Journal of Animal and Veterinary Advances 11 (21): 3921-3927, 2012
ISSN: 1680-5593

In vitro Digestibility of Diets Containing Different Parts of *Andrographis paniculata* Using Rumen Fluid from Goats

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Abstract: The study was carried out to determine the effect of diets containing different parts of *Andrographis paniculata* on cumulative gas production, dry matter digestibility, methane and volatile fatty acids production in goats using *in vitro* gas production technique. The leaves, stems and roots of *Andrographis paniculata* (AP) were analysed for total polyphenols. Leaves of AP were found to contain the highest concentration of polyphenols; 3.08% Tannic Polyphenol (TP), 0.46% Hydrolysable Tannin (HT) and 0.19% Condensed Tannin (CT). The Non-Tannic Polyphenols (NTP) were estimated as 2.43% of the total polyphenols in the herb. Four diets were formulated three of which contained 1% each of the parts analysed (APL = leaves, APS = stems and APR = roots) and not containing *Andrographis paniculata* (APO) which was used as the control and tested for digestibility via *in vitro* gas production technique. The results indicated that VFAs, IVDMD, gas production and pH of the rumen fluid were not significantly affected ($p>0.05$) by the experimental diets except for ammonia ($p<0.05$). The results suggest that goats can utilize nutrients from feeds containing AP without compromising the production of VFA and dry matter digestibility.

Key words: Polyphenols, *Andrographis paniculata*, digestibility, goat, diets

INTRODUCTION

Livestock feeds may offer other benefits beyond basic nutrition as they also contain some chemical compounds known as polyphenols (Makkar *et al*., 2007). Polyphenols are widely distributed in almost all foods of plant origin (Serrano *et al*., 2009) and they are made up of active pharmaceutical ingredients whose primary sources are mainly the medicinal plants (herbs). There exist differences in polyphenolic composition among various plant species (Okuda, 2005) and their parts. The concentration may vary from low in grasses to higher in trees, shrubs and herbaceous leguminous plants (Frutos *et al*., 2004). Moderate level of these secondary metabolites in many plants may possibly reduce ruminal protein breakdown and increase duodenal protein flow (Carulla *et al*., 2005). On the contrary, higher levels may impact negatively on digestibility (Kariuk and Norton, 2008) and thereby, alter the fermentation characteristics (Frutos *et al*., 2004). On the other hand, the process of feed digestion in livestock have been reported as among the major source of anthropogenic methane (Wood and Knipmeyer, 1998) which is one of the most important threats to human lives in recent times (West *et al*., 2006). This process represents 5-14% significant loss of feed energy that increases costs of feed and at the same time contributing to climate change which results to global warming (West *et al*., 2006). The issue of global warming which have been affecting the environment in many ways such as extremes of temperature and rainfall causing heat waves, floods as well as drought have a long-term and direct effect on human mortality and displacement. Energy is the fuel that keeps the different body functions operating. It is a vital but among the most wasted and expensive components of feed. Therefore, everything should be done to conserve and enhance the utilization of dietary energy and enhance a safer environment.

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Several feeding strategies have been introduced such as addition of dietary fat by feeding diets containing crushed or whole oilseeds like cottonseed, sunflower seed, canola seed or flaxseed or dried corn distillers grain and ethanol by-products which saved up to 20% of the energy lost as methane. High quality forages such as corn silage and alfalfa and ionophores compound, an antimicrobial which target the ruminal bacterial population usually result in short time reduction of methane. Plant extracts (condensed tannins and saponins) from mangosteen peel and soapberry fruit (Poungchompu *et al*., 2009), essential oils and rumen modifiers such as yeast (Beauchemin *et al*., 2011) have also been used to reduce methane production.

There are new approaches to increase the effectiveness of digestion and metabolism of nutrients which will improve the quality of productivity and ensuring a safer environment. One economical, sustainable and safer approach is the use of herbs such as *Andrographis paniculata* which contain diterpenoids and polyphenols such as flavonoids and tannins (Chao and Lin, 2010). These secondary metabolites are among other potent anti microbial agents present in *Andrographis paniculata* such as alkaloids, glycosides, saponins, steroids and terpenoids (Sule *et al*., 2010). It is possible that the presence or combination of these plant bioactive compounds could improve the effectiveness of nutrients utilization in ruminants. Gas production technique is a relatively simpler, cheaper and direct measurement of microbial activities and reflects all nutrients fermented. Therefore, the purpose of this study was to determine the effects of diets containing *Andrographis paniculata* leaves, stems and roots on cumulative gas production, dry matter digestibility, methane and volatile fatty acids production using *in vitro* gas production technique.

