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Molecular weight and protein binding affinity of *Leucaena* condensed tannins and their effects on *in vitro* fermentation parameters

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ABSTRACT

The objectives of the current study were, firstly to determine the molecular weight of condensed tannins (CTs) of 62-2-8 *Leucaena*-hybrid Bahru (LLB) and relating it to its protein binding ability, using the local *Leucaena leucocephala* (LLL) as control and; secondly to evaluate the effect of different levels of CTs of the hybrid LLB on rumen fermentation parameters, including dry matter and nitrogen digestibility and methane gas production. The weight-average molecular weights (M_w) of purified CTs extracted from hybrid LLB and local LLL, determined using gel permeation chromatography were near similar, being 2737 and 2872 Da, respectively. However, the CTs of the hybrid LLB exhibited stronger protein binding ability than the local LLL (0.305 and 0.420 mg, respectively, for LLB and LLL to bind half maximum bovine serum albumin). The above results seems to suggest that molecular mass of CTs, at least for *Leucaena* forages, is not the sole factor in determining their capability to bind protein. Inclusion of about 20–40 mg of CT/g DM of the CTs from hybrid LLB effectively decreased ($P<0.05$) methane production and *in vitro* nitrogen digestibility. The above results suggested a potential of using CTs from the hybrid LLB to protect dietary protein from rumen degradation for better utilization in the small intestine of ruminant animals, and in addition to mitigate their enteric methane emission, often implicated as a source of greenhouse gases.

1. Introduction

Leucaena leucocephala is the most exploited member of the *Leucaena* genus for animal feed, as the leaves contain high crude protein (CP) content ranging from 20 to 30% and are highly digestible (Brewbaker, 1987). This 'giant' *Leucaena* was widely used as protein supplement in animal feed until the first outbreak of the *Leucaena* psyllid, *Heterosylla cubana* Crawford, which occurred in Florida in 1983. This pest which highly preferred to feed on *L. leucocephala* was later discovered in Hawaii in 1984 (Sorensson and Brewbaker, 1987) and subsequently caused devastating defoliation to *Leucaena* plants in the Asia Pacific region. After several generations of crossing and selection between *L. leucocephala* and *L. diversifolia* for acid tolerant and adaptability to high soil aluminum, resulted in the release of two hybrids, namely 40-1-18 *Leucaena*-hybrid

Abbreviations: BSA, bovine serum albumin; BW, body weight; CH_4, methane; CP, crude protein; CTs, condensed tannins; Da, Dalton; DM, dry matter; DP, degree of polymerization; IVDMD, *in vitro* DM degradability; IVND, *in vitro* nitrogen degradability; LLB, *Leucaena*-hybrid Bahru; LLL, local *L. leucocephala*; M_n, number average molecular weight; M_w, weight-average molecular weight; ND, nitrogen degradability; PDI, polydispersity index.

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Rendang (LLR) and 62-2-8 *Leucaena*-hybrid Bahru (LLB) in Malaysia (Wong et al., 1998). Since one of the parental lines was *L. diversifolia*, which is known to contain higher secondary compounds including tannins, it was suggested that the hybrids may be resistant to psyllid attack. Recent study (Liang and Khamseekhiew, 2006), however, has reported that these two hybrids exhibited lower dry matter (DM) and nitrogen digestibility than local *Leucaena* varieties, presumably due to higher content of secondary compounds, particularly condensed tannins (CTs).

Tannins, one of the many secondary compounds found in plants, are oligomeric with multiple structure units containing free phenolic groups with molecular weight ranging from 500 to >20,000 Dalton (Da) (Mané et al., 2007). Tannins are categorized into two groups based on their structures; hydrolyzable tannins and CTs. The latter, also known as proanthocyanidins, are the most common type of tannins found in forage legumes, and structurally are complexes of oligomers and polymers of flavanoid units linked by carbon–carbon bonds (Hagerman and Butler, 1991). Condensed tannins have both beneficial and adverse effects depending on their concentration (Aerts et al., 1999a). Protein binding ability of CTs, particularly to protect protein from rumen fermentation for better utilization of the dietary protein in ruminant animals had received much attention. Molecular size (McNabb et al., 1998; Aerts et al., 1999b), structure (Perez-Maldonado et al., 1995) and pH (Makkar and Singh, 1995) were among the factors examined for their influence on protein binding ability of CTs.

