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# Nutritional evaluation of some Indian tree leaves and herbs as fodder and defaunating agent in sheep

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ABSTRACT

Nutritional evaluation as a fodder and defaunating agent of four multipurpose tree leaves namely *Ficus religiosa* (Pipal), *Ficus bengalensis* (Bargad), *Mangifera indica* (Mango), *Enterolobium timoba* (Jungle jalebi) and two herbs namely *Agave americana* (Ramkanta) and *Plantago major* (Isafghol) was done *in vitro*. The mean content of OM, CP, EE, NDF, ADF, cellulose and lignin of these tree leaves and herbs were 88.6, 12.6, 2.4, 46.2, 33.5, 25.8 and 7.3% on DM basis, respectively. *Enterolobium timoba* leaves contained highest amount of CP (22.5%) while highest amount of ADF and lignin content was observed in *Ficus bengalensis* (41.1% / 12.1%) leaves. Total rumen protozoa as well as Holotrich and spirotrich protozoa number became zero due to inclusion of *Agave americana* and *Enterolobium timoba* leaves in the incubation media. Total volatile fatty acids (TVFA) and propionate production was higher where as $NH_3$–N production was lower due to addition of *Agave americana* leaves in the incubation media. Highest IVTDMD and IVTOMD (61.4% / 64.1%) were observed for the *Agave americana* followed by *Enterolobium timoba* (59.8% / 62.5%) and *Plantago major* (57.5% / 59.2%) leaves. Activity of polysaccharide degrading enzymes like carboxymethyl cellulase and xylanase improved due to addition of *Agave americana* and *Enterolobium timoba* leaves in the incubation media. However, activity of β-glucosidase enzyme was similar among all the tested tree leaves and herbs. As a defaunating agent (removal of rumen protozoa / anti ciliate protozoal activity), *Agave americana* leaves were more effective in comparison to *Plantage major* leaves. The results indicated that among the tested tree leaves and herbs, *Agave americana, Enterolobium timoba* and *Plantago major* were good tree fodder for feeding to the animals and leaves of *Agave americana* and *Enterolobium timoba* could be used as defaunating agent for reducing rumen protozoal population to improve animal productivity.

**Key words**: Ciliate protozoa, Enzyme profile, Fodder quality, Herbs, *In vitro* rumen fermentation, Tree leaves

Shortage of animal feed is one of the important causes for poor production potential of Indian livestock. Due to the ever increasing human population and the consequent increase in the demand of food for human consumption, livestock feed tends to be derived from residues and by-products of the food industry (Bakshi and Wadhwa 2012). In India, the prominent role of multipurpose trees is related to fodder and fuel (Datt *et al*. 2008). The livestock of semi-arid zone of India mostly depend on fodder tree leaves, shrubs and herbs as well as grasses available in the forest (Bakshi and Wadhwa 2007). However, information about the nutritive value of such feed resources is limited.

Rumen protozoa are predators of rumen bacteria and decrease the intestinal flow of amino acids, mainly those of bacterial origin by 20–30% (Ivan *et al*. 2000). Moreover, rumen ciliate protozoa contribute significantly to ruminal production of methane and the associated loss of dietary energy. Methanogens associated with ciliate protozoa were responsible for 9–25% of methanogenesis in the rumen (Newbold *et al*. 1995). Therefore, elimination of protozoa from the rumen is desirable for efficient utilization of dietary protein and energy (Jouany and Ushida 1999). Elimination of ciliate protozoa from rumen (defaunation) results in an increased microbial protein synthesis (Jouany 1996), reduced ruminal methanogenesis and improved growth rate of young ruminants (Bird and Leng 1984, Santra and Karim 2000). Defaunation is presently not practical due to unavailability of a suitable defaunating agent commercially (Ivan *et al*. 2004). There is a need to identify feed additives with potential to manipulate/modify rumen fermentation by reducing or completely removing rumen protozoa, thereby enhancing the efficiency of feed utilization while decreasing methane emission and nitrogen excretion. Recently, there is increasing interest in the use of plants, plant extracts and particularly tree leaves for manipulating rumen fermentation for improving animal productivity (Bhatta *et al*. 2017). Tropical plants containing plant secondary metabolites like tannins/saponins have been

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shown to suppress or eliminate ciliate protozoa from rumen and reduce methane and ammonia production (Patra and Saxena 2010, Singh *et al*. 2018). However, effectiveness of plant to manipulate rumen fermentation varied depending upon the source and type of secondary metabolites present in these plants. The screening of these plants is an important step for the discovery of new compounds and their development as feed additives to mitigate rumen methanogenesis and nitrogen turnover as well as to remove rumen protozoa (defaunation). Therefore, the present experiment was conducted to evaluate some tropical tree leaves and herbs as fodder as well as for their anti-protozoal activities to manipulate the rumen fermentation for improving animal productivity.