MATERIALS AND METHODS

Sample preparation and tannins extraction: The plants were harvested, freeze dried as recommended by Orians (1995) they were separated into leaves, stem and roots. Each of the separate parts were continuously ground and sieved with 1 mm diameter sieve until no residue was obtained and prepared for analysis according to the procedure of Barman (2004). About 400 mg of ground sample was poured into a test tube; 40 mL diethyl ether containing 1% acetic acid (v/v) was added and mixed to remove the pigment material. After 5 min, the supernatant was discarded and 20 mL of 70% aqueous acetone was added and the flask was sealed with cotton plug, covered with aluminium foil and kept in electrical shaker for 2 h to enable extraction. Then, the sample was filtered through Whatman filter paper No. 1 and kept in refrigerator at 4°C until analysis.

Estimation of total phenol and tannins: To estimate total phenol and tannins, the following reagents were prepared:

- Folin Ciocalteu reagent (1 N) was prepared by diluting Folin Ciocalteu reagent (2 N) with equal volume of distilled water and the resultant solution was kept in a brown colour bottle and stored in refrigerator at 4°C
- Sodium carbonate (20%) was prepared by dissolving 50 g of sodium carbonate ($NaCO_3 10H_2O$) in distilled water which gave a volume of 250 mL
- Standard tannic acid solution (0.5 mg mL^{-1}) was freshly prepared by dissolving 25 mg of tannic acid (T-0125) obtained from the Sigma Company, USA in 50 mL of distilled water

The estimation of total phenol and tannins were done following the procedure of Makkar *et al*. (1993). About 50 μL of tannins extract was taken in a separate test tube and distilled water was added which gave a volume of 1.0 mL. Then, 0.5 mL of already prepared Folin Ciocalteu reagent was added and mixed properly by shaking electronically. Then, 2.5 mL of 20% sodium carbonate solution was added, mixed by shaking electronically and kept for 40 min at room temperature. Optical density was taken at 725 nm in spectrophotometer and concentration was estimated from the standard curve. Total phenol was estimated as tannic acid equivalent and expressed on dry matter basis.

Estimation of non-tannin phenol: Non-tannins phenol was estimated by precipitating tannins with Polyvinyl Polypyrrolidone (PVPP) which binds tannins. About 200 mg polyvinyl polypyrrolidone was measured into a test tube and then 2.0 mL distilled water and 2.0 mL tannins extract were added. The mixture was vortexed and kept in refrigerator for 15 min at 4°C. Then, the mixture was again vortexed and filtered through a Whatman filter paper No. 1. The filtrate was used for the estimation of non-tannin phenol. This was done by measuring 150 μL of filtrate into another test tube and distilled water was added which gave a volume of up to 1.0 mL and then processed as done for total phenol estimation. Concentration of non-tannin phenol was calculated from the standard curve and expressed on DM basis.

Total tannins were calculated by subtracting non-tannin phenol from total phenol. The standard was prepared from the stock solution of tannic acid (0.5 mg mL^{-1}) using 0, 10, 20, 30, 40 and 50 μL in test tubes

and volume was made to reach 1.0 mL. It gave a tannic acid concentration of 0, 5, 10, 15, 20 and 25 μg, respectively. Then, 0.5 mL Folin reagent and 2.5 mL 20% sodium carbonate were added. Whole content was mixed properly and after 40 min, reading was taken at 725 nm in spectrophotometer.

