Effect of *Acacia* purified tannins extract and polyethylene glycol treatment on *in vitro* ruminal fermentation pattern and methane production

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Abstract. Condensed tannins are polyphenolic compounds which can exert beneficial effects in ruminants. They have the ability to bind proteins and decrease their degradation. They have also been reported to reduce methanogenesis and improve ruminants performances. The present work aimed to study the effect of purified condensed tannins from *Acacia horrida* extract on fermentation parameters, gas and methane production. In this context, seven substrates were selected: four *Acacia* species (*Acacia pycnantha*, *Acacia dealbata*, *Acacia horrida* and *Acacia cyanophylla*) and three plant by-products (date palm leaves, grenade peel and artichoke stems). Biological activity of tannins was evaluated by the incubation of the substrates *in vitro* with Polyethylene glycol (PEG). Tannins decreased concentration of all fermentation parameters (gas ($p < 0.0001$), methane (CH_4) ($p = 0.0983$), ammonia (N-NH_3) ($p = 0.0382$), Volatile Fatty Acids (VFA) ($p = 0.0009$), acetate ($p < 0.0001$), propionate ($p = 0.1024$), butyrate ($p = 0.0373$), isobutyrate ($p = 0.0046$), valerate ($p < 0.0001$), isovalerate ($p = 0.0032$)). Substrates were also characterized by their content in main nutrients (dry matter (DM), organic matter (OM), ash, crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL)) and in secondary metabolites (total phenols (TP), total tannins (TT) and condensed tannins (CT)). All substrates presented high CP content except by-products (> 100 g/kg DM) and moderate cell-wall components. CT content was comprised between 120.5 and 680.4 g/kg DM in plants and between 23.1 and 170.4 g/kg DM in plant by-products. *A. horrida* presented the highest biological activity. Thus, their CT were extracted and purified on a sephadex LH-20 column. Purified tannins from *A. horrida* were incubated with two different forages alfalfa hay and barley straw at three different concentrations: 50, 100, 150 mg/g DM. Results showed that purified tannins from *A. horrida* had no effect on fermentation parameters ($P > 0.05$). The effects of *A. horrida* tannins may be strongly linked to their structure and their molecular weight more than to their concentration. For this reason, it is interesting that this work may be completed by physical characterization of these tannins.

Keywords: *Acacia horrida*, *Acacia* spp., plant by-products, purified tannins

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INTRODUCTION

Tannins are secondary metabolites with high molecular weight (Hao *et al.*, 2011) which are widely distributed in plants. Their principal role consists on defense against herbivore predators, and microorganisms (Sliwinski *et al.*, 2002). They are divided into two groups condensed tannins and hydrolysable tannins (Krueger, 2010; Naumann *et al.*, 2017).

In ruminant's nutrition, tannins can have beneficial and harmful effects; harmful effects are due to their ability to bind to macro-moieties mainly proteins (Ningrat *et al.*, 2016; Naumann *et al.*, 2018). They are considered as antinutritional factors that decrease feed intake and nutrient digestibility (Bravo, 1998; Goel *et al.*, 2005; Mezzomo *et al.*, 2011). However, tannins have been reported to modulate fermentation pattern, inhibit methanogenesis, reduce ruminal protein degradation and, consequently, increase its outflow to the intestine (Frutos *et al.*, 2004 ; Liu *et al.*, 2011; Patra and Saxena, 2011) indirectly by proteolytic enzymes inhibition (Makkar, 2003) and/or directly through their molecular coating; via the formation of tannins- proteins complexes. This application represents a biotechnological pathway to improve animal production.

The present work aims at highlighting this biotechnological pathway, thus seven substrates rich in tannins were selected: four plants (*Acacia pycnantha*, *Acacia dealbata*, *Acacia horrida* and *Acacia cyanophylla*) and three plant by-products (date palm leaves, grenade peel and artichoke stems). *Acacia* species are leguminous shrubs commonly found in the arid and semi-arid zones of Algeria. They are adapted to low rainfall, drought and soil salinity of these regions. They are generally rich in proteins, energy and minerals. They produce wood, edible seeds, valuable gums and can provide important protein supplements in ruminant's nutrition especially during the dry season when fresh fodder availability is limited (Fikri Benbrahim *et al.*, 2014). Likewise, plant by-products containing tannins can represent an important source of protein supplement in ruminants diet (Makkar, 2003). However, the potential use of these substrates as protein supplements for ruminants requires knowledge of concentration and biological activity of tannins since these antinutritional factors can cause

negatives effects on ingestion and digestibility (Makkar, 2005). Thus, the objectives of the present work were: (1) to evaluate the biological activity of substrates by the *in vitro* gas production method in presence of PEG, the nutrient composition and antinutritional factors content. (2) to study the effects of purified condensed tannins extract from the substrate with the highest tannins biological activity on ruminal fermentation pattern and methane production.