## MATERIALS AND METHODS

*Collection and processing of leaves:* Three tree leaves of multipurpose trees namely Pipal (*Ficus religiosa*), Bargad (*Ficus bengalensis*), Mango (*Manigifera indica*) and leaves of two herbs namely Isafghol (*Plantago major*) and Ramkanta (*Agave Americana*) were collected from Tonk District of Rajasthan whereas tree leaves of multipurpose tree. Jungle jalebi (*Enterolobium timoba*) were collected from Dehradun, Uttarakhand. All the tested trees and herbs are evergreen. Each collected tree leave and herb were dried at 50°C for 72 h in a forced hot air oven. Dried tree leaves and herbs were grinded in a hammer mill and passed through 1 mm sieve. Ground plant materials were stored in an air tight container for further chemical and biochemical analysis. These ground plant materials were tested for their nutritional evaluation as an animal feed as well as to observe their effect on ruminal fermentation characteristics and ciliate protozoal population *in vitro.*

In vitro *incubation:* Rumen liquor was collected just before morning feeding from two cannulated Malpura ram (about one year of age), fed on a diet (total mixed ration) containing *Cenchrus ciliaris* dried grass and concentrate mixture in 1:1 ratio. After collection of rumen liquor, it was strained through muslin cloth, was pooled and used as the source of inoculums. The inoculum/incubation medium was prepared by mixing rumen liquor with buffer (McDough buffer) in the ratio of 1:2. Each air-equilibrated milled (<1.0 mm) leave/herb 1000±5 mg was incubated with 100 ml of buffered rumen inoculums in a 250 ml conical flask under anaerobic condition and placed in an orbital shaker incubator at 39°C (Tilley and Terry 1963). The incubations were conducted in triplicate for each tree leaves for 24 h and these were repeated three times at a 15 days interval.

*Use of leaves as feed additives:* On the basis of 24 h incubation studies, two tree leaves e.g. *Enterolobium timoba* and *Agave americana* were selected for their antiprotozoal activity for further testing them as feed additives to reduce rumen protozoal population in sheep. Control substrate i.e., 1000±10 mg of air dried milled (<1.0 mm) *Cenchrus ciliaris* straw was replaced by 0, 5, 10, 15, 20, 25, 30, 35 and 40 mg of each selected tree leaves (*Enterolobium timoba* and *Agave americana*) to observe their effect as a feed additive to manipulate rumen fermentation for reducing rumen ciliate protozoal population. Each sample was incubated with 100 ml of buffered rumen inoculums in a 250 ml conical flask and placed in an orbital shaker incubator at 39ºC (Tilley and Terry 1963). The incubations were conducted in triplicate for each sample for 2 h and 4 h. These were repeated three times at a 15 days interval.

*Enumeration of rumen ciliate protozoa:* At the end of incubation (24 or 4 or 2 h), the contents of the conical flask were mixed properly and 1 ml sample was mixed with 1 ml brilliant green formal saline solution. The stained sample was kept overnight at room temperature and protozoa were counted microscopically (Veira *et al.* 1983). Rumen ciliates were identified according to Hungate (1966). Spirotrichs were not identified to generic level were classified into small spirotrichs (mainly Entodinia with an average size 42 mm × 23 mm) and large spirotrichs (mainly Diplodinia with an average size of 132 mm × 66 mm).

*Chemical analysis:* Each tree leaf sample was analyzed for organic matter (OM) by ashing at 550°C for 4 h and crude protein (CP) by Kjeldahl technique (AOAC 1995). The neutral detergent fibre (NDF), acid detergent fibre (ADF) and lignin were estimated following the method of Van Soest *et al.* (1991). Total volatile fatty acids (TVFA) estimation of incubation medium was carried out as described by Barnett and Reid (1957) and fractionation of VFA as well as estimation of the enzymes activity was done as described by Patra *et al*. (2006a). Ammonia nitrogen concentration in the syringe content was estimated as per method of Weatherburn (1967).