Approximately, 2–15% of feed energy is lost via the release of methane from livestock production (Johnson and Johnson, 1995). Relative to CO_2, methane has a global warming potential that is 23 times higher (IPCC, 2001). It was reported that methane contributes to 20% of greenhouse gases effect, and about 15% of the methane are contributed by ruminants with a 1% increase annually (Crutzen, 1995; Moss et al., 2000). Therefore, mitigation of methane emission through nutrient manipulation in ruminants has two potential roles; alleviating the effect of greenhouse gases and enhancing the efficiency of feed utilization. Although Beauchemin et al. (2007) found that feeding up to 20 mg/g of the dietary DM quebracho tannin extract failed to reduce enteric methane emissions from growing cattle, other studies (Tavendale et al., 2005; Animut et al., 2008) suggested otherwise. The above discrepancy thus reaffirmed that the effects of CTs on rumen fermentation (Getachew et al., 2008; Ammar et al., 2009) and perhaps protein binding ability as well, are influenced by multi-factors.

The current study consisted of two experiments with the following objectives; firstly to determine the molecular weight of CTs of 62-2-8 *Leucaena*-hybrid Bahru (LLB) and relating them to their protein binding ability, using the local *L. leucocephala* (LLL) as control and, secondly to evaluate the effect of different levels of CTs of the hybrid LLB on rumen fermentation parameters, including DM and nitrogen digestibility and methane gas production.

2. Materials and methods

2.1. Experimental forages

62-2-8 *Leucaena*-hybrid Bahru (LLB) and local *L. leucocephala* (LLL) were harvested from the research farm of Department of Animal Science, Universiti Putra Malaysia (3°00′18.88″N, 101°42′15.05″E) between 09:00 to 10:30 h by cutting tips of about 30 cm from the youngest fully expanded leaves of the main stems and branches from several trees. The harvested samples were immediately frozen on pellet dry ice (Dalzell and Shelton, 1997), freeze-dried prior to grinding through a 1.0 mm sieve and stored at 4 °C in air tight dark container pending further analysis. *Panicum maximum* forage was also harvested from the same site, oven dried (60 °C) overnight prior to grinding through 1.0 mm sieve, stored for later use as substrate in the gas production study.

2.2. Extraction and purification of CTs

Condensed tannins were extracted from the freeze-dried hybrid LLB and local LLL using aqueous acetone/diethyl ether as described by Terrill et al. (1992) and purified (Terrill et al., 1990) with a 40 cm × 16 mm id XK 16 column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) packed with Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Low molecular weight fractions and other polyphenols were eluted with 400 ml/l methanol and the CTs with 800 ml/l aqueous acetone. After evaporating traces of aqueous acetone using Büchi rotary evaporator (Büchi Labortechnic, Flawil, Switzerland), the purified CTs were lyophilized and stored at 4 °C in the dark.

2.3. Molecular weight determination by gel permeation chromatography

Molecular weight of the purified CTs was determined by Gel Permeation Chromatography (Waters, Milford, USA) with columns (HR 0.5, HR 1 and HR 2, for molecular mass ranges of 0–1000, 100–5000 and 500–20,000 Da, respectively) connected in series. Purified CTs was dissolved in tetrahydrofuran which was also used as solvent at 1 ml/min (25 °C). Relative molecular weight was calculated after calibration with polystyrene (molecular weight standards) in the range of 162–22,000 Da. Weight-average molecular weights (M_w) were used to represent relative molecular weights of purified CTs. Polydispersity index (PDI), is a measure of the distribution of molecular mass in a given polymer sample in organic chemistry which indicates the distribution of individual molecular mass in a batch of polymers. The PDI was calculated as the weight-average molecular weight (M_w) divided by the number average molecular weight (M_n): PDI = M_w/M_n. Degree of polymerization (DP) was also estimated on the basis of a constituent proanthocyanidin peracetate M_w of approximately 500 (Williams et al., 1983).