MATERIALS AND METHODS

The study was carried out at the Department of Biochemistry and Cell and Molecular Biology, Faculty of Nature and Life Sciences, University of Mentouri Brothers, Constantine, Algeria and at the Zaidin Experimental Station, Granada, Spain from February 2018 to February 2019.

Experimental substrates

Seven substrates were studied: leaves from four plants species (*Acacia pycnantha*, *Acacia dealbata*, *Acacia horrida* and *Acacia cyanophylla*) and three plant by-products: date palm (*Phoenix dactylifera*) leaves, artichoke (*Cynara scolymus*) stems and pomegranate (*Punica granatum*) peel. The plants leaves and the plant by-products were collected on February 2016 from semi-arid zones of the north east of Algeria. Date palm leaves were collected from arid zone. The samples were air-dried and ground to calibrated particles of 1 mm.

Nutrients composition

Substrates were evaluated for dry matter (DM), organic matter (OM) according to the Association of Official Analytical Chemists (AOAC) (1990). Crude (CP) protein by Kjeldhal procedure, neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were estimated following the procedure of Van Soest *et al.* (1991).

Tannins extraction and purification

Extraction

An amount of 200 mg of dried substrate were added to 10 ml of acetone 70% (acetone: water, 70:30) and subjected to ultrasonic treatment in an

Ultrasonic Processor (Bioblock Scientific vibracell 75043) for 20 min at ambient temperature (25°C). The mixture was then centrifuged at 3000 x g and 4°C for 20 min. The supernatant was kept on ice and the pellet was resuspended in 10 ml of acetone 70% and again subjected to ultrasonic treatment for 20 min. The extraction was repeated until the supernatant become colourless indicating the end of extraction process (Makkar *et al.*, 1993).

Total phenols and tannins determination

Total phenols (TP) were determinate by Folin-Ciocalteu method. Total tannins (TT) were evaluated by the difference between the total phenols before and after treatment with tannins agent binding, the polyvinylpyrrolidone (PVPP) according to Makkar *et al.*, (1993); a volume of 1.0 ml of distilled water was added to 1.0 ml of the extract and 100 mg of PVPP, the mixture was kept at 4°C for 15 min then was centrifuged at 3000 x g and 4°C for 10 min. Phenolic content of the supernatant (other than precipitated tannins with PVPP) was determinate by Folin-Ciocalteu method. Absorbance was measured at 725 nm. TP and TT contents were expressed as tannic acid equivalent ($y = 0005x$, $r^2 = 0.9927$).

Total Condensed tannins (TCT) were determinate by butanol-HCl method at 550 nm and expressed as catechin equivalent ($y = 0.3188x$, $r^2 = 0.9874$) (Porter *et al.*, 1986).

Purification

An amount of 50 g of *Acacia horrida* leaves were extracted with 500 ml of aqueous acetone (70%+1g ascorbic acid) with stirring for 40 min. The mixture was filtered into vacuum flask with a porcelain funnel provided with filter paper ($\varnothing = 15$ cm). The filtrate (250 ml) was then taken into a separating funnel and mixed with 250 ml of dichloromethane. After decantation, the lower phase was discarded (containing pigments and lipids). However, the upper phase was washed again two times with dichloromethane. This phase was recovered and evaporated at 37°C to remove acetone, the aqueous residue was freeze dried (Terrill *et al.*, 1992).

About 1 g of tannin extract was dissolved in methanol (50%) and applied to Sephadex LH-20 column (length 13 cm and diameter 2.3 cm)

equilibrated with methanol (50%). The column was washed with the same solvent (650ml) and then tannins were eluted with aqueous acetone (70%, 450ml). The eluates were checked by thin-layer chromatography (TLC). The fractions containing tannins were combined, rotary evaporated and kept on ice until use.

In vitro gas production essay

The study was divided into two experiments: the first was conducted to select the substrate with the highest tannins biological activity. This was assayed by adding polyethylene glycol (PEG) as tannins binding agent (Makkar *et al.*, 1995). The second experiment was aimed to study the effect of purified tannins on ruminal fermentation parameters, gas and methane production.

