NUTRITIONAL EVALUATION AND METHANE PRODUCTION OF SOME FODDER PLANTS USING IN VITRO GAS PRODUCTION TECHNIQUE

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ABSTRACT

The aim of this study was to evaluate the nutritive value of four salt tolerant plants (Pennisetum americanum, Acacia saligna, Leucaena leucocephala and Kochia indica) based on their chemical composition, in vitro gas production and fermentation kinetics. The chemical composition of A. saligna and L. leucocephala showed the higher content of crude protein and non-protein nitrogen (NPN) as well. Total tannins, phenols and saponin contents in A. saligna and L. leucocephala was higher compared to K indica. While P americanum was free of total tannins and saponin. A significant differences were observed in short-chain fatty acids and acetic acid for the tested plants. Cumulative gas production for A. saligna, L. leucocephala and K indica showed pronounced methane inhibition compared to P americanum. The results indicated that the salt-tolerant plants used in the experiment could be promising feed resources to decrease energy loss as methane in ruminant diets. However, the presence of secondary metabolites and protein nitrogen (NPN) should be taken into consideration when formulating diets containing salt-tolerant forages for small ruminants.

Keywords: salt-tolerant plants, digestibility, rumen fermentation, methane production
INTRODUCTION

In Egypt, the availability of forage feeds is more restricted particularly in areas with dry to semi-dry climate. Halophytes and other salt-tolerant plants have the advantage of tolerating high salt levels in the saline lands and drought conditions (Helal et al., 2013). These plants can provide great potentialities particularly as sources of livestock fodders and can fill up the feed gaps in the summer (Aderao et al. 2018). In the current study four fodder plants, Acacia saligna, Leucaena leucocephala, Kochia indica and Pennisetum americanum will present as salt-tolerant plants which could be used for feeding ruminants.

Acacia and Leucaena species are belonging to family Fabacea, and characterized as a drought resistant, moderately salinity tolerant, have high production of green biomass and high crude protein content (El Shaer 2010 & Shaker et al., 2014). Kochia indica are annual shrubs which belong to the family Chenopodiaceae, these plants are adapted to be grown under drought and/or salt-affected lands (El Shereef 2016). Besides, P americanum is a salt and drought tolerant grass that could be used successfully and safely for feeding ruminants in semi-arid regions (Fahmy et al., 2010). Because of that, there is a need to use the available forages from such plants (shrubs, trees, and grasses) for feeding livestock with low feed costs under desert conditions. So, the purpose of this study is to assess the nutritional values of these plants by evaluate nutrient digestibility, ruminal fermentation profiles and methane production at in vitro level.

Material and methods

Sample collection and preparation
Plant samples (P americanum, A saligna, L leucocephala and K indica) were collected from six different sites randomly selected in South Sinai of Egypt, Sinai Peninsula (200 km South East of Cairo), Egypt. Laboratory work was conducted at the Laboratory of Climate Change and Livestock Production of FMVZ-UADY, Mexico. The experimental procedures were approved and complied with the ethical standards set by the faculty. For each species, the plants were cut into small pieces (3-5 cm). Samples were then dried at 50°C for 48 h using a forced air oven to prevent enzymatic degradation of the phenolic compounds present in the plant matter (Makkar et al. 1993b). Once dry, 400 g of plant matter was ground in a Lab-Willey Grinder (code MSW-342- IN; 10122740) and sieved through a 2-mm screen. The grounded material was mixed well and then 100 g were sub-sampled, reground and passed through a 0.5-mm screen sieve. These finely ground subsamples were used in tannin analysis while the rest of the material was used for in vitro gas production analysis.
The proximate analysis
Dry matter (DM), crude protein (CP), crude fiber (CF) and ash of feed ingredients were determined according to AOAC (2007). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined using the ANKOM fiber technology technique (Robinson et al. 1999) without using alpha-amylase. The non-protein nitrogen (NPN) was obtained by precipitation of true protein in the filtrate with tungstic acid (10% sodium tungstate solution) and determined as the difference between total N and the N content of the residue after filtration. Ammonia nitrogen were determined by Warner (1964). Determine of saponins was according to the method of Segal et al. (1966). Measurement of microbial protein was analyzed using the spectrophotometric method of Zinn & Owens (1986) after incorporating suggested modifications of Makkar & Becker (1991) and Obispo & Dehority (1999). Microbial counts as bacteria and protozoa of ruminal fluid were determined using a counting cell (Hawskley, UK) as described by Demeyer (1981). Alkaloids concentration was determined according to Shamsa et al. (2007). Total oxalate was determined by HPLC method (Savage et al., 2000). 1 mL of 50% sulphuric acid was added and they were frozen at -20 ºC until analysis fractionation of SCFA’s according to Erwin et al. (1961). Calcium was determined by spectrophotometer (Gindler and King, 1972). Inorganic phosphorus was determined by atomic absorption spectrophotometer according to Chapman and Pratt (1961). Sodium (Na) and potassium (K) were determined by using the standard flame photometry (Jackson, 1958) while copper (Cu), and zinc (Zn) concentrations were tested using atomic absorption techniques.

