Molecular Weight, Protein Binding Affinity and Methane Mitigation of Condensed Tannins from Mangosteen-peel (*Garcinia mangostana L*)

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ABSTRACT: The objectives of this study were to determine the molecular weight of condensed tannins (CT) extracted from mangosteen (*Garcinia mangostana L*) peel, its protein binding affinity and effects on fermentation parameters including total gas, methane (CH_4) and volatile fatty acids (VFA) production. The average molecular weight (M_w) of the purified CT was 2,081 Da with a protein binding affinity of 0.69 (the amount needed to bind half the maximum bovine serum albumin). *In vitro* gas production declined by 0.409, 0.121, and 0.311, respectively, while CH_4 production decreased by 0.211, 0.353, and 0.549, respectively, with addition of 10, 20, and 30 mg CT/500 mg dry matter (DM) compared to the control ($p < 0.05$). The effects of CT from mangosteen-peel on *in vitro* DM degradability (IVDMD) and *in vitro* N degradability was negative and linear ($p < 0.01$). Total VFA, concentrations of acetic, propionic, butyric and isovaleric acids decreased linearly with increasing amount of CT. The aforementioned results show that protein binding affinity of CT from mangosteen-peel is lower than those reported for *Leucaena* forages, however, the former has stronger negative effect on IVDMD. Therefore, the use of mangosteen-peel as protein source and CH_4 mitigating agent in ruminant feed requires further investigations. (**Key Words:** Condensed Tannins, Mangosteen-peel, Methane Production, Molecular Weight, Protein Binding Affinity)

INTRODUCTION

Microbial fermentation of feedstuff in the rumen produces volatile fatty acids (VFA) and microbial protein to provide the bulk of the energy and protein required by the host animal. Methane (CH_4) is produced during the fermentation process and represents 0.020 to 0.120 of the dietary gross energy loss (Johnson and Johnson, 1995). Methanogenic organisms use the H_2 produced during carbohydrate fermentation to reduce CO_2 to CH_4, therefore, compounds that inhibit the activity of methanogens are likely to reduce ruminal CH_4 production, which has been implicated as a source of greenhouse gases affecting global warming and climate change. Because CH_4 production has a negative correlation with energy exploitation in ruminants, there have been considerable efforts to reduce its production to enhance VFA and microbial mass yields.

Tannins are polyphenols found in many plants (Terril et al., 1992). They can be subdivided into hydrolysable tannins (HT) and condensed tannins (CT). Condensed tannins usually have higher molecular weights (1,000 to 20,000 Da) than HT (500 to 3,000 Da) (Frutos et al., 2004). One of the main features of CT is its affinity to bind and precipitate proteins which are affected by type, molecular mass, structure of tannins (Huang et al., 2011b) and quality of fiber (Tiemann et al., 2008a). Research has suggested that CT suppresses methanogenesis by reducing methanogen population in the rumen directly or via reduction of protozoa population (McAllister et al., 1994; Ngamsaeng et al., 2006; Tan et al., 2011) and decreased NH_3-N (Makkar et al., 1997; Sahoo et al., 2010).

Mangosteen (*Garcinia mangostana*) is a common indigenous fruit in the South-east Asian countries, including Thailand, and its peel (a byproduct from the fruit processing industry) contains a high crude tannins content (0.070 to

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0.150 of dry matter [DM]) and crude protein (CP; 0.083 to 0.215 of DM) (Ngamsaeng et al., 2006; Suchitra and Wanapat, 2008). Because of its high CP content, mangosteen-peel could serve as a feed ingredient for ruminant animals. However, the presence of CT may affect the availability of the protein to the animals as it has been reported that CT, particularly high molecular weight CT exhibits strong protein binding affinity and inhibits CH_4 production (Huang et al., 2011a,b). The objectives of this study were to determine the concentration and molecular weight of CT of mangosteen peel, and examine the effects of the CT from mangosteen-peel on protein binding affinity, CH_4 production and other fermentation parameters *in vitro*.