Animals

Four female Murciano-granadina goats equipped with permanent rumen fistula were used as donors of rumen liquor for both experiments. Animals were kept in individual pens and had free access to tap water. They were fed of forage: concentrate (50:50 of alfalfa hay/concentrate) served in two equal meals twice a day at 08:00 h and 16:00 h.

Rumen liquor

Rumen juice presented the inoculum was collected before morning feeding from the four goats and immediately was taken in prewarmed thermal flasks to the laboratory, where it was strained through two layers of cheesecloth under a continuous stream of CO₂ at 39°C. Rumen juice was then mixed with prewarmed artificial saliva in a 1:3 proportion.

Experiment 1: Screening of the substrate with the highest tannins biological activity

In order to select the substrate with the highest tannins biological activity, amount of 400 mg of each substrate without or with 1 g of PEG (MW 4000) were incubated with 40 ml of medium culture (rumen fluid (10 ml) + artificial saliva (30 ml)) in 120 ml glass bottles (Theodorou *et al.*, 1994). The bottles were incubated in water bath at 39°C. For each substrate, four replicates were done. Under the same conditions, four blanks were incubated (medium culture without substrate).

Experiment 2: The effect of purified tannins on ruminal fermentation parameters, gas and methane production

Different concentrations of purified tannins (0, 50, 100, 150 mg/g DM of forage) were added to 500 mg of alfalfa hay (DM 91.0%, OM 90.3%, NDF 51.8%, ADF 33.8%, ADL 9.54%, CP 19.7%) and barley straw (DM 92.7%, OM 93.7 %, NDF 64.5%, ADF 38.1%, ADL 6.65%, CP 7.0%) and 40 ml of medium culture. Samples were incubated for 24 h at 39°C.

Gas and methane measurements and calculation

The gas pressure in each bottle was recorded at 2, 4, 6, 12, 24 and 48 h using a Wide Range Pressure Meter (Sper Scientific LTD, Scottsdale, AZ, USA). The gas produced was adjusted to the monophasic exponential model $y = A*[1 - e^{-(ct)}]$ of France *et al.* (2000), where y represents the gas production (GP, ml); t represents the time of incubation (h); A represents the asymptote (ml); and c represents the rate of gas production (h^{-1}). After 24 h, a gas sample of 5 ml from each bottle were taken and injected in evacuated tubes (Terumo Europe N.V., Leuven, Belgium) to determinate CH_4 production by gas chromatography (HP Hewlett 5890 Packard Series II gas chromatograph, Waldbronn, Germany).

Determination of ammonia and volatile fatty acids

After 24 h, 0.8 and 0.2 ml of medium culture were taken from bottles then transferred to eppendorf tubes and added to 0.2 ml of deproteinising solution (4 mg/ml crotonic acid and 20 mg/ml of metaphosphoric acid dissolved in 0.5M HCl) for volatile fatty acids (VFA) determination and to 0.05 ml of Trichloroacetic Acid (TCA, 25%), for ammonia ($N-NH_3$) determination. Samples were frozen at -20°C until analysis. Before analysis, samples were centrifuged at 4000 x g and 4°C for 20 min. The VFA were analyzed by gas chromatography (Auto system Perkin-Elmer Cor., Norwalk, Connecticut, USA) following the procedure pronounced by Arco-Pérez *et al.* (2017). Ammonia was determined using the Colorimetric method of Weatherburn (1967).

Statistical analysis

Data was subjected to one-way analysis of variance (ANOVA-one way) using the SAS (Institute Inc, 2003). At $P < 0.05$, differences were estimated significant. When significant differences were found means were compared by the Tukey test.

RESULTS AND DISCUSSION

Nutrient composition

The nutrient composition of plants and plant by-products is represented in Table 1. All *Acacia* species presented high CP content ranging from 178.0 to 201.6 g/kg DM. Thus, these species can be used as protein supplement in the diet of ruminants feeding low quality forage (Makkar *et al.*, 1995). The CP content recorded in *Acacia* species was comparable to the one found by Rubanza *et al.* (2005) and higher than the one found by Al-Soqeer (2008). Differences recorded between studies may be due to variations in the stage of growth, the part of the plant studied (twigs, leaves, stem, pods), the type of soil and season (Melesse *et al.*, 2017).

Plant by-products presented low CP content ranged from 48.1 to 95.8 g/kg DM. The highest amount was recorded in date palm leaves (95.8 g/kg DM) and the lowest was recorded in *Punica granatum* peel (48.1 g/kg DM). The result recorded in date palm leaves was higher than those reported by (Pascual *et al.*, 2000; Ziaei and Sharifi Hosseini 200; Boufennara *et al.*, 2016). The CP concentration of *Punica granatum* peel was similar to those noted by (Shabtay *et al.*, 2008; Sadq *et al.*, 2016; Yurtseven *et al.*, 2018) and higher than the one recorded by Mirzaei-Aghsaghali *et al.* (2011).