*Statistical analysis:* Data were analyzed by the methods described by Snedecor and Cochran (1994). The data were subjected to analysis of variance (ANOVA) and significant treatment effect was determined by comparing the means with Duncan's multiple range test (Duncan 1955).

## RESULTS AND DISCUSSION

*Chemical composition of leaves:* Organic matter (OM) content of the tested leaves ranged between 82.8% (*Mangifera indica*) to 92.3% (*Enterolobium timoba*) (Table 1). The crude protein content (CP) was highest in *Enterolobium timoba* leaves (22.5%) followed by *Plantago major* (12.9%) and *Ficus religiosa* (11.1%) tree leaves. The overall mean contents of OM, CP and EE were 88.7, 12.6 and 2.8%, respectively. The tree leaves evaluated in the present study, had varying levels of nutrients, which could sufficiently support the nutrient requirement of rumen microbes for optimum rumen fermentation. The feed resources with 8% CP are recommended to be appropriate to maintain the desired rumen metabolic activity for rumen fibre degradation (Leng 1990). All the tested tree leaves contained more than 9% CP below which the rumen fermentation is adversely affected (Datt *et al*. 2007). In general, most of the tree leaves were rich in CP and contained higher CP concentration than grasses and cultivated fodder (Datt *et al.* 2007). The values obtained in

Table 1. Chemical composition (on % DM basis) of different tree leaves

| Tree leaves | | Family | Chemical composition (on % DM) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Local name | Scientific name | | Organic matter | Crude protein | Ether extract | Neutral detergent fibre | Acid detergent fibre | Cellulose | Lignin |
| Pipal | *Ficus religiosa* | Moraceae | 85.3 | 11.1 | 2.8 | 49.7 | 36.8 | 27.4 | 8.9 |
| Bargad | *Ficus bengalensis* | Moraceae | 89.1 | 10.1 | 2.6 | 58.9 | 41.1 | 28.7 | 12.1 |
| Ramkanta | *Agave americana* | Asparagaceae | 90.6 | 9.1 | 1.5 | 37.1 | 28.4 | 23.7 | 4.3 |
| Mango | *Manigifera indica* | Anacardiaceae | 82.8 | 9.6 | 1.3 | 48.4 | 34.9 | 25.9 | 8.4 |
| Jungle jalebi | *Enterolobium timoba* | Fabaceae | 92.3 | 22.5 | 3.2 | 40.1 | 29.6 | 24.3 | 4.7 |
| Isafghol | *Plantago major* | Plantaginaceae | 91.6 | 12.9 | 2.9 | 42.9 | 30.4 | 24.8 | 5.3 |

the present study were in the range as reported in other investigations in different parts of the India (Bakshi *et al*. 2007, Datt *et al*. 2007).

The NDF and ADF content varied between 37.1 and 58.9%, and 28.4 and 41.1%, respectively. *Agave americana* and *Enterolobium timoba* tree leaves ained less amount of ADF e.g. 28.4 and 29.6%, respectively among the tested tree leaves (Table 1). Cellulose content varied between 23.7 to 28.7% among the tested tree leaves. The leaves of *Ficus bengalensis* had the highest amount of lignin content (12.1%) followed by *Ficus religiosa* (8.9%) and *Mangifera indica* (8.4%). However, *Agave americana* and *Enterolobium timoba* leaves had the lowest lignin content. The cell wall analysis based on detergent extraction can predict the nutritional value of fibrous feed resources, because voluntary dry matter intake and its digestibility are related to cell wall constituent NDF (Bakshi and Wadhwa 2007). The leaves of *Ficus bengalensis* were highly fibrous (highest NDF and ADF contents) which is an indicator of their potential for low voluntary dry matter intake. *Agave americana* and *Enterolobium timoba* tree leaves content less amount of ADF among the tested tree leaves, indicating good potential as livestock feed stuffs. The values for cell wall constituents of the tree leaves were comparable with those of other workers (Bakshi and Wadhwa 2007, Datt *et al*. 2007).