MATERIALS AND METHODS

Extraction and purification of condensed tannins

Pre-dry manogsteen-peel powder sold commercially in Thailand was used for this study. The manogesteen-peel producers normally collect the raw material (a waste-product) from local fruit processing plants from different locations which in turn purchased the mangosteen fruits from different fruit orchards. Condensed tannins were extracted from the above mentioned mangosteen-peel using aqueous acetone/diethyl ether following the protocol of Terrill et al. (1992) and purified (Terrill et al., 1990) with a 40 cm×16 mm id 16 column (GE Healthcare Bio-Science AB, Uppsala, Sweden) packed with Sephadex LH-20 (GE Healthcare Bio-Science AB, Sweden) as described by Huang et al. (2010). Low molecular weight tannins and other polyphenols were eluted with 400 mL/L methanol and the CT with 800 mL/L aqueous acetone. After evaporating traces of aqueous acetone using Buchi rotary evaporator (Buchi Labortechnic, Flawil, Switzerland), the purified CT were lyophilized and stored at 4°C in the dark for molecular weight, protein binding affinity and *in vitro* rumen fermentation studies.

Molecular weight determination

Molecular weight of the purified CT was determined using Gel Permeation chromatography (Waters, Milford, DE, USA) with columns (25 mM borate, 25 mM potassium chloride, pH 8.5. Column bed dimensions: 16 mm diameter ×~16 cm. Flow rate: ~3 mL/h) 0.5, HR 1, and HR 2, for molecular mass ranges of 0 to 1,000, 100 to 5,000, and 5,000 to 20,000 Da, respectively) connected in series as previously described by Huang et al (2010). Purified CT was dissolved in tetrahydrofuran which was also used as solvent at 1 mL/min (25°C). Relative molecular weights were calculated after calibration with polystyrene (molecular weight standards) in the range of 162 to 22,000 Da. Weight-average molecular weights (M_w) were used to represent relative molecular weights of the 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 M_w divided by the number average molecular weight (M_n): PDI = M_w/M_n. Degree of polymerization was also estimated on the basis of a constituent proanthocyanidin peracetate M_w of approximately 500 (Williams et al., 1983).

Protein binding affinity of condensed tannins

Protein binding affinity of the purified CT was determined using protein precipitation assay with bovine serum albumin (BSA) to determine the relative capacities of the extracted CT to bind protein (Makkar et al., 1987) with minor modification (Huang et al., 2010). Different concentrations of BSA, from 0 to 1.2 mg/mL were used to generate the standard curve. The 0.5 mL of BSA buffer (1 mg/mL of BSA dissolved in 0.2 M acetate buffer [pH 5.0] containing 0.17 M NaCl) and 50 mL/L methanol were added to the CT at concentrations of 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, and 1.6 mg, vortexed at room temperature for 15 min and centrifuged at 5,000×*g* for 20 min. The supernatant was discarded and the unbound protein was removed by washing with 1.5 mL of 0.2 M acetate buffer, centrifuged at 5,000 *g* for 20 min, dried in the oven 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 cooled after 1 mL acetic acid was gently added and an aliquot of 0.1 mL was added to 1 mL of ninhydrin solution. Then incubated in a 100°C water bath for 20 min and 5 mL of deionized water added after the tube was cooled. The absorbance was recorded at 570 nm after vortexing. Equation for protein binding data was analyzed using a non-linear regression procedure. The curve for CT was fitted in a sigmoid curve: $Y = a/(1+b \times exp^{(-cx)})$, where Y = mg of BSA precipitated, *X* = mg of CT incubated. Protein binding affinity of CT was expressed as b-value, which represents the amount of CT used to bind half of the maximum BSA.

In vitro fermentation parameters

Gas production was determined according to Menke and Steingass (1988). Rumen fluid was collected from two rumen fistulated cattle (fed a 0.4 concentrate: 0.6 forage diet) prior to the morning meal. The rumen fluid was immediately transported to the laboratory and homogenized with a kitchen-blender, filtered through four layers of cheese-cloth and continuously purged with CO_2. The well-mixed and CO_2-flushed rumen fluid was added to the buffered rumen fluid solution, which was maintained in a water bath at 39°C. Standard hay (University of Hohenheim, Stuttgart, Germany) with a predictable gas production of 49.61 mL/g DM used as a standard to calibrate the *in vitro*

gas production system. About 500 mg of *Panicum maximum* substrate with 0 (control), 10, 20, and 30 mg CT/500 mg DM were incubated for 24 h with 40 mL buffered rumen fluid under continuous CO_2 reflux in 100 mL calibrated glass syringes (Haberle Labortechnik, Lonsee-Ettlenschieb, Germany) in a water bath maintained at 39°C. Samples were incubated in triplicate together with three syringes containing only incubation medium (blank).