In vitro gas production
Gas production (GP) was determined following Theodorou et al. (1994) technique. Rumen fluid was collected from Pelibuey hair sheep in pre-warmed insulated bottle. Samples weighing 0.999 g of each plant then placed in 125 ml serum glass bottles, and approximately 90 ml of buffered rumen fluid was added to each bottle. Once closed, the bottles were gently shaken and placed in a water bath at 39 ºC. Gas production measurements, for the samples incubated 72 h, were taken hourly up to 8 hours after incubation, then every four (from 12- to 28), eight (from 36-to 60) and 72 h post incubation. After 24 and 72 h, the incubation residue, respectively, was analyzed for digestibility of dry matter (DMD), organic matter (OMD) and the digestibility of neutral detergent fiber (DNDF) content using the ANKOM fiber technology technique (Robinson et al. 1999).
Phenols content was determined using the Folin-Ciocalteu method and tannins were measured using polyvinylpolypyrrolidone (PVPP) as described by Makkar et al. (1993b).

Methane measurement
Using a gas-tight syringe, gas samples were collected from each bottle at 24 h post-incubation as in Bhatta et al. (2015) and Kaya et al. (2016). After the volume of gas
was recorded, and the sample removed for methane analysis, the remaining gas was released. The CH4 content was determined by injecting 1 ml of gas into a Perkin Elmer gas chromatograph (model: Clarus 500 series) equipped with a flame ionized detector (FID). Separation was achieved using an Elite-Q Plot Capillary Column (Perkin Elmer) packed with a 60/80 mesh carboxenTM-1000 stationary phase. Nitrogen was used as the carrier gas with a flow rate of 30 mL/min, an isothermal oven temperature of 50 oC, and an injector temperature of 250 oC. The calibration curve using a regression equation was completed with standard CH4 (99.99 % from ALTECH).

Statistical analysis
The data obtained from each plant were analyzed for variance using an ANOVA procedure according to SAS (2000) using the following model: Yij = µ + ai + εij where Yij is observation, µ is overall mean, ai is plant species (i = 1 to 4), and εij is error. Tukey’s test was used for the multiple comparisons among mean values for the four plants and the significance level was set at p < 0.05.

Results and discussion

Proximate analysis
The chemical composition is the first step to evaluate the nutritive value of such plants to be a feed for animals. Although A. saligna and L. leucocephala were high in CP (113 and 147 g/kg), the nitrogen richness of such plants may not be fully used by ruminants since non-protein nitrogen (NPN) represents 46 and 52 % of CP content, respectively (Table 1). The NPN could not be metabolized and converted to protein in the rumen if there is not sufficient energy source or some of these compounds would be converted to ammonia in the rumen, which is absorbed and converted to urea then excreted in the urine (SCA, 2007). Otherwise K indica had moderate contents of CP and NPN %. While P. americanum had the lowest CP, NPN and ADL contents compared with the other plants. In this regards, forages with CP content of less than 70 g /kg DM require protein supplementation to offset limitations on voluntary feed intake as recommended by Melaku et al. (2003). The present study suggest that crude protein (CP) is inadequate estimation for salt tolerant plants of true protein because it is based on the assumption (certainly untrue) that all nitrogen in the biomass will become protein; i.e. CP (%) = nitrogen (%) * 6.25.

The differences in cell wall constituents as NDF, ADF and ADL could be due to species genotypic differences for the tested plants and their values were agreed with Shawket et al. (2010), Shaker et al. (2014) and El Shereef (2016).

