum peltatum	113.0	20.0	191.0	183.0	200.0	146.8	15.7	122.6

All samples except[a] were leaf samples.
[a] Neutral detergent fibre.
[b] Acid detergent fibre.
[c] As tannic acid equivalent.
[d] As leucocyanidin equivalent.
[e] On PEG addition.
[f] Cut on 20 August 2007.
[g] Cut on 20 July 2007.
[h] Was a single root sample.

tannin-containing plant materials with and without PEG (molecular weight 6000) in buffered rumen fluid in the *in vitro* Hohenheim gas method (Makkar et al., 1995). The increase in gas on addition of PEG is a measure of tannin activity, where the protocol used was similar to that described in Makkar et al. (1995). For this assay, rumen liquor was collected just before morning feeding from two cannulated Friesian Holstein cows fed a total mixed ration based on grass silage. The rumen liquor, strained through a 100-μm nylon net, was pooled and used as the source of inoculum.

2.3. Gas and methane determination

After 24 h of incubation, total gas was recorded from visual assessment of the calibrated scale on the syringe. Methane was measured using an infrared (0–30 ml methane/100 ml gas range) methane analyser (Pronova Analysentechnik GmbH & Co. KG, Berlin, Germany) calibrated against 10.6 ml methane/100 ml gas (Goel et al., 2008). After measuring gas volume, the tubing of the syringe outlet was inserted into the inlet of the methane analyser. The display on the methane analyser displays methane as percent of total gas.

2.4. Observations and calculations

Net methane and gas productions were calculated from the differences of the methane and gas in the test syringe and the corresponding blank, and the methane concentration was determined as:

$$\frac{\text{Net methane production}}{\text{Net gas production}} \times 100$$

Methane production reduction potential (MRP) was calculated by taking net methane values for the control (hay) as 100%:

$$\text{MRP} = \frac{\%\,\text{Net methane in control} - \%\,\text{Net methane in the test}}{\%\,\text{Net methane in control}} \times 100$$

The other parameter calculated was percent increase in methane production after PEG addition, which was calculated as:

$$\text{Methane increase (\%)} = \frac{\text{CH}_4 \text{ with PEG addition (ml)} - \text{CH}_4 \text{ without PEG addition (ml)}}{\text{CH}_4 \text{ without PEG addition (ml)}} \times 100$$

2.5. Statistical analysis

Data were analysed using correlation and regression options in Minitab software (Minitab, 2000). Multiple linear regression was used to establish relationships between various tannin assays and other chemical composition parameters and methane production parameters (*i.e.*, methane concentration, MRP and percent increase in methane production after PEG addition). The method used was backward elimination of parameters not contributing significantly (*i.e.*, $P<0.05$) to the variation. The predictors which have multicollinearities such as between total phenols and total tannins, and between NDF and ADF, were taken into consideration (*i.e.*, when one was included in the stepwise regression the other was not). The total n for all regressions was 17.

3. Results

The CP content (g/kg DM) varied widely, from 32.7 to 245.9, with the highest being for *Taraxacum officinale* leaves and lowest for *Bergenia crassifolia* roots (Table 1). Total phenols (TP) content in *Vaccinium vitis idea*, *R. typhina*, *B. crassifolia* and *P. peltatum* leaves and *B. crassifolia* roots was >200 g/kg DM, and total tannins (TT) content in these samples was >100 g/kg DM. Activity of tannins in these samples, as represented by the increase in gas on addition of PEG, was also high (Table 1). The methane concentration, MRP and percent increase in methane on addition of PEG are in Table 2. The MRP of *B. crassifolia* leaves and roots and *P. peltatum* leaves was >40% (Table 2). The MRP of zero for *Seratula centauroides* meant that the methane concentration for this sample was the same as that of the control hay. Similarly, zero values of percent increase in methane on addition of PEG for other materials illustrate that tannins in these samples do not contribute to methane reduction.

Amongst the nutrients, NDF and ADF were positively correlated with methane concentration, with r-values of 0.86 and 0.80, respectively ($P<0.001$), while no relationships existed for CP and ether extract. Negative relationships ($P<0.001$) occurred between NDF or ADF and the MRP, as well as with methane increase on PEG addition ($P<0.05$) (Table 3).

There were negative relationships of TP, TT and tannin activity with methane concentration. The correlation (r) values ranged from −0.59 to −0.75 with $P<0.05$ for these relationships (Table 3). The correlations for TP were generally higher than for TT, suggesting a contribution of non-tannin phenols in reducing methane production. A very weak relationship occurred between CT and methane concentration. There were positive relationships between TP, TT or tannin activity and the MRP, as well as with methane increase with PEG addition. Amongst the tannin assays, the highest correlation of 0.79 ($P<0.001$) occurred between tannin activity and MRP. Relationships between tannin assays were stronger for methane increase with PEG than for MRP (Table 3). The correlation between MRP and methane increase after PEG addition was 0.73 ($P<0.001$). On addition of PEG, the methane concentration in materials showing high tannin activity by the tannin bioassay increased to almost 81% of the control hay level (results not shown).