2.4. Protein binding affinity of CTs

Protein binding affinity of the purified CTs from hybrid LLB and local LLL was determined using protein precipitation assay of Makkar et al. (1987) with minor modification. Bovine serum albumin (BSA) was used to determine the ability of the CTs to bind protein. Different concentrations of BSA, from 0 to 1.2 mg/ml were used to generate the standard curve. Half milliliter of the CTs at concentrations of 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 and 1.6 mg/ml were dissolved in 500 ml/l aqueous methanol and mixed with 0.5 ml BSA buffer (1 mg/ml of BSA in 0.2 M acetate buffer with 0.17 M NaCl at pH 5.0) for sample analysis. After centrifuged at 5000 × *g* for 20 min, the supernatant was carefully discarded and the unbound protein was removed by washing with 0.2 M acetate buffer. All the tubes were then oven dried at 100 °C overnight. The hydrolysis was carried out by using 13.5 M NaOH in the oven at 120 °C for 20 min. The tubes were then cooled after 1 ml acetic acid was gently added and an aliquot of 0.1 ml was added to 1 ml of ninhydrin solution. After 20 min of incubation in 100 °C water bath, the tubes were cooled and 5 ml of deionized water was added. The absorbance was recorded at 570 nm after vortex. Equation for protein binding data was analyzed using a non-linear regression procedure. The curve for purified CTs was fitted for sigmoid curve: $Y = a/(1 + b \times \exp^{(-c \times x)})$, where Y = mg of BSA precipitated, X = mg of extracted CTs incubated. Protein binding affinity of CTs was expressed as b-value which represented the amount of CTs used to bind half of the maximum BSA.

2.5. In vitro determination of rumen fermentation parameters

Two near matured rumen fistulated Kedah–Kelantan male cattle (averaged 209 kg BW) fed with 0.025 of BW DM/day containing *P. maximum* and commercial cattle concentrate (60:40) were used as donors of rumen liquor. 0.5 g of oven dried *P. maximum* (substrate) with 0 (control), 10, 15, 20 and 25 mg of CTs were incubated for 24 h with the rumen liquor (40 ml) in 100 ml calibrated gas syringe (Häberle Labortechnik, Lonsee-Ettlenschieß, Germany). Standard hay (University of Hohenheim, Stuttgart, Germany) with an estimated gas production of 49.61 ml/g DM was used as a standard to calibrate the *in vitro* gas production system. A total of six syringes for each treatment were used for total gas and methane production measurements. After that, the contents of three syringes were used for *in vitro* DM degradability (IVDMD) and pH analysis, and the remaining three syringes were used for the determination of *in vitro* nitrogen degradability (IVND). The above procedures were performed in three separate runs.

In vitro gas production was measured according to Menke and Steingass (1988) modified by Makkar et al. (1995). The gas produced in the head space of glass syringes were recorded at the end of 24 h incubation at 39 °C. Methane gas production was measured by injecting 500 μl of the gas from each of the above syringes into gas chromatograph (Agilent 6890 Series Gas Chromatograph, Wilmington, DE, USA) equipped with thermal conductivity detector. Separation was achieved using a HP-Plot Q column (30 m × 0.53 mm × 40 μm) (Agilent Technologies, Wilmington, DE, USA) with nitrogen (MOX, Kuala Lumpur, Malaysia) as the carrier gas at the flow rate of 3.5 ml/min. An isothermal oven temperature of 50 °C was adopted in the separation. Calibration was completed using standard methane prepared by Scott Specialty Gases (Supelco, Bellefonte, PA, USA). All the procedures were replicated three times. The pattern of non-linear gas production were obtained by fitting data of cumulative gas production to the exponential equation described as $p = a + b[1 + e^{-c(t)}]$ (Ørskov and McDonald, 1979); where p represents the cumulative gas production at time t, c is the rate of gas production (h^{-1}), and $(a + b)$ equals the potential accumulate gas production.