Plant secondary metabolites (PSM) and minerals
PSM is a group of chemical bioactive compounds such as tannins, saponins, alkaloids, flavonoids, glucosides, etc., that are not involved in the primary biochemical processes of growth and reproduction, but play a vital role in the interaction between plants and the
environment (Kliebenstein 2013). A saligna and L leucocephala showed higher alkaloids, saponin, and phenols components compared to the other plants (Table 2). Both plants had alkaloids content above 40 g/kg DM. Ventura et al. (2000) recorded the negative correlation between feed intake and alkaloids content. Besides, Abd El-Rahman (2003) reported that Halocnemum strobilaceum and Hammada elegans which consider non palatable plants was related to high level of alkaloids (31.1 and 61.6 g/kg DM). Likewise, saponins are characterized by a bitter taste and foaming properties (Kumar, 2011). Thus the present results of alkaloids and saponins concentrations could explain the lower dry matter intake from A saligna and L leucocephala in different studies (Shawket et al., 2010, Helal et al. 2013 and Hassan et al. 2015) compared to traditional rations. Concerning condensed tannins (CT), it was reported that moderate levels (30 to 40 g/kg DM) of CT may result in nutritional advantages by increased bypass protein availability and bloat suppression in cattle. The mode of action of condensed tannins (at low concentrations) was noticed by bind with plant protein at nearly neutral pH in the mouth and rumen to form tannin-protein complexes which are stable and insoluble at pH 3.6 -7.0, but dissociate and release protein at pH <3.5 in the abomasums (Soltan et al., 2013). Therefore, the presence of high contents of CT should be taken into consideration when formulating diets containing the tested plants for feeding ruminants.

Total oxalate was higher in K indica compared to the other plant species. El Shereef et al. (2016) reported a significant inhibition for Ca concentration in blasma for sheep fed K indica silage, the authors suggests that Ca bioavailability may decrease as result of the binding with Ca to form calcium oxalate, a non-soluble and non-digestible compound. P americanum was free of total tannins, condensed tannins and saponin. In general, the concentrations of alkaloids, saponin, total tannins, total oxalate and condensed tannins contents for the experimental plants were comparable to that obtained by El Shereef et al. (2016) and Fahmy & Ibrahim (2005) for K indica and P americanum while these values was above than that reported by Bueno et al. (2005) and Soltan et al. (2012) for A saligna and L leucocephala. This variation could be due to many factors like temperature, drought, salinity, seasonality, altitude and light, metal ions, wounding and nutrient deficiencies can affect their concentration and these are also dependent on the growing conditions and metabolic pathways of related PSM (Gouvea et al. 2012).

In respect to minerals values (Table 2), it seems that A saligna and L leucocephala could be good resource of Ca for feeding animals. K indica and A saligna surpassed the other plants in Na contents while P americanum had the highest P concentration. This finding was agreed with Helal et al., (2013). A wide variation of the other minerals concentrations are recorded for the experimental plants, these was in harmony with the finding by
El Shaer (2016) when he set that salt tolerant plants are characterized by moderate digestible crude protein, soluble carbohydrates and high mineral contents, particularly Na, K, Cl and Ca concentrations.

**In vitro fermentation profiles**

As expected, in vitro fermentation parameters differed (P<0.05) among plant species (Table 3). *A saligna* and *L leucocephala* releasing a lower ruminal NH3-N concentration resulting a significant decreasing in pH values compared to *K indica* plant. This may be associated with the protection of dietary protein from microbial activity by binding tannin-protein complexes which are stable and insoluble at pH 3.6 -7.0 as mention in Table 2. While *P americanum* releasing the lowest ruminal NH3-N and lower pH which may be attributed to their lower nitrogen components (CP and NPN) as shown in Table 1.