Stepwise regression results of tannin assays and other dietary compounds showed that the tannin bioassay contributed significantly in most of the algorithms derived to predict methane production parameters (Table 4). Furthermore, tannin bioassay was the only significant parameter ($P<0.05$) for estimation of methane increase with PEG addition, with an adjusted R^2 of 0.835. Total phenols also

Table 2
Methane concentration, Methane production reduction potential (MRP) and methane increase by PEG addition from some plant materials.

Plant	Methane (%)	MRP (%)	Methane increase with PEG addition (%)
Artemisia frigida[a]	17.5	5.9	0.0
Tanacetum vulgare	15.8	15.1	0.0
Iris lactea	17.1	8.1	0.0
Rhenum undulatum	0	100	137.5
Thymus gobicus	15.1	18.8	0.0
Seratula centauroides	18.6	0	4.3
Stellera chamaejasme	15.9	14.5	0.0
Taraxacum officinale	15.5	16.7	13.5
Delphinum elatum	17.3	7.0	2.3
Artemisia frigida[b]	18.5	0.5	2.4
Vaccinium vitis idea	13.7	26.3	92.7
Salsola laricifolia	22[d]	−18.3	23.4
Bergenia crassifolia	9.5	48.9	215.8
Bergenia crassifolia[c]	4.4	76.3	417.4
Salix alba	12.5	12.1	7.3
Rhus typhina	10.4	27.1	16.4
Peltiphyllum peltatum	5.7	60.0	78.8
SEM	0.98	5.10	18.78
LSD	1.14	6.22	15.05

All samples except [a] were leaf samples. For calculation of methane (%), MRP (%) and methane increase with PEG addition (%), see Section 2.

[a] Cut on 20 August 2007.
[b] Cut on 20 July 2007.
[c] Was a single root sample.
[d] Methane concentration was higher than the control hay.

Table 3
Correlation coefficients (r) between chemical composition and various tannin assays and methane production parameters.

Predictor	Methane (%)	MRP (%)	Methane increase with PEG addition (%)
Crude protein	0.29^{ns}	-0.36^{ns}	-0.62^{**}
Ether extract	0.20^{ns}	-0.27^{ns}	-0.35^{ns}
Neutral detergent fibre	0.86^{***}	-0.79^{***}	-0.55^{*}
Acid detergent fibre	0.80^{***}	-0.71^{***}	-0.53^{*}
Total phenols	-0.59^{*}	0.57^{*}	0.78^{***}
Total tannins	-0.60^{*}	0.54^{*}	0.62^{**}
Condensed tannins	-0.07^{ns}	0.09^{ns}	0.24^{ns}
Tannin bioassay	-0.75^{***}	0.79^{***}	0.92^{***}

^{ns} Not significant.
* $P<0.05$.
** $P<0.01$.
*** $P<0.001$.

contributed significantly to some regression equations, but to a lesser extent than the tannin bioassay. NDF and ADF contributed to the predictive equations, but exhibited a response opposite to the tannin assays (*i.e.*, increased methane concentration and decreased the MRP).

4. Discussion

Tannin-containing plants have been shown to reduce ruminal methanogenesis (Carulla et al., 2005; Puchala et al., 2005; Tavendale et al., 2005), although some reports suggest that tannins have no effect on rumen methane production. For example, Oliveira et al. (2007) reported that there was no effect of tannin levels from diets containing sorghum silages with higher or lower tannin concentrations on methane production. Beauchemin et al. (2007) also reported that feeding a diet-containing quebracho tannins extract up to 20 g/kg DM failed to reduce enteric methane emissions in growing cattle, although the protein-binding effect of the quebracho tannin extract was evident.

Table 4
Stepwise multiple linear regression of various tannin assays and other dietary compounds.

Response	Regression equations	Adjusted R^2 (%)
CH_4 (%)	$3.07 + (0.0305 \times TP) - (0.0733 \times bioassay) + (0.0262 \times NDF)$[a]	83.3
	$-3.89 + (0.0915 \times TP) - (0.137 \times bioassay) + (0.0531 \times CP) - (0.259 \times EE) + (0.0434 \times ADF)$[b]	86.7
	$-4.38 + (0.0345 \times CP) + (0.0337 \times NDF)$[c]	79.5
	$6.60 - (0.0368 \times bioassay) + (0.0269 \times ADF)$[d]	71.8
MRP (%)	$132 - (0.514 \times TP) + (0.133 \times CT) + (0.707 \times bioassay) - (0.319 \times CP) + (1.07 \times EE) - (0.186 \times NDF)$[a]	93.7
	$98.1 - (0.545 \times TP) + (0.885 \times bioassay) - (0.268 \times CP) + (1.40 \times EE) - (0.183 \times ADF)$[b]	87.6
	$54.3 + (0.220 \times bioassay) - (0.0980 \times NDF)$[c]	71.4
	$7.39 + (0.362 \times bioassay)$[d]	59.1
Methane increase with PEG addition (%)	$-14.4 + (1.55 \times bioassay)$[a,b,c,d]	83.5