All *Acacia* species had high NDF and ADL contents, their concentration ranged between 379.1-553.9 g/kg DM and between 140.6-264.7 g/kg DM, respectively. NDF results were similar to those found by Al-Soqeer (2008), whereas those of ADL were higher than those noted by the same author. The highest NDF content recorded in *A. cyanophylla* was comparable to those found by (Bensalem *et al.*, 1997; Bensalem *et al.*, 1999; Moujahed *et al.*, 2000). The ADF content ranged from 109.5 to 353.7 g/kg DM. The highest amount was noted in *A. pycnantha* which was

comparable to the one found by Hanafy *et al.*, (2014) and the lowest in *A. horrida* which was similar to the amount noted by Tefera *et al.*, (2008). Variation in cell-wall components between *Acacia* species may be due to the stage of growth.

A considerable variation in cell-wall components was recorded in plant by-products. The highest amounts were recorded in date palm leaves; this can be attributed to the high temperature which generally favors accumulation of the cell wall structural materials (Abebe *et al.*, 2012). The result of NDF was similar to the one observed by Pascual *et al.* (2000) and lower than the one noted by Boufennara *et al.* (2016). However, ADF and ADL contents were higher than those noted by the same studies.

Phenolic compounds analysis

Phenolic compounds composition of plants and plant by products is also represented in Table 1. All *Acacia* species had high TP content; amounts ranged between 591.2 and 798.4 mg/g DM. They were also characterized by their high TT content which was greater than 300 mg/g DM. The TP and TT contents recorded in our study were similar to those obtained by Rubanza *et al.* (2005). Tested *Acacia* species had high TCT content, the highest amount was recorded in *A. horrida* (680.4 mg/g DM) and the lowest was recorded in *A. pycnantha* (120 mg/g DM). The amount recorded in *A. pycnantha* was similar to the one reported by Grainger *et al.* (2009) in *Acacia mearnsii*. Variation in phenolic compounds between studies may be due to stage of growth, the collection period of plants, extraction method and technique used to determine phenolic contents.

In plant by-products, TP and TT contents levels ranged between 585.9-799.2 mg/g DM and between 351.3-517.9 mg/g DM, respectively. The highest amounts of both TP and TT were recorded in *Punica granatum*. Plant by-products showed low TCT content except date palm leaves which presented amount of 170.4 mg/g DM.

Screening of plant with the highest tannins biological activity

Effect of tannins on gas production

The effect of tannins on Gas Production (GP) is exposed in Table 2. The results showed that tannins affected GP in all substrates ($P < 0.0001$).

In absence of PEG, the highest GP was recorded in *C. scolyumus* stems (159.8 ml) and the lowest in *A. dealbata* (29.6 ml). However, in presence of PEG, the lowest GP was recorded in date palm leaves (70.96 ml).

In plants, the largest effect of tannins was observed in *A. dealbata*. GP was increased by 210.16% in presence of PEG, followed by *A. horrida* (92.5%), *A. cyanophylla* (40.7%) and *A. pycnantha* (19.0%) (Figure1). In plant by-products, the impact of tannins was more pronounced for *C. scolyumus* stems (27.6%) than grenade peel (13.8%) and date palm leaves (1%) (Figure 2).

Results showed that in absence of PEG, low GP was recorded in *Acacia* species particularly in *A. dealbata*, plant with the highest TT content determinate by chemical method. Low GP recorded in *Acacia* species can be attributed to the anti-nutritional effects of tannins, indeed tannins can bind to carbohydrates and inhibit their degradation and thus reduce GP, since GP is the result of carbohydrates degradation (Njida and Nasiru, 2010; Abdallah Hassan Sallam *et al.*, 2010). This result was similar to the one reported by Bhatta *et al.*, (2009), these authors noted that GP was reduced in samples containing tannins. Likewise, Rodriguez *et al.*, (2014) reported that species with high tannins concentration showed the lower GP.

In plant by-products, the lowest GP was recorded in date palm leaves; by-product with the highest tannins content, but there was no increment in gas production with PEG inclusion suggesting the low biological activity of its tannins (Guerrero *et al.*, 2012).