Six syringes for each treatment were used for total gas, CH_4, CO_2, and H_2 production. The volume of gas produced was determined by subtracting the volume of gas displaced in the headspace of the syringes with the initial volume (prior to incubation). The concentrations of CH_4, CO_2, and H_2 in the headspace gas phase of syringes were determined by injecting 500 μL of the gas into the 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. The retention times for CH_4, CO_2, and H_2 were determined using a mixtures of gases prepared by Scott Specialty Gases (Supelco, Bellefonte, PA, USA) which contain 10 mL/L CH_4, CO_2, O_2, and H_2. The calibration of standards for the gases were carried out using pure CH_4 (990 mL/L), CO_2 (990 mL/L) (MOX, Kuala Lumpur, Malaysia) and H_2 (10 mL/L) respectively.

For calibration of the gas chromatograph, the models of non-linear gas production were obtained by fitting data of cumulative gas production to the exponential equation described as $p = b[1-e^{-c(t)}]$ (Ørskov and McDonald, 1979); where *p* represents the cumulative gas production at time *t*, *c* is the rate of gas production (per h), and *b* equals the potential gas production. To do this, the amount of cumulative gas produced at 2, 4, 8, 10, 12, and 24 h incubation were determined.

After 24 h of incubation, the contents of three syringes were centrifuged at 3,500×g for 15 min and the pellet was used for *in vitro* DM degradability (IVDMD) and the supernatant for VFA analysis. Another set of three syringes were used for the determination of *in vitro* nitrogen degradability (IVND). The IVDMD and IVND were determined using methods of Jones et al. (2000) modified from Tilley and Terry (1963) by comparing the weight of residues before and after drying in oven at 60°C for IVDMD and residues from the other set of incubation syringes for IVND determination were washed with distilled water, centrifuged at 3,000×g for 10 min at 25°C and incubated at 39°C for 24 h in 40 mL of acid pepsin. Residues were then dried at 45°C for 72 h and the dried residues were weighed for the IVND. The concentrations of

VFA were determined by gas chromatography (Agilent Technologies, USA, Model GC6890) with a flame ionization detector and fused silica capillary column. The above *in vitro* procedure was performed in two runs. The three analytical replicates for each parameter (i.e., total gas, CH_4, CH_4/total gas, IVDMD, rate of gas production) from each run were averaged and used as replicate in the statistical analysis.

Statistic analysis

The data were analyzed by analysis of variance using the general linear model procedure of SAS. Orthogonal polynomial contrasts were used to test for linear, quadratic and cubic effects of the different levels of CT. Significant differences were defined using p≤0.05. The rate of gas production was analyzed using the non-linear procedure of SAS by fitting data of cumulative gas production to the exponential equation described above.

RESULTS

The concentration of CT in mangosteen-peel was estimated to be 0.154 (DM basis) while the molecular weight, defined as M_w, was 2,081 Da and M_n was 1,133. The M_w value was about twice the M_n, thus, the calculated PDI (M_w/M_n) was 1.84. Protein binding affinity of the CT from mangosteen-peel was estimated to be 0.690 (Figure 1).

Although the rates of gas production (*c*) were not different among treatments, ranging from 0.004 to 0.016, total gas production (24 h) decreased linearly (p<0.01) with increased addition of CT. Similarly, addition of CT also resulted in a linear decrease (p<0.01) in CH_4, CO_2 productions and CH_4: total gas for the CT free control and the 30 mg CT treatment group. However, addition of CT did not affect H_2 production but resulted in linear decrease (p<0.01) in IVDMD and IVND (Table 1).

Total VFA and concentration of acetic, propionic, butyric, isovaleric, and valeric acids decreased linearly with addition of CT (Table 2). Acetic acid concentration decreased by 0.177 from 35.01 mmol in the CT-free control group to 28.83 mmol for the 30 mg CT treatment group, while those for propionic acid decreased by 0.208 from 7.87 to 6.23 mmol, respectively, for the control and 30 mg CT groups, thus resulting in a linear increase in the acetic: propionic ratio with addition of CT.