IVDMD and IVND were determined using methods of Jones et al. (2000) modified from Tilley and Terry (1963). Each determination was replicated three times as described before.

2.6. Statistical analysis

The data were analyzed by the general linear model procedure of SAS v. 9.0 (SAS Inst. Inc., Cary, NC). Orthogonal polynomial contrasts were used to test for linear, quadratic and cubic effects of levels of CTs. Significant differences were accepted if $P<0.05$.

3. Results

3.1. Molecular weight distribution of purified CTs from LLB and LLL

The M_w, PDI and degree of polymerization of the purified CTs extracted from LLB and LLL are presented in Table 1. The molecular weights of the purified CTs from hybrid LLB and local LLL, defined as M_w, were 2737 and 2871 Da, respectively. The molecular weight profiles of CTs from LLB and LLL (Fig. 1) showed normal distribution and similar distribution curve characteristic. The M_w values were about twice that of the M_n, therefore, the calculated PDI (M_w/M_n) were 1.81 and 1.77, respectively for LLB and LLL.

Table 1
Molecular weight distribution of the purified CTs extracted from hybrid (LLB) and local (LLL) *Leucaena*.

Source of CT	M_w[a] (range)	DP[b]	PDI[c]	Peak width[d]
LLB	2737 (251–31,623)	5.47	1.81	31.4
LLL	2871 (282–31,623)	5.74	1.78	31.2

[a] M_w: weight-average molecular weight (Da).
[b] Values represent degree of polymerization (DP) rounded to the nearest whole number, estimated as M_w/500, on the basis of M_w of a proanthocyanidin peracetate unit of approximately 500 (Williams et al., 1983).
[c] PDI = polydispersity (M_w/M_n).
[d] Calculated as $(DP_{max} - DP_{min})/2$.

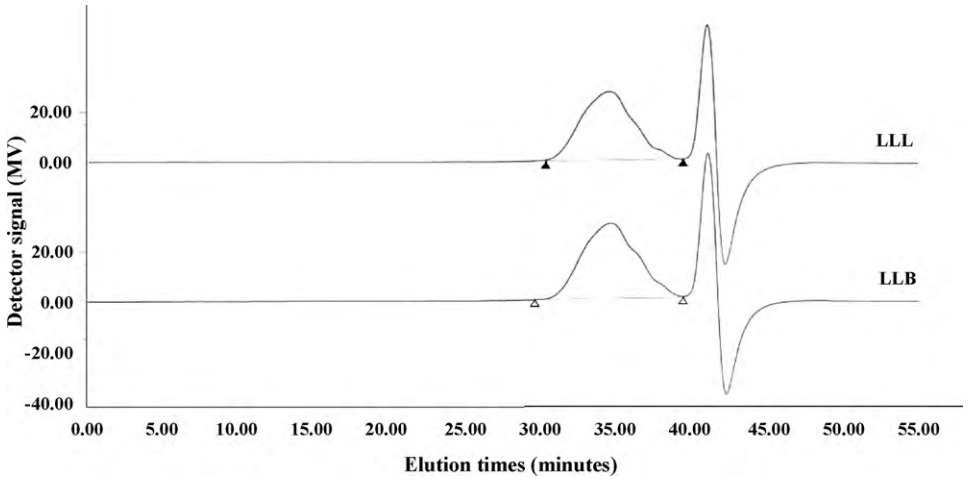

Fig. 1. Chromatogram of the gel permeation chromatography (GPC) determination of molecular weight of CT from LLB (△) and LLL (▲).

3.2. Protein binding affinity of purified CTs

Standard curve obtained from the different concentrations of BSA was $Y = 0.783X$ ($r^2 = 0.999$), where Y represents the optical density at 570 nm and X was the BSA concentration. *b*-value, the amount of CTs used to bind half of the maximum BSA is used to denote the protein binding affinity of CTs, *i.e.* protein binding affinity of the CTs is stronger when its *b*-value is smaller. The *b*-value of the CTs extracted from LLB was 0.305 ± 0.05, while that for LLL was 0.420 ± 0.02, indicating stronger protein binding affinity of CTs of LLB than that of LLL (Fig. 2).