Effect of tannins on methane production

Tannins' effect on CH₄ production is also exposed in Table 2. The results indicated that tannins influenced methane production differently among substrates. However, these differences were statistically comparable ($P > 0.05$). The highest CH₄ reduction was observed in *A. pycnantha* (54.5%). Tannins content of *C. scolyumus* and *A. horrida* had no effect on methane production.

Table 1. Nutrient composition and secondary compounds content of experimental plants and plant by-products expressed as g/kg and mg/g of dry matter.

Substrate	Nutrient Composition									Secondary compounds content		
	DM	OM	Ash	CP	NDF	ADF	Hemicellulose	Cellulose	ADL	TP	TT	TCT
<i>A. horrida</i>	730.7	899.9	100.1	182.8	379.1	191.4	187.7	50.8	140.6	785.1	521.3	680.4
<i>A. cyanophylla</i>	632.4	881.0	119.0	186.6	553.9	253.3	300.6	80.8	172.5	778.7	442.3	439.7
<i>A. dealbata</i>	623.9	950.1	49.9	201.6	429.4	238.3	191.1	73.2	165.1	798.4	536.4	464.5
<i>A. pycnantha</i>	613.5	919.7	80.3	178.0	520.5	345.3	175.2	80.6	264.7	591.2	395.3	120.5
<i>Cynara scolymus</i>	702.3	821.7	178.3	81.7	351.0	230.4	120.6	201.5	28.9	594.4	376.0	23.1
<i>Punica granatum</i>	784.9	967.4	32.6	48.1	301.6	109.5	192.1	40.9	68.6	799.2	517.9	50.9
<i>Phoenix dactylefera</i>	932.7	833.4	166.6	95.8	533.8	353.7	180.1	126.0	127.7	585.9	351.3	170.4

DM: Dry Matter, OM: Organic Matter, CP: Crude Protein, NDF: Neutral Detergent Fiber, ADF: Acid Detergent Fiber, ADL: Acid Detergent Lignin, TP: Total phenols, TT: Total tannins, TCT: Total Condensed Tannins.

Table 2. *In vitro* tannins effects on fermentation characteristics.

Item	-PEG								+PEG								SEM	p-value
	Plants				Plant by-products				Plants				Plant by-products					
	<i>A. horrida</i>	<i>A. cyanophylla</i>	<i>A. dealbata</i>	<i>A. pycnantha</i>	<i>Cynara scolymus</i>	<i>Punica granatum</i>	<i>Phoenix dactylifera</i>	<i>A. horrida</i>	<i>A. cyanophylla</i>	<i>A. dealbata</i>	<i>A. pycnantha</i>	<i>Cynara scolymus</i>	<i>Punica granatum</i>	<i>Phoenix dactylifera</i>				
GP (ml/g OM)	54.51 ^b	61.12 ^b	29.60 ^b	60.91 ^b	159.81 ^a	73.67 ^b	70.21 ^b	104.94 ^b	86.02 ^b	91.81 ^b	72.49 ^b	203.99 ^a	83.89 ^b	70.69 ^b	5.02	<0.0001		
CH ₄ (ml/g OM)	25.49 ^a	22.58 ^a	25.75 ^a	20.73 ^a	33.57 ^a	32.28 ^a	24.86 ^a	24.51 ^a	26.61 ^a	31.17 ^a	32.04 ^a	28.73 ^a	36.31 ^a	28.61 ^a	2.67	0.0983		
NH ₃ mg/100ml	18.97 ^{ab}	17.67 ^{ab}	18.12 ^{ab}	20.47 ^a	18.52 ^{ab}	14.05 ^b	17.97 ^{ab}	22.40 ^{ab}	21.35 ^{ab}	25.95 ^{ab}	27.45 ^a	14.25 ^{ab}	14.90 ^b	20.37 ^{ab}	2.28	0.0382		
VFA mM/g OM	49.68 ^b	51.83 ^{ab}	37.33 ^{bc}	48.00 ^{bc}	72.18 ^a	51.85 ^b	44.15 ^c	58.46 ^b	54.38 ^{bc}	54.80 ^{bc}	55.06 ^{bc}	68.08 ^a	57.85 ^b	45.73 ^c	2.42	0.0009		
Acetate %	33.05 ^b	36.21 ^b	24.67 ^c	33.54 ^b	48.82 ^a	33.61 ^b	30.25 ^c	37.63 ^b	38.62 ^b	35.48 ^c	37.37 ^b	46.24 ^a	37.36 ^b	31.40 ^c	1.59	<0.0001		
Propionate %	11.7 ^{ab}	10.80 ^{bc}	8.22 ^{bc}	9.77 ^{bc}	14.72 ^a	11.49 ^{ab}	7.78 ^c	14.06 ^{ab}	9.70 ^{bc}	11.92 ^{bc}	11.26 ^{bc}	14.35 ^a	12.80 ^{ab}	7.98 ^c	1.55	0.1024		
Butyrate %	2.66 ^b	2.53 ^b	2.47 ^b	2.67 ^b	4.82 ^a	4.63 ^a	3.57 ^{ab}	3.47 ^b	3.17 ^b	3.85 ^b	3.32 ^b	4.23 ^a	5.13 ^a	3.65 ^{ab}	0.92	0.0373		
Isobutyrate %	0.89 ^a	0.87 ^a	0.80 ^a	0.64 ^a	1.26 ^a	0.64 ^a	0.95 ^a	1.26 ^a	0.84 ^a	1.24 ^a	1.07 ^a	1.14	1.00	1.03	0.52	0.0046		
Valerate %	0.47 ^b	0.5 ^b	0.38 ^b	0.41 ^b	1.22 ^a	0.57 ^b	0.43 ^b	0.72 ^b	0.77 ^b	0.85 ^b	0.67 ^b	1.08 ^a	0.59 ^b	0.49 ^b	0.36	<0.0001		
Isovalerate %	0.89 ^a	0.88 ^a	0.77 ^a	0.94 ^a	1.32 ^a	0.89 ^a	1.14 ^a	1.29 ^a	1.27 ^a	1.42 ^a	1.34 ^a	1.02 ^a	0.95 ^a	1.17 ^a	0.52	0.0032		
C ₂ :C ₃	2.82	3.35	3.00	3.43	3.32	2.93	3.89	2.68	3.98	2.98	3.32	3.22	2.92	3.93				