Estimation of condensed tannin (proanthocyanidin): Condensed tannin was estimated according to the method of Porter *et al*. (1986):

- Firstly, a butanol-HCl reagent was prepared by mixing 950 mL n-butanol with 50 mL of concentrated HCl which gave butanol-HCl, 95:5 (v/v) reagents
- Secondly, ferric reagent was also prepared by diluting 16.6 mL concentrated HCl to 100 mL of distilled water to make 2 N HCl in which 2.0 g ferric ammonium sulphate was dissolved which gave (2% ferric ammonium sulphate in 2 N HCl). The reagent was stored in an amber coloured bottle

Analysis: About 0.5 mL of tannins extract was taken into test tubes in triplicates and 3.0 mL of prepared butanol-HCl reagent and 0.1 mL of ferric reagent were added. The tube was vortexed to ensure thorough mixing. The mouth of the tube was covered with glass marble and then boiled for 60 min in a water bath. Similarly, blank was also prepared for each sample but the reagent was not subjected to heating. The tube was allowed to cool to room temperature and reading was taken at 550 nm using spectrophotometer. Condensed tannin as leucocyanidin equivalent was calculated by the equation:

$$\text{Condensed tannins (\%)} = \frac{\text{A550 nm} \times 78.26 \times \text{Dilution factor}}{\text{Dry matter (\%)}}$$

Determination of hydrolysable tannin: Hydrolysable tannin was estimated by subtracting condensed tannins from total tannin phenol.

***In vitro* digestibility**

Experimental design: The study was conducted by using 3 fistulated goats as rumen liquor donors. The rumen fluid was then used to evaluate the effects of these different combinations on *in vitro* cumulative gas production, methane production, rumen fluid pH and volatile fatty acids.

Substrates: Four experimental diets formulated to contain the Leaves (APL), Roots (APR) and Stem (APS) in addition to the control diets (AP0) were used as substrates. The level of inclusion of the various parts of the plant was 1% (w/w). They were ground to fine pieces and were sieved through a 1 mm sieve. They were then stored in air-tight containers.

Preparation of buffer solutions

Phosphate buffer: Phosphate buffer was prepared by dissolving 28.80 g of disodium phosphate dehydrate ($Na_2HPO_4.12H_2O$), 6.10 g of monosodium phosphate monohydrate ($NaH_2PO_4.H_2O$) and 1.40 g of ammonium chloride (NH_4Cl) powder completely in 1 L of distilled water. The buffer solution was saturated with carbon dioxide (CO_2) by rapid bubbling of CO_2 through the solution. After saturation by CO_2, the pH was then adjusted to 6.8 and stored at 38-40°C until subsequent use.

Bicarbonate buffer: Bicarbonate buffer was prepared by dissolving 39.21 g of sodium bicarbonate ($NaHCO_3$) powder completely in 1 L of distilled water. It is then stored at 38- 40°C until subsequent use.

Preparation of syringes for incubation: After estimating the polyphenol in the herb, 100 g of 4 isocaloric and isonitrogenous diets were formulated as follows:

- Basal diet–Control (AP0)
- Basal diet + 1% leave powder of *Andrographis paniculata* (w/w) (APL1%)
- Basal diet + 1% roots powder of *Andrographis paniculata* (w/w) (APR 1%)
- Basal diet + 1% stem powder of *Andrographis paniculata* (w/w) (APS1%)

These feeds were then tested via *in vitro* incubation techniques to determine their extent of fermentability and digestibility in the laboratory. About 0.25 g from each formulated feeds was weighed using an electronic scale and then placed in 100 mL gas syringes (Haberle Labortechnik, Lonsee-Ettlenschieβ, Germany) according to the four treatment groups while the syringes for the blank remained empty. Plungers lubricated with vaseline were inserted carefully into all syringes to fix the measured feed within the wall of the syringes in order to avoid spillage. The syringes were kept at room temperature until were used.

Sampling and preparation of rumen liquor: Rumen liquor was collected from three fistulated Kajang crossbred male goats fed with the oil palm fronds (50%) and concentrates (50%), early in the morning before morning feeding. The rumen fluid from the three experimental goats was first pooled and mixed in a thermal jar and then immediately transferred to the laboratory. In the laboratory, the rumen

fluid was squeezed through four layer of cheese cloth to remove coarse materials and feed particles. Following to that the rumen fluid/buffer suspension was prepared by mixing the filtrate and phosphate and bicarbonate buffer at 1:4 ratios. After mixing and during inoculation of gas syringes, the suspension was continuously stirred using magnetic stirrer and kept warm on a hot plate at 40°C.