*Effect of different leaves on rumen ciliate protozoal population:* Ciliate protozoa present in the rumen liquor as well as incubation media was B type population due to the presence of *Epidinium* sp and the absence of *Polyplastron multivesiculatm* (Coleman 1980). The protozoal number (both holotrich and spirotrich) and total protozoal was zero due to addition of *Agave americana* and *Enterolobium timoba* leaves in the incubation medium (Table 2). However, total as well as different rumen protozoal counts were similar among the others tested leaves. The inhibitory effect of *Agave americana* and *Enterolobium timoba* leaves on rumen protozoa might be due to saponin content in those two tree leaves. All types of saponins reduce the rumen protozoal population (Patra and Saxena 2009). The antiprotozoal effect of saponins is attributed to the binding of saponins to cholesterol in the protozoal cell membrane, causing cell lysis (Cheeke 2000). Numerically spirotrich protozoa comprised more than 80% of total protozoal population in the present experiment similar to the earlier findings (Santra *et al*. 1998, Santra *et al*. 2013). In the present experiment, on an average, holotrich protozoa comprised 3.3% and spiroteich protozoa comprised 96.7% of the total rumen protozoal population.

*Effect on rumen fermentation and feed degradability:* pH of the incubation media after termination of incubation at 24 h, was lowest (P<0.05) due to inclusion of *Agave americana* leaves followed by *Enterolobium timoba* and *Plantago major* leaves (Table 3). It has been suggested that protozoa exert a stabilising effect on pH, because their partial or total removal results in a lower and more variable rumen pH (Veira *et al*. 1983). Rumen protozoa engulf excess starch present in the rumen media, resulting in lower starch degradation in the rumen which leads to check the rapid fall in rumen pH. Lower pH of the incubation media due to inclusion of *Agave americana* and *Enterolobium timoba* leaves, might be due to complete elimination of rumen protozoa.

TVFA was highest (P<0.01) due to inclusion of *Agave americana* leaves followed by *Enterolobium timoba* and *Plantago major* leaves while, TVFA production was lowest (P<0.01) due to inclusion of *Ficus bengalensis* followed by *Ficus religiosa* and *Mangifera indica* leaves (Table 3). The variability in the VFA production pattern with the six tested tree leaves might be due to the difference in their chemical composition as well as the active principles e.g. plant secondary metabolites present in those leaves. Tannin has been found to reduce production of total volatile fatty acids (Hristove *et al*. 2003). Decreased TVFA production due to addition of *Ficus bengalensis* and *Mangifera indica* leaves could be due to the presence of tannin. Higher (P<0.01) TVFA production by *Agave americana* and *Enterolobium timoba* tree leaves might be due to their higher DM and OM degradation in the rumen. Acetate production was decreased (P<0.05) and propinate production increased (P<0.05) due to inclusion of *Agave americana* in comparison to other tested leaves. However, butyrate production was not influenced by the inclusion of any of the tested leaves. The ratio of acetate to propionate was lowest by the addition of *Agave Americana followed* by *Enterolobium timoba* leaves in the incubation medium. Hess *et al.* (2003) reported that reduced rumen protozoal number was associated with increase in propionate and decrease in

Table 2. Effect of different tree leaves on rumen protozoal population (× $10^3$/ ml) *in vitro*

| Parameter | Different tree leaves | | | | | | SEM |
|---|---|---|---|---|---|---|---|
| | *Ficus religiosa* | *Ficus bengalensis* | *Agave americana* | *Manigifera indica* | *Enterolobium timoba* | *Plantago major* | |
| Large Holotrich protozoa | $0.3^{b}$ | $0.1^{a}$ | 00 | $0.2^{ab}$ | 00 | $0.3^{b}$ | 0.05 |
| Small Holotrich protozoa | $1.2^{ab}$ | $1.3^{b}$ | 00 | $1.2^{ab}$ | 00 | $1.1^{a}$ | 0.07 |
| Total holotrich protozoa | 1.5 | 1.4 | 00 | 1.4 | 00 | 1.4 | 0.09 |
| Large Spiotrich protozoa | $6.2^{a}$ | $7.1^{b}$ | 00 | $7.5^{b}$ | 00 | $6.8^{ab}$ | 0.39 |
| Small Spirotrich protozoa | 36.9 | 37.3 | 00 | 35.9 | 00 | 38.1 | 1.13 |
| Total Spirotrich protozoa | 43.1 | 44.4 | 00 | 43.4 | 00 | 44.9 | 1.38 |
| Total rumen protozoa | 44.6 | 45.8 | 00 | 44.8 | 00 | 46.3 | 1.79 |