PEG: Polyethylene Glycol, GP: Gas Production, CH₄:Methane, N-NH₃:Ammonia,VFA:Volatile Fatty Acids, C₂:C₃: Acetate:Propionate, SEM: Standard Error Mean, Means in the same row with different superscript letters differ (p < 0.05).

Figure 1. Gas production profile of plants in presence and absence of PEG.

Figure 2. Gas production profile of plant by-products in presence and absence of PEG.

Results showed that tannins affected CH₄ production in different ways. This effect is mainly independent of tannins concentrations. Our finding was in disagreement with Rira *et al.*, (2015). These authors recorded that CH₄ production was reduced by 37.5 % and 56.25% with incorporation of *A. cyanophylla* tannins by 30% and 60%, respectively. However, similar results were noted by Gameda and Hassen (2015), these authors reported that tannins of nineteen tanniferous browse plants reduced methane production. In our study, it appears that tannins structure was more responsible of their biological activity than their concentration (Arhab *et al.*, 2009; Rodriguez *et al.*, 2014). This observation was confirmed by the result obtained in *A. horrida* (-3.84%); this plant had the highest condensed tannins content but had no effect on CH₄ production. Such finding was similar to that recorded by Bhatta *et al.*, (2012), these authors reported that *T. chebula* with the highest TT content, showed the lowest reduction of methane production.

Tannins act through two mechanisms to decrease CH₄ produced by ruminants: (i) indirectly through a reduction of fiber digestion, which reduce H₂ production; and (ii) directly through suppression of methanogens (Wanapat *et al.*, 2013). In the present study, reduction of CH₄ production was likely the result of a decrease in fiber degradation by bacteria consortium in the ruminant rumen.

Effect of tannins on fermentation parameters **Ammonia production**

The effect of tannins on ruminal fermentation parameters are demonstrated in Table 2. It indicated that ammonia (N-NH₃) production was significantly decreased by tannins ($p=0.0382$). The highest decrease was noted in *A. dealbata* (43.2%) and the lowest in grenade peel (6.0%). This effect can be due to the formation of tannins- proteins complexes which inhibit proteins degradation and reduce ammonia production. Bhatta *et al.* (2014) reported a reduction in N-NH₃ production in samples containing CT and hydrolysable tannins (HT).