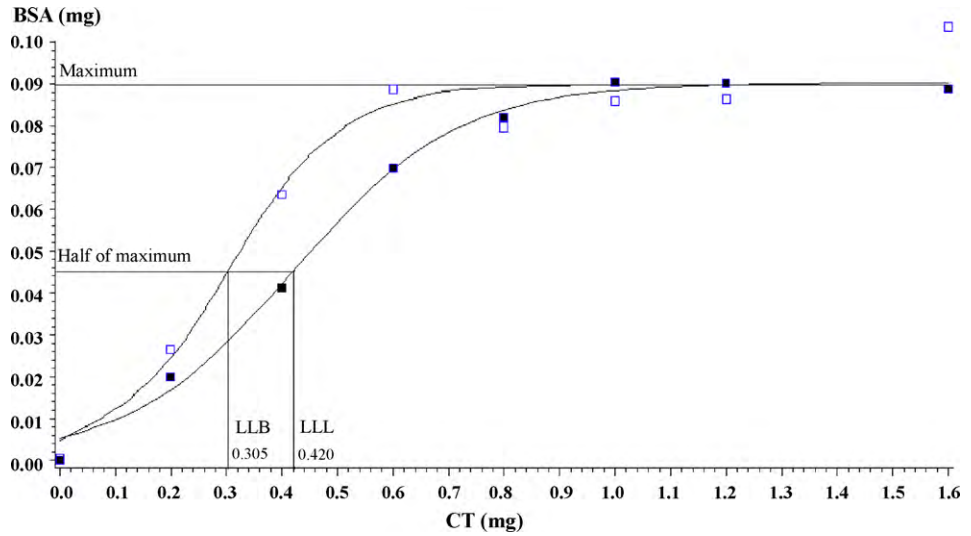

Fig. 2. Protein binding affinity of purified CTs from LLB (□) and LLL(■) (*Y*-axis represents BSA precipitated, while *X*-axis represents extracted CTs incubated).

Table 2
Effects of CTs concentrations extracted from *Leucaena*-hybrid Bahru (LLB) on *in vitro* rumen fermentation parameters using *Panicum maximum* as the substrate.

Parameters	Different levels of CTs (mg/500 mg DM)					SEM	*P*=		
	0 mg (control)	10 mg	15 mg	20 mg	25 mg		L[a]	Q[b]	C[c]
Gas Production (ml/g DM)	68.89	55.33	49.39	45.72	48.11	2.0708	<0.0001	0.0013	0.8170
CH_4 (ml/g DM)	8.83	6.31	5.18	4.85	5.04	0.5011	0.0002	0.0077	0.5953
CH_4/total gas (v/v)	0.13	0.11	0.10	0.11	0.10	0.0021	≤0.0001	0.0004	0.4447
pH	7.06	7.07	7.09	7.09	7.09	0.0240	0.2986	0.5905	0.8309
IVDMD[d]	0.35	0.36	0.34	0.33	0.26	0.0150	0.0013	0.0190	0.7206
IVND[e]	0.51	0.42	0.42	0.42	0.42	0.0065	≤0.0001	≤0.0001	0.0008
c (h^{-1})[f]	0.034	0.030	0.032	0.025	0.030	0.0021	0.0587	0.2674	0.3879

[a] L: linear effect.
[b] Q: quadratic effect.
[c] C: cubic effect.
[d] IVDMD: *in vitro* DM degradability.
[e] IVND: *in vitro* nitrogen degradability.
[f] *c*: the rate of gas production (h^{-1}).