[a,b]Mean with different superscripts in a row differ significantly among treatment at P<0.05

Table 3. Effect of different tree leaves on rumen fermentation, enzyme activity and feed digestibility *in vitro*

| Parameter | Tree leaves | | | | | | SEM |
|---|---|---|---|---|---|---|---|
| | *Ficus religiosa* | *Ficus bengalensis* | *Agave americana* | *Manigifera indica* | *Enterolobium timoba* | *Plantago major* | |
| *p*H | $6.73^{e}$ | $6.81^{f}$ | $6.32^{a}$ | $6.65^{d}$ | $6.44^{b}$ | $6.58^{c}$ | 0.013 |
| TVFAs (MEq/dl) | $4.5^{B}$ | $4.1^{A}$ | $6.6^{F}$ | $4.9^{C}$ | $5.9^{E}$ | $5.2^{D}$ | 0.11 |
| Acetate (% of TVFA) | $68.4^{c}$ | $68.5^{c}$ | $67.1^{a}$ | $68.4^{c}$ | $67.8^{b}$ | $67.9^{b}$ | 0.21 |
| Propionate (% of TVFA) | $22.3^{a}$ | $22.1^{a}$ | $23.8^{d}$ | $22.4^{a}$ | $23.1^{c}$ | $22.7^{b}$ | 0.11 |
| Butyrate (% of TVFA) | 9.3 | 9.4 | 9.1 | 9.2 | 9.1 | 9.4 | 0.13 |
| Acetate : Propionate ratio | $3.06^{d}$ | $3.09^{d}$ | $2.81^{a}$ | $3.05^{d}$ | $2.94^{bc}$ | $2.99^{cd}$ | 0.06 |
| Total nitrogen (mg/dl) | $19.8^{A}$ | $18.5^{A}$ | $18.9^{A}$ | $18.2^{A}$ | $28.5^{B}$ | $21.1^{A}$ | 1.58 |
| $NH_3$–N (mg/dl) | $10.7^{B}$ | $11.2^{B}$ | $7.5^{A}$ | $10.9^{B}$ | $80.3^{A}$ | $11.5^{B}$ | 0.75 |
| *Enzyme activity (IU/dl/h)* | | | | | | | |
| Carboxy methyl cellulase | $18.9^{A}$ | $19.3^{A}$ | $27.5^{B}$ | $20.5^{A}$ | $25.7^{B}$ | $17.9^{A}$ | 0.97 |
| Xylanase | $26.9^{A}$ | $25.3^{A}$ | $34.1^{B}$ | $26.8^{A}$ | $32.6^{B}$ | $27.3^{A}$ | 2.17 |
| β–glucosidase | 7.7 | 6.9 | 8.2 | 7.5 | 7.9 | 7.4 | 1.5 |
| Amylase | 137.8 | 139.5 | 140.7 | 142.9 | 132.4 | 135.7 | 15.9 |
| Dry matter degradability (%) | $50.2^{B}$ | $47.6^{A}$ | $61.4^{D}$ | $51.7^{B}$ | $59.8^{C}$ | $57.5^{B}$ | 0.39 |
| Organic matter degradability (%) | $52.5^{B}$ | $49.3^{A}$ | $64.1^{D}$ | $53.5^{B}$ | $62.5^{C}$ | $59.2^{B}$ | 0.41 |

Mean with different superscripts in a row differ significantly among treatment [$^{ABC}$(P<0.01), $^{abc}$(P<0.05)].

A:P ratio. The inhibition of protozoal population also resulted in the inhibition of methanogenesis (Goel *et al*. 2008) which leads to more TVFA and propionate production. Increased propionate production due to inclusion of *Agave americana* followed by *Enterolobium timoba* leaves in the incubation media might be due to complete elimination of rumen protozoa from the incubation media. Further, higher propionic acid production might be due to a shift in rumen fermentation towards propionate at the expense of acetate production (Shingfield *et al*. 2008).