Tannins are characterized by their ability to bind to proteins through hydrogen bonds and hydrophobic interactions. This property of tannins can be attributed to the presence of great number of hydroxyl groups (Martinez *et al.*, 2004;

Shimada, 2006). In the rumen, where pH is neutral, proteins cannot be cleaved from these bonds, so they are not degraded by microorganisms, thus, CT reduce ammonia production. In the abomasums where the pH is acidic (2-3), some of the tannin-protein bonds are split and consequently proteins can be broken down into amino acids and then absorbed into the intestine (Piñero-Vázquez *et al.*, 2015). Furthermore, tannins can reduce ammonia production by inhibiting growth of proteolytic bacteria (Patra and Saxena, 2010; Hao *et al.*, 2011). Molan *et al.* (2001) demonstrated that CT from *L. corniculatus* and *L. pedunculatus* have inhibited the growth of proteolytic rumen bacteria *in vitro*. Otherwise, ammonia reduction can be caused by proteolytic enzymes deactivation (Makkar, 2003). Effectively, Nsahlalai *et al.* (2011) reported that tannins from *Acacia sieberiana* and *Acacia nilotica* have depressed protein digestibility in the rumen by deactivating of proteolytic enzymes.

Volatile fatty acids (VFA) production

The impact of tannins on total and individual volatile fatty acids production is also demonstrated in Table 2. This effect was statistically significant ($P < 0.001$). The highest increase of total volatile fatty acids production after PEG inclusion was noted in *A. horrida* (58.4%) and the lowest in date palm leaves (3.57%).

Except for *C. scolymus*, tannins affected acetate production ($P < 0.0001$). It was increased when PEG was added; the highest increment was recorded in *A. dealbata* (43.8%) and the lowest in date palms leaves (3.8%). Likewise, tannins affected propionate production. The highest increments were recorded in *A. horrida*, *A. dealbata*, and *A. pycnantha*. Despite that this effect was statistically not significant ($P > 0.05$). The impact of tannins was significantly different for butyrate and valerate productions ($P < 0.05$).

The reduction of total and individual VFA concentrations was comparable to the findings recorded by Rodriguez *et al.* (2014) and Tiemann *et al.* (2008). This reduction is due to tannins inhibition of fiber degradation. These results are consistent with those of methane production; inhibition of fiber degradation diverted VFA profile from acetate and resulted in less hydrogen

and lower methane production (Bhatta *et al.*, 2012).

There is an inverse relationship between the acetate: propionate (C₂:C₃) ratio and VFA and CH₄ production (Tavendale *et al.*, 2005). However, in our study the ratio C₂:C₃ was not influenced and propionate production was decreased in the absence of PEG. These results were in disagreement with other studies. Rira *et al.* (2015) noted an increase in the propionate concentration with *A. cyanophylla* supplementation. Bhatta *et al.* (2009) also reported an increase in propionate production with supplementation of CT from quebracho and mimosa.

Impact of A. horrida purified tannins on in vitro fermentation

The effects of *A. horrida* purified tannins incubated with two different forages: alfalfa hay and barley straw at three different concentrations: 50, 100 and 150 mg/g DM on fermentation parameters are represented in Table 3. The results indicated that *A. horrida* purified tannins had no effect on fermentation parameters except GP. This parameter was decreased with all purified tannins doses compared to the control. The higher decrease was observed for the low concentration (50 mg/g DM). These results are different from those reported by Hassanat and Benchaar (2012), these authors studied the effect of purified tannins from *A. mearnsii* at 20, 50 and 100 mg/g DM and reported that tannins concentration ≥ 50 mg/g reduced gas and methane production as well N-NH₃ and branched-chain VFA concentrations. Tiemann *et al.* (2008) also recorded lower GP and VFA concentration when purified tannins from tropical shrub legumes were added to forage at 25, 50, 75 and 100 mg/g DM. Likewise, Rira *et al.* (2015) noted that methane, VFA and fermented organic matter were decreased as tannins concentrations increased. Moreover, tannins effects may appear with lower concentration. In two separate studies conducted by Tan *et al.* (2011) and Huang *et al.* (2010) on *Leucaena leucocephala*, the concentration of gas, CH₄ and VFA were reduced at 0-30 mg/g DM, 20-50 mg/g DM, respectively. However, Jayanegara *et al.* (2011) in a meta-analysis reported that tannins

effects can only be predicted from concentration level >20 mg / g DM.

In the current study, the absence of tannins effects on the different fermentation parameters may be due to many factors other than concentration such as tannins source, chemical structure and polymerization degree. Indeed several authors documented varying biological effects at the same concentration of tannins from different sources (Tiemann *et al.*, 2008; Hassanat and Benchaar, 2012; Rira *et al.*, 2015). This situation was also supported by the work carried out by Gatechew *et al.* (2008), these authors studied the effect of supplementation of quebracho, tannic acid and gallic to alfalfa and reported that tannins effects on rumen fermentation and protein degradation did not only depend on their concentration but also on their type. Huyen *et al.* (2016) studied the effect of CT polymerization degree of eight plants extracts and found that the proportion of prodelphinidins had the greatest effect on fermentation pattern followed by average polymer size and percentage of cis-flavan-3-ols.