DISCUSSION

The concentration of CT from mangosteen-peel determined in the present study was 0.154. This value is consistent with those reported by Ngamsaeng et al. (2006) and Suchitra and Wanapat (2008) (0.168 and 0.156, respectively). Concentration of CT in different species of
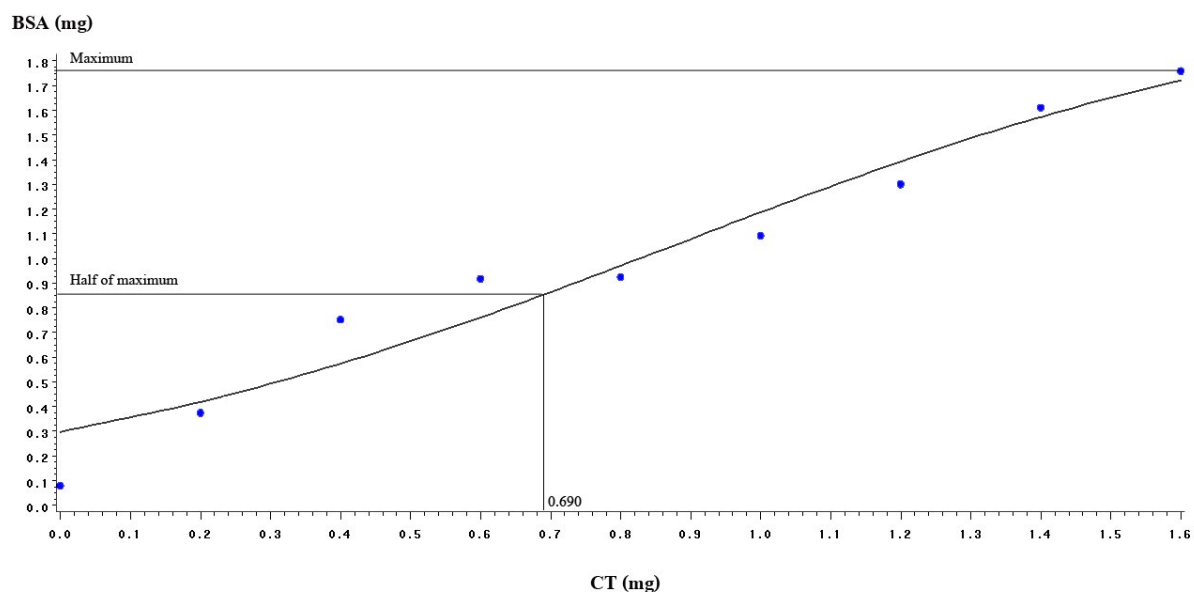
Figure 1. Protein binding affinity of condensed tannin from mangosteen peel as determined by the amount needed to bind half of the maximum bovine serum albumin (BSA) was 0.69.

Leucaena, a widely used forage supplement for ruminant animals in the tropics and sub-tropics, was reported to range from very low to 0.310 (Castillo et al., 1997; McNeill et al., 1998). The relative concentration of CT is one important factor for tannin-protein interaction in a feed material (Waterman and Mole, 1994), with the general rule that a feed ingredient with higher CT content has a stronger protein binding affinity which can prevent it being rapidly fermented in the rumen resulting in higher outflow of dietary protein into the small intestine.

Table 1. Effects of different levels of condensed tannin (CT) from mangosteen-peel on *in vitro* rumen fermentation parameters

| | CT (mg/500 mg DM) | | | | SEM | p value | | |
	0	10	20	30		L	Q	C
Total gas (mL/g DM)	70.48	67.60	62.00	48.58	1.763	0.0006	0.1547	0.7483
CH_4 (mL/g DM)	6.72	5.30	4.35	3.03	0.134	$\leq$0.0001	0.8621	0.4913
CH_4/total gas	0.10	0.08	0.07	0.06	0.001	$\leq$0.0001	0.0111	0.2046
H_2 (mL/g DM)	0.02	0.02	0.03	0.04	0.004	0.0713	1.0000	1.0000
CO_2 (mL/g DM)	5.82	5.67	5.12	3.44	0.179	0.0004	0.0546	0.6593
IVDMD	0.14	0.10	0.08	0.06	0.005	$\leq$0.0001	0.2814	0.4802
IVND	0.43	0.38	0.34	0.31	0.005	$\leq$0.0001	0.4954	0.7940
c (h^{-1})	0.016	0.004	0.008	0.010	0.0034	0.6653	0.2746	0.5709

DM, dry matter; SEM, standard error of the mean; L, linear effect; Q, quadratic effect; C, cubic effect; IVDMD, *in vitro* DM degradability; IVND, *in vitro* nitrogen degradability; c, the rate of gas production (h^{-1}).