3.3. Effect of purified hybrid CTs on in vitro total gas and methane production

Total gas production (24 h) from the incubation substrate (*P. maximum*) in all CTs inclusion treatments decreased linearly and quadratically ($P<0.05$) as compared to the control (Table 2). Gas production declined with increasing amounts of CTs inclusion, with a 19.68% reduction for the 10 mg treatment and continued to decline to a maximum of 33.63% reduction for the 20 mg treatment, thereafter the effect was reduced. The constant rate of gas production (*c*) was in the range of 0.25–0.34 with the inclusion of CTs.

Methane production decreased ($P<0.05$), irrespective of the level of inclusion of CTs from hybrid LLB, being 8.83 ml/g DM for the control (0 mg CTs) and 6.31, 5.18, 4.85, 5.04 ml/g DM for the 10, 15, 20 and 25 mg/500 mg DM inclusion, respectively (Table 2). The 15, 20 and 25 mg/500 mg DM treatment groups were more effective in inhibiting methane production, equivalent to 41.34, 45.07 and 42.92% reduction, respectively, followed by the 10 mg/500 mg DM group, which recorded reduction of 28.54%. Linear and quadratic changes ($P<0.05$) were recorded for both, methane production (ml CH_4/g DM) and ratio of CH_4 to total gas production, as the amount of CTs inclusion increased.

3.4. In vitro DM and nitrogen degradability

The effect of purified CTs on IVDMD was according to linear and quadratic changes ($P<0.05$). At the inclusion rate of 25 mg/500 mg DM substrate, IVDMD decreased by 25.71% of the control.

In vitro nitrogen degradability for the 0 (control), 10, 15, 20 and 25 mg CTs treatments were 0.51, 0.42, 0.42, 0.42 and 0.42, respectively (Table 2). Irrespective of inclusion levels, CTs exhibited linear, quadratic and cubic reduction ($P<0.05$) in IVND. Reduction of 17.65% in the IVND was recorded irrespective of levels of CTs inclusion.

4. Discussion

The present study was a follow up of the previous work (Liang and Khamseekhiew, 2006) conducted in this laboratory which suggested that the lower DM and nitrogen digestibility of 40-1-18 *Leucaena*-hybrid Rendang and 62-2-8 *Leucaena*-hybrid Bahru (LLB) recently released in Malaysia (Wong et al., 1998) as compared to the local varieties could be due to the higher molecular weight CTs of the hybrids. Therefore, the objectives of this study were two folds; firstly to determine the molecular weight of CTs of the 62-2-8 *Leucaena*-hybrid Bahru (LLB) and relating them to their protein binding ability, using the local *L. leucocephala* (LLL) as control and, secondly to evaluate the effect of CTs of the hybrid LLB on rumen fermentation parameters.

4.1. Molecular weight and protein binding affinity of CTs from hybrid LLB and local LLL

In general, molecular mass of polyphenol in plants ranges from 100 to 10,000 Da (Yanagida et al., 2002), while those for tannins ranges from 500 to >20,000 Da (Mané et al., 2007). McAllister et al. (2005) examined nine different temperate plants and reported that the molecular weight of CTs ranged between 3036 and 7143 Da. Other studies (Foo et al., 1996, 1997; Maie et al., 2003), however, reported much lower values; ranged between 1300 Da for fresh willow to 2200 Da for *Lotus pedunculatus*. The above data thus suggested genetic makeup and environmental influences on molecular mass of CTs. The M_w of CTs for hybrid LLB (2737 Da) and local LLL (2871 Da) determined in the present study, therefore, fall within the range reported for plant CTs in the literature. However, we do not know of any report on the molecular mass of CTs of *Leucaena* in the literature for comparison.