Tannins are characterized by their high molecular weight (MW) which can play an important role in their biological activity. In this context, Saminathan *et al.* (2014) have demonstrated that the effect of CT with high MW on CH₄ production was higher than other CT fractions extracted from *Leucaena leucocephala*. Likewise, Huang *et al.* (2011) noted that the highest inhibition of CH₄ production was recorded in the fraction with the highest MW. In the present study, our purified tannins may not have the appropriate MW or polymerization degree that allows them to decrease methane, VFA or ammonia production. These suggestions need to be confirmed through the determination of the structure of our purified condensed tannins. The determination of the structure should clarify the various structural details responsible for the effects recorded in our study.

Table 3. *In vitro* *A. horrida* purified tannins Effects on fermentation characteristics.

Item	Alfalfa hay forage						Item	Barley straw forage					
	Dose (mg/g DM)				SEM	P value		Dose (mg/g DM)				SEM	P value
	0	50	100	150				0	50	100	150		
Gas (ml/g OM)	479.10 ^a	21.17 ^a	166.85 ^a	327.52 ^a	5.03	0.9704	Gas (ml/24h)	350.50 ^a	67.62 ^a	172.65 ^a	280.07 ^a	5.46	0.9993
CH ₄ (ml/g OM)	31.03 ^a	37.41 ^a	35.90 ^a	41.64 ^a	2.80	0.2912	CH ₄ (ml/24h)	27.94 ^a	29.28 ^a	31.60 ^a	32.05 ^a	2.63	0.9130
N-NH ₃ (mg/100ml)	23.67 ^a	25.52 ^a	27.21 ^a	26.25 ^a	2.33	0.9168	N-NH ₃ (mg/100ml)	18.10 ^a	19.72 ^a	19.59 ^a	21.82 ^a	2.23	0.8854
VFA (mM/g OM)	68.24 ^a	69.04 ^a	70.52 ^a	68.09 ^a	2.61	0.9164	Total VFA (mM)	51.81 ^a	52.66 ^a	51.55 ^a	50.19 ^a	3.42	0.9976
Acetate %	44.49 ^a	44.98 ^a	45.92 ^a	44.49 ^a	2.34	0.9600	Acetate %	33.87 ^a	34.48 ^a	33.64 ^a	32.99 ^a	2.86	0.9988
Propionate %	13.46 ^a	13.65 ^a	13.98 ^a	13.53 ^a	0.91	0.6037	Propionate %	10.00 ^a	10.01 ^a	9.74 ^a	9.73 ^a	1.33	0.9951
Butyrate %	6.09 ^a	6.23 ^a	6.42 ^a	6.17 ^a	1.04	0.9631	Butyrate %	5.00 ^a	5.08 ^a	4.97 ^a	5.00 ^a	1.23	0.9995
Isobutyrate %	1.31 ^a	1.22 ^a	1.20 ^a	1.12 ^a	0.30	0.1142	Isobutyrate %	0.92 ^a	0.96 ^a	1.01 ^a	0.95 ^a	0.37	0.9188
Valerate %	1.20 ^a	1.22 ^a	1.25 ^a	1.11 ^a	0.48	0.9456	Valerate %	0.72 ^a	0.76 ^a	0.74 ^a	0.69 ^a	0.43	0.9831
Isovalerate %	1.66 ^a	1.71 ^a	1.73 ^a	1.65 ^a	0.47	0.9878	Isovalerate %	1.28 ^a	1.36 ^a	1.43 ^a	1.24 ^a	0.55	0.9209
C ₂ :C ₃	3.31	3.29	3.28	3.29			C ₂ :C ₃	3.38	3.44	3.45			

GP: Gas Production, CH₄: Methane, N-NH₃: Ammonia, VFA: Volatile Fatty Acids, C₂:C₃:Acetate:Propionate, SEM: Standard Error Mean, Means in the same row with different superscript letters differ (p < 0.05).

CONCLUSIONS

According to the present results, the supplementation of *A. horrida* purified tannins extract to two different forages at different concentrations did not cause significant changes in fermentation parameters and methane production *in vitro*, despite that this plant presents high condensed tannin content. This suggests, as was previously reported by other studies conducted on different tannins sources, that effects of tannins may be strongly linked to their structure and their molecular weight more than to their concentration. For this reason, it is interesting that this work may be completed by physical characterization of these tannins. Moreover, comprehensive *in vivo* studies have to be executed to evaluate the effects of these tannins and to better understand their action mechanism.

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