Bioassay: tannin bioassay, increase in gas production on PEG addition.
[a] Predictors: TP, NDF and CT, tannin bioassay, CP, EE.
[b] Predictors: TP, ADF and CT, tannin bioassay, CP, EE.
[c] Predictors: TT, NDF and CT, tannin bioassay, CP, EE.
[d] Predictors: TT, ADF and CT, tannin bioassay, CP, EE.

In the present study, the MRP was calculated with respect to methane concentration for the hay since most samples studied can be categorised as forages. This parameter reflects the total methane production reduction potential of the samples, while the increase in methane on addition of PEG shows the contribution of tannins only. High correlations between tannin activity using the tannin bioassay and the MRP, or the increase in methane on addition of PEG, and increases in methane concentration to near control hay values on addition of PEG supports the view that tannins decrease methane production. Tannin-containing plants, therefore, could be strategically used in diets to decrease methane emissions from ruminants. Incomplete reversal of methane production on addition of PEG might be due to the presence of other secondary metabolites in the plants studied, which were largely medicinal. Preliminary studies (data not shown) had shown that some of these samples have saponins and lectins.

A higher correlation of TP than TT with MRP and methane increase with PEG addition suggests a role of non-tannin phenols in methane reduction. It would be interesting to obtain direct evidence by isolating these non-tannin phenols and incubating them *in vitro*. These results, if confirmed, could have wide application since non-tannin phenols are not likely to decrease utilization of proteins and other nutrients, but could also have beneficial effects (*e.g.*, antioxidant, anticarcinogenic) associated with phenolic compounds (Makkar, 2003b; Makkar et al., 2007).

Other dietary components such as NDF and EE also contributed to explaining total variation in methane production. Higher NDF increases methane production by shifting short chain fatty acid proportion towards acetate which produce more hydrogen. Ether extract had no relationship with all methane expressions when it was correlated individually, but when it was included together with tannin assays and other dietary components, the effect was significant for some predictive equations. The role of lipids in decreasing methane is well illustrated, albeit at levels higher than present in most of the samples in this study (Bucher et al., 2008; Beauchemin et al., 2008). In addition, most of the samples in our study were forage in nature with low levels of ether extract. The nature of lipid and its form (*i.e.*, bound or free) are also determining factors in its effect on methane production (Beauchemin et al., 2008).

For screening plants for antimethanogenic activity, the most promising tannin assay was the tannin bioassay followed by TP and TT. Amongst the tannin assays, determination of TP is relatively simple and is possible using routine laboratory equipment. Screening of plants for TP could provide information on their antimethanogenic potential. If facilities for conducting tannin bioassay exist, this assay should be preferred.

All chemical tannin assays, except the tannin bioassay, measure tannins under conditions (*i.e.*, temperature, pH, ionic strength) that differ from those of the rumen. Thus the results have limited applicability for predicting the activity of tannins. Polyethylene glycol is considered to specifically bind tannins, and its use in the *in vitro* rumen assay is a better representation of tannin activity under rumen conditions. Similar results have been obtained when different assays were used to predict the nutritive value of tannin-containing feedstuffs; and tannin bioassay, protein precipitation capacity assay and TP (in the order mentioned) were good predictors of apparent N disappearance in ruminants, but CT was a very poor predictor (Makkar, 2005). The present study also showed that CT values are poor predictors of MRP. It is becoming increasingly evident (Makkar, 2005) that values obtained using the butanol–HCl–iron method for CT are not indicative of the biological activity of tannins, and the results obtained using this method should be interpreted with caution.

5. Conclusions

Tannins decrease methane production and, among the tannin assays, tannin bioassay (a reflection of tannin activity) was the best predictor of the methane production reduction potential of a plant. Total phenol and total tannins were also good predictors of methane production reduction potential. As chemical analysis of these parameters is relatively simple, they could be used to screen large numbers of samples in a relatively short time and, with commonly available laboratory facilities, for their antimethanogenic potential in the quest to obtain plants and plant products as alternatives to conventional antibiotic growth promoters. If the facilities for conducting tannin bioassay exist, this assay should be preferred. The leaves of *Rheum undulatum*, *V. vitis-idaea*, *B. crassifolia*, *R. typhina* and *P. peltatum*, and roots of *B. crassifolia* have considerable potential (*i.e.*, >25%) to decrease enteric methane production from ruminants.

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