The primary objective of many studies (Osborne and McNeill, 2001; Kariuki and Norton, 2008; Grabber, 2009) on plant CTs focused on the capability of the CTs to bind protein to protect it from microbial fermentation for better utilization of quality dietary protein in the small intestine in ruminant animals. It is now evident that the protein binding affinity of CTs differed among plant varieties (Waghorn and Shelton, 1995, 1997; Schofield et al., 2001) and within the *Leucaena* genus (Osborne and McNeill, 2001). The latter researcher reported that the larger-sized CTs (fractionated by size exclusion chromatography procedure) of *L. pallida* and *L. trichandra* had stronger protein binding than those of the smaller-sized, but the above relationship was not observed for *L. leucocephala*. The above findings thus suggest that molecular size of CTs is not the sole factor influencing protein binding ability. Although it is generally correct to assume that fractionation using size exclusion chromatography procedure separates the CTs into different molecular size fractions, the actual molecular sizes of *Leucaena* CTs in the study of Osborne and McNeill (2001) was not measured. In this study the molecular mass of the *Leucaena* genus were determined, being 2737 Da for the hybrid LLB and 2871 Da for the local LLL. The above results were rather surprising as it was expected that CTs of the hybrid LLB to be higher than those of the local LLL as the former exhibited much higher capability to bind protein (*b*-values of 0.305 and 0.420, respectively, for LLB and LLL). Osborne and McNeill (2001) and Kariuki and Norton (2008) reported *b*-values of 0.295 and 0.305, respectively, for *L. leucocephala* which are comparable to those in the present study.

The weaker protein binding ability of the local LLL is in agreement with several previous suggestions (Osborne and McNeill, 2001; Liang and Khamseekhiew, 2006; Kariuki and Norton, 2008) that the CTs of *L. Leucocephala* has "mild" protein binding affinity when compared with other species and hybrids. Results of the present study thus confirmed that molecular mass of CTs, at least for *Leucaen*a forages, is not the sole factor in determining their capability to bind protein. Perhaps, other factors including the chemical structure could also play a vital role in it.

4.2. Effect of CTs from hybrid LLB on rumen fermentation parameters

The *in vitro* gas production technique provides useful data on the fermentation parameters of feed with a high correlation with *in vivo* study (Menke et al., 1979; Menke and Steingass, 1988). Leinmüller et al. (1991) reported that CTs at concentration exceeding 60 mg/g of DM depressed voluntary feed intake, fiber and protein digestibility and eventually decreased the growth rate of ruminant livestock. However, more recent studies (Priolo et al., 2000; Hove et al., 2001; Vitti et al., 2005) presented contradictory results. Barry et al. (1986) reported that the formation of CTs-protein complexes occurred at CTs concentration smaller than 40 mg/g of the dietary DM. Results of the present study suggested that inclusion of CTs from hybrid LLB decreased total and methane gas productions, even at the lowest inclusion level of 10 mg/500 mg of dietary DM, while 15, 20 and 25 mg/500 mg inclusion levels exhibited more effective reduction as compared to the control. The proportion of methane in total gas decreased from 0.13 in the control (without CTs) to between 0.1 and 0.11 for the various treatment groups further suggesting a direct effect of CTs on methane production. Animut et al. (2008) reported significant reduction in methane production in goats fed with an equivalent of 7–20 mg CT/500 mg DM.

Although IVDMD was not affected by the addition of less than 20 mg/500 mg CTs, IVND was reduced about 9 units (Table 2), irrespective of CTs levels. The above results thus suggested possible formation of CTs–protein complexes even at 20 mg/g (10 mg/500 mg DM) inclusion of CTs from hybrid LLB. Taking into considerations of the effectiveness of CTs to enhance protein utilization and mitigating the emission of methane from ruminants, results of the present study seems to suggest an inclusion of 30 mg/g (15 mg/500 mg DM) to 40 mg/g (20 mg/500 mg DM) is appropriate. However, how much of the unfermented protein could eventually be digested and absorbed in the small intestine for use by the host animals requires further investigations.

5. Conclusion

The present study reported the molecular mass of CTs extracted from *L. leucocephala* and its hybrid LLB. Results from the present study suggested that CTs of the *L. leucocephala* × *L. diversifolia* hybrid (LLB) had stronger protein binding ability than the local *L. leucocephala* (LLL) but, the stronger protein binding affinity was not due to the larger molecular size of the CTs. The above finding thus suggests that, at least for *Leucaena,* the molecular mass of CTs is not the sole factor determining their capability to bind protein, and knowing their chemical structures could provide better understanding to the above.

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