Table 2 Effects of different levels of condensed tannins (CT) from mangosteen-peel on *in vitro* volatile fatty acids (VFA) production

| | CT (mg/500 mg DM) | | | | SEM | p value | | |
	0	10	20	30		L	Q	C
Acetic acid	35.01	33.40	30.79	28.83	0.581	0.0016	0.8846	0.7566
Propionic acid	7.87	7.29	6.79	6.23	0.141	0.0010	0.9742	0.9003
Butyric acid	3.44	3.11	3.02	2.77	0.096	0.0304	0.8267	0.6608
Acetic:propionate	4.45	4.59	4.53	4.64	0.025	0.0491	0.1100	2.3700
Isobutyric acid	0.48	0.66	0.38	0.37	0.065	0.3143	0.4744	0.2370
Isovaleric acid	1.10	0.92	0.83	0.82	0.041	0.0272	0.3189	0.9534
Valeric acid	0.46	0.40	0.38	0.37	0.018	0.0995	0.5885	0.9352
Total VFA	48.36	45.78	42.19	39.40	0.883	0.003	0.884	0.922

DM, dry matter; SEM, standard error of the mean; L, linear effect; Q, quadratic effect; C, cubic effect.

However, several researchers examined the effect of molecular weight of CT on protein binding affinity (Aerts et al., 1999) and *in vitro* gas including CH_4 production (Huang et al., 2010) and reported that besides its concentration, CT of larger molecular weight has stronger influence on the above mentioned parameters. In general, molecular weight of polyphenol in plants ranged from 100 to 10,000 Da (Yanagita et al., 1999). Among fruits, it was reported CT from apple juice ranged from 1,754 to 2,907 Da (Shoji et al., 2006) and from 465 to 2,194 Da for grape seed (Yang and Chien, 2000). The molecular weight of CT from mongosteen-peel estimated in this study (2,081 Da) falls within the published range of the various plant materials. However, the molecular weight of the CT from mangosteen-peel is about 0.25 lower than those reported by Huang et al. (2010) for Malaysian *Leucaena leucocephala* (2,737 Da) and its hybrid (*62-2-8 Leucaena leucocephala hybrid-Bahru; 2,871 Da*). The lower molecular weight of CT from mangosteen-peel compared to the *Leucaena* forages could explain the lower protein binding affinity of the former (*b* = 0.690) as compared to the *Leucaena* forages (*b* = 0.305 to 0.420) previously reported (Huang et al., 2010) using similar protocol.

Total gas production declined by the fraction of 0.041, 0.121, and 0.311, respectively, with the 10, 20, and 30 mg CT treatments as compared to the control. The above results suggested that CT from mangosteen-peel has a weaker inhibition effect on fermentation rate than those reported for *Leucaena leucocephala* hybrid (0.197 and 0.336, respectively for 10 and 20 mg CT treatments) by Huang et al. (2010). Similarly, addition of CT also resulted in linear decrease (p<0.01) in CH_4 production by fractions of 0.211, 0.353, and 0.549, respectively, with addition of 10, 20, and 30 mg CT compared to the control (p<0.05). Although the effect of tannins from mangosteen-peel on CH_4 production has been reported by several authors (Poungchompu et al., 2009; Pilajun and Wanapat, 2011; Thanh et al., 2012), they used whole mangosteen-peel powder, which reportedly containing tannins, rather than pure CT as the CH_4 inhibitor in this study. The present results indicate that the inhibitory effect of CT from mangosteen-peel (based on similar CT dosage) on CH_4 production is lower than that of CT from *Leucaena* reported by Huang et al. (2010). The latter examined the additions of 10, 15, 20, and 25 mg CT/500 mg DM, reported reductions of 0.285 for 10 mg CT/500 mg DM and 0.451 for 20 mg CT/500 mg DM groups. The lower inhibitory effect of CT from mangosteen-peel as compared to that from *Leucaena* on CH_4 production could be explained by the smaller molecular weight of the former or different molecular structure of the two sources (Huang et al., 2011a).