The presence of protozoa in the rumen ecosystem is associated with increased recycling of microbial nitrogen in the rumen and therefore, decreased protozoal population in the rumen are usually associated with lowered ammonia concentrations, primarily as a result of a decrease in proteolysis of bacterial protein by ruminal protozoa (Hristov *et al*. 2005). Ammonia nitrogen concentration was lower (P<0.01) due to addition of *Agave americana* and *Enterolobium timoba* leaves and this is might be due to lower rumen ciliate protozoal population. Moreover, these two tree leaves might be contained saponin which reduced the rumen ciliate protozoal population *in vitro* and a number of studies have reported that saponins or saponin containing plant decreased rumen ammonia nitrogen concentrations *in vitro* and *in vivo* (Patra and Saxena 2009).

The activity of carboxymethyl cellulase and xylanase was higher (P<0.05) due to addition of *Agave americana* and *Enterolobium timoba* leaves in incubation media (Table 3). However, activities of β–glucosidase and amylase enzyme were not influenced by any of the tested leave. The lingo–cellulosic feed stuffs are degraded in the rumen by the synergistic activities of the bacteria, protozoa and fungi, with bacteria and fungi contributing approximately 80% of the degradative activity, and the protozoa only 20% (Dijkstra and Tamminga 1995). Agarwal *et al.* (1991) reported that 38% of cellulase activity in the rumen, is associated with protozoa fraction of rumen liquor. Ruminal fungi produce a broad array of enzymes and generally degrade a wide range of substrates than do rumen bacteria (Trinci *et al*. 1994). Furthermore, ruminal fungi are able to degrade the most resistant plant cell wall polymers and the cellulase and xylanase produce by them are among the most active

fibrolytic enzymes (Forsberg and Cheng 1992). The ruminal protozoa also contribute to the degradation of plant cell wall polymers, but their contribution in fibre degradation is considered not as important as that of the bacteria and fungi (Lee *et al*. 2000). Rumen bacterial and fungal population become increased due to complete removal of rumen protozoa as evident from defaunated animals or due to reduction in rumen protozoal number which is believed to be as a consequence of reduce engulfment of these micro-organisms by the rumen protozoa (Williams and Coleman 1997). It had been reported that cellulolytic bacterial counts had been shown to increase due to feeding *Enterolobium cyclocarpum* which contained saponins (Diaz 1993) and reduced rumen ciliate protozoal population. Higher activity of carboxymethyl cellulase and xylanase enzyme due to addition of *Agave americana* and *Enterolobium timoba* leaves in incubation media might be due to higher population of rumen fungi and rumen bacteria due to absence of rumen protozoa. Patra *et al*. (2006b) reported that plant parts rich in tannin did not alter carboxy-methyl cellulose enzyme activity in the rumen.

*In vitro* true dry matter degradability (IVTDMD) and *in vitro* true organic matter degradability (IVTOMD) of the tested tree leaves varies from 47.6 to 61.4% and 49.3 to 64.1%, respectively (Table 3). Highest (P<0.01) IVTDMD and IVTOMD was observed in *Agave americana* tree leaves followed by *Enterolobium timoba* and *Plantago major* tree leaves while it was lowest (P<0.01) in *Ficus bengalensis* (47.6% and 49.3%) followed by *Ficus religiosa* (50.2% and 52.5%) and *Mangifera indica* (51.7% and 53.5%) tree leaves. Majority of the tested leaves possessed IVTDMD/ IVTOMD values of more than 50% level which indicated that they might be used in the diet of ruminant animals as good fodder. The data on IVTDMD and IVTOMD in the present experiment is comparable to that reported earlier (Bakshi *et al*. 2007, Datt *et al*. 2007, Bhatta *et al*. 2017). Dry matter as well as organic matter digestion of forages is highly dependent on structural fraction such as the relative proportion of cell type present in the plant tissue and higher lignifications reduce the digestibility of both dry matter and organic matter (Bhatta *et al*. 2017). Lowest IVTDMD and IVTOMD value of *Ficus bengalensis* tree leaves followed by *Ficus religiosa* and *Mangifera indica* tree leaves might be due to higher lignin content of those tree leaves.