***In vitro* incubation:** In each syringe prepared earlier, 25 mL of rumen fluid/buffer 1:4 (v/v) was measured with a cylinder and poured into a funnel connected to a plastic tube attached to each of the syringes by which the suspension was sucked via the plastic tube. Excess bubbles were expelled from the gas syringes by carefully pushing the plungers. All syringes were incubated in the oven at temperature of 39°C for 24 h. Each of the treatments were replicated six times and performed in two separate runs to determine the cumulative gas production, methane production, *In Vitro* Dry Matter Degradability (IVDMD) and rumen fluid pH and total VFA production. The *in vitro* gas production was measured according to Menke and Steingass (1988) as modified by Makkar *et al.* (1995). The gas produced in the head space of the syringes was recorded at 2 h interval until 12 h and final reading was taken at the end of the 24 h incubation.

Methane production: Methane gas production was measured by injecting 1 mL of the gas from each of the above syringes into gas chromatograph (Agilent 5890 Series Gas Chromatograph, Wilmington, DE, USA) equipped with FID detector. Separation was achieved using a HP-Plot Q column (30 m ×0.53 mm ×40 m) (Agilent Technologies, Wilmington, DE, USA) with nitrogen (Dominick Hunter) as the carrier gas at the flow rate of 3.5 mL min^{-1}. An isothermal oven temperature of 50°C was adopted in the separation. Calibration was completed using standard methane prepared by Scotty Specialty Gases (Supelco, Bellefonte, PA, USA). All the procedures were replicated twice.

***In Vitro* Dry Matter Digestibility (IVDMD):** The *in vitro* dry matter digestibility was determined using the methods of Tilley and Terry (1963) and modified by Jones *et al.* (2000). At the end of the 24 h incubation, the content of the gas syringes for all treatments including the blank were transferred into beakers pre-dried in a 40°C oven overnight where each individual dry weight of the beaker was recorded and labelled accordingly. Distilled water was used to rinse the interior and the plunger of the syringes to reduce chances of underestimation or overestimation of IVDMD. The beakers were then incubated at 100°C until their content dried up and their weight had become stable prior to weight determinations. Each determination was replicated twice as described earlier.

Rumen liquor pH measurement and fixation: The rumen liquor pH was measured immediately using a Mettler-Toledo pH meter (Mettler-Toledo Ltd. England) after the 24 h incubation.

Determination of volatile fatty acids in rumen liquor: The volatile fatty acid profile of the rumen liquor was determined using gas liquid chromatography. The rumen liquor at the end of the 24 h incubation was fixed with 4:1 (v/v) metaphosphoric acid in water, centrifuged at 4000 rpm for 10 min and the supernatant was collected. The supernatant (0.5 mL) was added with an equal volume of 20 mM methyl n-valeric acid (Sigma Chemical Co., St. Louis, Missouri, USA). Separation was done on a Quadrex 007 Series (Quadrex Corporation, New Haven, CT 06525 USA) bonded phase fused silica capillary column (15 m, 0.32 mm ID, 0.25 μm film thickness) in a 6890A Hewlett-Packard Gas-Liquid Chromatograph (Hewlett-Packard, Avondale, PA) equipped with a flame ionization detector. The injector/detector temperature was programmed at 220/230°C. The column temperature was set at the range of 70-150°C with temperature programming at the rate of 7°C min^{-1} increment to facilitate optimal separation. The identification of the peaks was made by comparison with authentic standards of acetic, propionic, butyric, isobutyric, valeric, isovaleric and 4-methyl-n-valeric acids.

RESULTS AND DISCUSSION

The leaves of *Andrographis paniculata* (APL) were higher in total polyphenols (3.08%), tannic polyphenols (hydrolysable 0.46%, condensed 0.19%) as well as non-tannic polyphenols 2.43% as compared to (the stem (APS) and roots (APR). The percentage of total polyphenols (3.08%) and non-tannic polyphenol (2.43%) in the leaves of *Andrographis paniculata* observed from this study (Fig. 1), conforms to 2.39% and (Sule *et al.*, 2010) who discovered both tannic and non-tannic polyphenols in AP and the presence of tannins in AP have also been earlier confirmed by Chao and Lin (2010). APS has the least concentration of polyphenols, except for hydrolysable tannins which was the lowest in APR (0.36%).