The significantly lower CH_4:total gas production ratios between the CT supplemented groups compared to the

control implies that CT had direct effect on reduction of CH_4 and the reduction in CH_4 was not due to the lower total gas production. This observation suggests that CT of mangosteen-peel has stronger effect on CH_4 than overall fermentation rate. Although the present study did not examine changes in the microbial population, including the methanogens, to explain for the effect of CT from mangosteen-peel on CH_4 production, previous study conducted in our laboratory (Tan et al., 2011) suggested CT from *Leucaena* suppresses CH_4 production by reducing methanogens and protozoa populations. Interesting, Pilajun and Wanapat (2011) reported that the effect of mangosteen-peel on CH_4 reduction was lower than that of coconut oil or coconut oil-mangosteen-peel mixture. The authors also reported no differences in methanogens population except minor differences in the methanogen diversity among treatments.

The present results clearly suggest that CT from mangosteen-peel had strong negative effect on IVDMD, nearly by 0.300 even at the lowest inclusion rate of 10 mg/500 mg DM as compared to the control. In agreement with our results, Tiemann et al. (2008b) reported that although inclusion of tannin-rich plants reduced CH_4 emission, the reduction in CH_4 seems to have been mostly the result of a reduced organic matter and fiber digestion.

The primary objective of many studies (Osborne and McNeill, 2001; Kariuki and Norton, 2008) on CT focused on the capability of the CT to bind protein to protect it from microbial fermentation for more efficient utilization of quality dietary protein in the small intestine in ruminant animals. The approximately 0.120 to 0.280 reduction in IVND due to addition of CT recorded in the present study thus suggests that a significant amount of protein could be protected from being fermented in the rumen which could eventually be hydrolyzed and absorbed in the small intestine for the use by the host animals. However, further studies are needed to overcome the strong negative effect of CT from mangosteen-peel on IVDMD if mangosteen-peel is to be used as source of slowly fermentable dietary protein or CH_4 inhibitor in ruminants.

Just like total gas production, *in vitro* VFA production data provides a useful assessment of the extent of feed, particularly carbohydrate, fermentation in the rumen. Total VFA production decreased linearly from 48.36 (control) to between 45.78 to 39.40 mmol, equivalent to between 0.053 to 0.185 reduction from 10 to 30 mg treatment groups. This was expected as addition of CT has resulted in significant negative effect on IVDMD, an indication of its negative effect of microbial activity. However, the ratio of CH_4 per unit VFA production (the latter indicating fermentation rate) reduced from for 13.89 mL/g DM for the control to 11.58, 10.41, and 7.69 mL/g DM, respectively for 10, 20, and 30 mg treatment groups. The above result, in agreement with

that of CH_4 per unit total gas production (see earlier discussion) suggests that CT from mangosteen-peel has stronger inhibition effect on CH_4 production in relation to total gas and total VFA productions. Addition of CT from mangosteen-peel reduced the concentrations of acetic acid, propionic acid, butyric acid, isovaleric acid and increased the acetic:propionic ratio. In contrast, Poungchompu et al. (2009) reported supplementation of 0.121 of a mixture of soapberry fruit and mangosteen-peel increased total VFA and propionate concentration but that of acetate decreased. The inconsistent results between the two studies could be because the supplement used in the latter study contained a mixture of saponins and tannins.

CONCLUSION

Because of the relatively high CP content and the presence of tannins in the mangosteen-peel, there is an increasing interest to use this byproduct from the fruit-processing industry as a source of non-degradable protein and CH_4 mitigating agent in ruminants. To the best of our knowledge, the present study is the first to purify the CT from mangosteen-peel and determine its molecular weight and further examine its efficacy to bind protein and to inhibit CH_4 production *in vitro*. Since CT from mangosteen-peel has strong negative effects on IVDMD, the use of this material as feed ingredient in ruminant production requires further investigations including *in vivo* feeding trials.

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