*Effect of tree leaves as additives on rumen protozoal population:* The total rumen protozoal number become gradually reduced due to addition of *Enterolobium timoba* or *Agave americana* tree leaves as a feeds additive in the incubation media (Table 4). Rumen protozoal number after 2 h of incubation was $41.5\times10^3$, $20.3\times10^3$ and $4.7\times10^3$/ml incubation media due to inclusion of 0, 20 and 30 mg *Enterolobium timoba* leaves in the incubating substrate and it was $41.5\times10^3$, $3.8\times10^3$ and $00\times10^3$/ml incubation media due to inclusion 0, 20 and 30 mg of *Agave americana* tree leaves in the incubating substrate, respectively. Rumen protozoal number become zero due to replacement of 20 mg of substrate by *Agave americana* tree leaves as well as 30 mg of substrate replaced by *Enterolobium timoba* tree laves after 4 h of incubation. Similarly, no rumen protozoa was found in the incubation media after 2 h of incubation due to replacement of 25 mg substrate by *Agave americana* or 35 mg substrate by *Enterolobium timoba* tree leaves. A number of studies have demonstrated that all type of pure saponins and saponin containing plants have inhibitory effects on protozoa depending upon the concentration in the diet (Patra and Saxena 2009) and have been identified as possible defaunating agents in the rumen (New bold *et al.* 1997). The two feed additives in the present experiment e.g. leaves of *Enterolobium timoba* and *Agave americana* completely remove rumen protozoa when their doses as feed additive increased in the control substrate might be due to higher content of saponin in the experimental substrate as doses of those two tested tree leaves increased gradually. The dose-dependent effect of the saponins on rumen protozoa has clearly been observed in many studies (Agarwal *et al*. 2006, Pen *et al*. 2006). The oral administration of saponins from the plant *Biophytum petersianum* decreased protozoal numbers in goats by 35 to 40% at the dose of 13 and 19.5 mg/kg body weight, respectively, beyond which protozoal counts did not further reduced (Santoso *et al.* 2007). Further, the results of present experiment indicated that leaves of *Agave americana* had strong anti-protozoal activity than *Enterolobium timoba* tree leaves.

Table 4. Effect of different tree leaves as feed additives on rumen total protozoal number ($\times 10^3$/ ml) after 2 h and 4 h incubation *in vitro*

| Additive (mg) | Tree leaves/Incubation period (h) | | | | SEM |
|---|---|---|---|---|---|
| | *Enterolobium timoba* | | *Agave americana* | | |
| | 2 h | 4 h | 2 h | 4 h | |
| 00 | $41.5^{u}$ | $41.3^{t}$ | $41.5^{t}$ | $41.3^{s}$ | 1.47 |
| 5 | $40.7^{u}$ | $39.5^{t}$ | $38.2^{s}$ | $37.9^{r}$ | 1.35 |
| 10 | $36.8^{Dt}$ | $29.4^{Cs}$ | $18.7^{Br}$ | $12.9^{Aq}$ | 1.18 |
| 15 | $26.7^{Ds}$ | $23.2^{Cr}$ | $9.3^{Bq}$ | $5.2^{A\,p}$ | 1.03 |
| 20 | $20.3^{Cr}$ | $13.5^{Bq}$ | $3.8^{Ap}$ | 00 | 0.75 |
| 25 | $11.5^{Bq}$ | $5.7^{Ap}$ | 00 | 00 | 0.29 |
| 30 | $4.7^{p}$ | 00 | 00 | 00 | 0.08 |
| 35 | 00 | 00 | 00 | 00 | – |
| 40 | 00 | 00 | 00 | 00 | – |

$^{pqrstu}$Means with different superscripts in a column differ significantly among levels (P<0.05); $^{ABCD}$Means with different superscripts in a row differ significantly among treatments (P<0.01).

Based on the results, leaves of *Agave americana*, *Enterolobium timoba* and *Plantago major* could be considered of good quality and those of *Mangifera indica*, *Ficus religiosa* and *Ficus begalensis* are medium quality fodder for feeding to the animals. Further, the results of this experiment indicate that the plant secondary metabolites appear to have a potential to manipulate rumen fermentation favourably. Dried powder of *Agave americana* and *Enterolobium timoba* leaves have the potential to reduce

rumen protozoal population and improve propionate production *in vitro*. *Agave americana* was a strong defaunating agent than *Enterolobium timoba* leaves. However, further studies are needed to explore chemical nature of the active principles responsible for the anti-protozoal activity and testing of these plants products in *in vivo* experiments for their effect on ruminal fermentation and nutrient utilization by the ruminant animals so that they can be used as a herbal feed additive to manipulate rumen fermentation for improving animal productivity.

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