In comparison with the APO (control diet), neither VFAs nor the dry matter digestibility were affected by the 1% level of inclusion of APL, APR and APS in the diet (Table 1). This was also confirmed by Mir Ishtiyak *et al.*

Table 1: Differences in the digestibility of diets containing different parts of *Andrographis paniculata*

	Treatments (n = 12)			
Parameters	APL	APR	APS	AP0
Acetic acid	19.29±0.680	18.98±0.79	19.83±1.14	18.04±0.730
Propionic acid	5.30±0.270	5.46±0.28	5.80±0.44	4.73±0.120
Butyric acid	0.71±0.050	0.80±0.07	0.87±0.10	0.67±0.020
Total VFA	31.35±1.360	31.45±1.52	33.20±2.26	28.67±0.910
Capronic acid	3.67±1.090	3.50±0.07	3.48±0.12	3.83±0.160
IVDMD	45.33±5.680	55.00±6.07	53.67±5.62	48.67±5.950
Ammonia	20.23 ±1.17^{b}	19.00±1.11^{bc}	16.04±1.20^{c}	23.87 ±1.39^{a}

Mean±standard error; means with different superscripts within a row differ significantly at p<0.05. IVDMD: *In Vitro* Dry Matter Digestibility; APL: *Andrographis paniculata* Leaves; APR: *Andrographis paniculata* Roots; APS: *Andrographis paniculata* Stem; AP0: Control diet (without *Andrographis paniculata*)

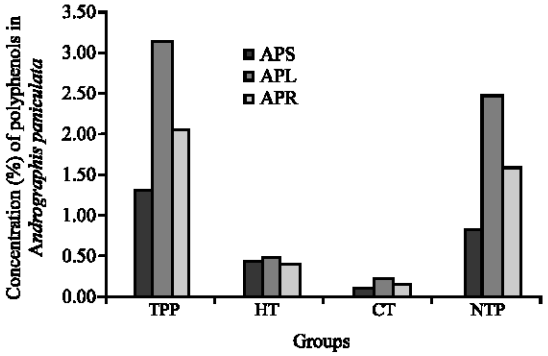

Fig. 1: Concentrations (%) of polyphenols in different parts of *Andrographis paniculata* APS: *Andrographis paniculata* Stem; APL: *Andrographis paniculata* Leaves; APR: *Andrographis paniculata* Roots (TPP: Total Polyphenol; HT: Hydrolysable Tannin; CT: Condensed Tannin; NTP: Non-Tannin Polyphenols)

(2010) that addition of herbs (*Melia azedarach* (fruit), *Pimpinella anisum* (seed), *Cuminum cyminum* Linn (seed), *Murraya koenigii* (leaves), *Emblica officinalis* (fruit), *Allium sativum* Linn (bulb), *Terminalia arjuna* (bark), *Sapindus trifoliatus* (seed), *Zingiber officinale* (rhizome), *Trigonella foenun-graecum* (seed) at three dose levels (1.5, 2.0 and 3.0% of DM) had no significant effect on the *in vitro* dry matter digestibility.

Even though, dry matter digestibility did not differ statistically as confirmed by Mir Ishtiyak *et al.* (2010) who reported that addition of the herbs at 1.5% level had no significant (p>0.05) effect on *in vitro* dry matter digestibility, the slight lower value (45.33%) observed in the APL could partly be due to the higher concentration of polyphenols up to 2.39% in the leaves according to research review. Hence, 3.08% observed in this study, could have altered the fermentation characteristics

Table 2: Differences in gas production and rumen pH among different dietary treatments

	Treatments (n = 12)			
Parameters	APL	APR	APS	AP0
Total GP (mL)	28.08±1.29	28.08±0.88	26.47±1.37	25.67±0.37
Rate GP (mL h^{-1})	1.17±0.05	1.17±0.04	1.10±0.06	1.07±0.12
Methane (mL g^{-1} DM)	17.53±0.62	17.34±0.84	18.03±0.80	17.45±1.00
pH (unit)	7.21±0.01	7.20±0.01	7.20±0.02	7.19±0.01

Mean±standard error; means with different superscripts within a row differ significantly at p<0.05. GP: Gas Production; APL: *Andrographis paniculata* Leaves; APR: *Andrographis paniculata* Roots; AP: *Andrographis paniculata* Stem; AP0: Control diet (without *Andrographis paniculata*)

(Kariuk and Norton, 2008) and due to the presence of those polyphenols mentioned earlier (Sule *et al.*, 2010) which could be responsible for the slightly low dry matter digestibility and protein breakdown as compared to the control diet (AP0), APS and APR (Table 1). Mir Ishtiyak *et al.* (2010) also observed slight improvement in *in vitro* dry matter digestibility at 2% and significant increase (p<0.05) at 3% level of inclusion of Trigonella foenungraecum (51.97) as compared to control (43.95).

As regards to VFAs, a reduction in ruminal protein breakdown of diets, a decrease in acetic acid and an increase in propionic acid in the rumen of animals fed diet containing polyphenolic compound has been reported and documented (Carulla *et al.*, 2005; Poungchompu *et al.*, 2009) and these contradicted the current study. Although, not significant, the result of propionic acid from the current study is in agreement with a previous study on the effect of dietary saponins and tannins from mangosteen peel and soapberry fruit (Poungchompu *et al.*, 2009).

Ammonia was significantly higher (p<0.05) in the rumen liquor of the control diet (AP0) than those of the APL, APR and APS. However, among the diets containing AP, significant differences in ammonia were only observed between APL and APS. In general, the differences in ammonia between the diets containing AP and the control are in line with (Carulla *et al.*, 2005).

Although, there were no significant differences seen in all the parameters studied (p>0.05), the total gas production shown by the AP0 (control) was numerically lower (25.67±0.37) compared to the other treatments (Table 2). Irrespective of the parts, the inclusion of *Andrographis paniculata* did not significantly reduce the gas production. However, a slightly lower (26.47±1.37) value was observed in the APS treatment. Moreover, the rate of gas production was also slower in the control diet (AP0) with a slightly higher methane concentration observed in the APS. The results indicate that the low total and slow rate of gas production did not affect the pH and concentration of methane in the gas produced under

an average pH range between 7.19±0.01-7.21±0.01 which was also similar across all treatments. This is in line with the finding of Mir Ishtiyak *et al.* (2010) that addition of herbs {*Melia azedarach* (fruit), *Pimpinella anisum* (seed), *Cuminum cyminum* Linn (seed), *Murraya koenigii* (leaves), *Emblica officinalis* (fruit), *Allium sativum* Linn (bulb), *Terminalia arjuna* (bark), *Sapindus trifoliatus* (seed), *Zingiber officinale* (rhizome), *Trigonella foenun-graecum* (seed)} at three dose level (1.5, 2.0 and 3.0% of DM) had no significant effect on the *in vitro* fluid pH.

Although, not significant, the differences observed in gas production from this study contradicted the earlier findings reported by Beauchemin *et al.* (2011) that plant extracts such as condensed tannins and saponins which were reportedly to be present in *Andrographis paniculata* (Chao and Lin, 2010; Sule *et al.*, 2010) could reduce gas production. Poungchompu *et al.* (2009) reported that tannin reduced methane production in ruminants. Hess *et al.* (2006) classified acetone and methanol extracts of *E. globulus* and aqueous extract of *S. mukorossi* and *E. globulus* as the best inhibitors of methane production while Carulla *et al.* (2005) documented that supplementation of Acacia mearnsii tannins at a level of approximately 0.025% of the diet had significantly reduced methane emissions by 13%.

CONCLUSION

The inclusion of *Andrographis paniculata* irrespective of its parts in the diets improved digestibility by reducing ruminal protein breakdown. The slightly higher methane and low gas production demonstrated in this study could possibly be explained by low level of the inclusion of the plant in the diets. Future research should be carried out to determine if higher level of inclusion could further improve the digestibility and reduce methane production without any adverse effects. Generally, the results from the present study suggest that goats can utilize nutrients from feeds containing AP without compromising the production of VFA and dry matter digestibility.

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