Total short chain fatty acid (SCFA’s) and acetic acid was higher (P<0.05) for *K indica* and *P americanum* compared to the other plants. Otherwise the results showed that, *A saligna* , *L leucocephala* and *K indica* presented a pronounced CH4 inhibition while *P americanum* significantly decreased ruminal bacteria. The major challenge of utilize salt-tolerant plants is that their high cell wall contents, phenolic compounds, with variable mineral concentration. As indicated in the present study for the experimental plants species which have a considerable proportion of condensed tannins that may form complexes with proteins and carbohydrates resulting in reduction of their ruminal fermentation. Even though, this generally increases efficiency of ruminal N utilization and intestinal input of N, it can restrict fiber digestion in the rumen, resulting in unsynchronized availability of N and energy to microbes for synthesis VFA (Attia et al. 2018) . So, it could be explain the lower SCFA’s and acetic acid for rich phenolic plants (Table 2). The variable responses of in vitro gas production among plants could be due to variable levels of PSM. It is noticeable the higher tannins and saponins contents for *A saligna* and *L leucocephala* plants (Table 2) that reflect in reduction of total gas, methane production and total number of ruminal Bacteria as well (Table 3). In this regards, the anti-methanogenic activity of tannins has recently been reported by many studies (Goal and Makkar, 2012 and Liu and Zhou, 2011) the mechanism of tannins may be due to inhibit ruminal microorganisms through bactericidal or bacteriostatic activities, the growth or activity of rumen methanogens and protozoa. However, Saponins have a potent antimicrobial activity and limit the H2 availability for methanogenesis in the rumen, thereby could reduce CH4 production (Bodas et al. 2012 and Patra & Saxena, 2009).

**In vitro nutrients digestibility**

The variations of in vitro digestibility values (Table 4) could be due to variable levels of phenolic, tannin activity and cell wall content among the experimental plants. The high IVDM, IVOMD and IVNDFD for *P americanum* could be due to the lower
phenolic components, absence of condensed tannin and saponin. The lower digestibility of *A. saligna*, *L leucocephala* could be attributed to the higher concentration of condensed tannins through formation of complexes with dietary carbohydrates which is associated with reduction in organic matter digestibility. Moreover, other researchers (Min *et al*. 2003 and Ammar *et al*. 2005) reported that concentrations of condensed tannins are negatively correlated with *in vitro* dry matter degradability. The result of all nutrients digestibility of the experimental plants was lower than that recorded by Hassan *et al*. (2015) and Shawket *et al*. (2010) at in vivo level, this mainly attributed to supplemented the salt-tolerant plants with different energy resources that could improve ruminal microbes for better utilization of their nutrients.
Table 1. Chemical composition and fiber fraction of the experimental plants

| parameters | Plants         |
|------------|---------------|
|            | \(P_{americanum}\) | \(A_{saligna}\) | \(L_{leucocephala}\) | \(K_{indica}\) |
| DM (g/kg)  | 427           | 435           | 383           | 337           |
| CP (g/kg DM) | 54           | 113           | 147           | 87            |
| NPN * (CP %) | 13           | 46            | 52            | 43            |
| CF (g/kg DM) | 287           | 268           | 240           | 276           |
| NDF (g/kg DM) | 560           | 460           | 410           | 584           |
| ADF (g/kg DM) | 361           | 350           | 260           | 392           |
| ADL (g/kg DM) | 64            | 108           | 147           | 95            |
| Ash (g/kg DM) | 115           | 95            | 75            | 141           |

DM = dry matter, CP = crude protein, CF = crude fiber, NDF = nutrient detergent fiber, ADF = acid detergent fiber, ADL = acid detergent lignin, * The percentage of crude protein of the plant that is non-protein nitrogen x 6.25.
Table 2. Secondary metabolites and some minerals contents of the experimental plants

| parameters                      | Plants                  |
|---------------------------------|-------------------------|
|                                 | *P. americanum* | *A. saligna* | *L. leucocephala* | *K. indica* |
| Alkaloids (g/kg DM)             | 11.9        | 77.9        | 42.4            | 21.0        |
| Total oxalate (g/kg DM)         | 12          | 18.0        | 26.0            | 34.0        |
| Saponin (g/kg DM)               | 0           | 105.6       | 64.8            | 38.1        |
| Total phenols (g/kg DM)         | 5.6         | 95.1        | 103.0           | 34.7        |
| Total tannins (g/kg DM)         | 0           | 75.0        | 89.0            | 48.3        |
| Condensed Tannins (g/kg DM)     | 0           | 68.0        | 59.0            | 32.0        |
| **Mineral contents**            |             |             |                 |             |
| Cu (mg/kg)                      | 35.8        | 17.0        | 7.8             | 6.5         |
| Zn (mg/kg)                      | 60.1        | 41.8        | 30.8            | 29.4        |
| K (g/kg DM)                     | 15.0        | 21.5        | 17.5            | 6.5         |
| Na (g/kg DM)                    | 10.5        | 15.0        | 2.0             | 15.5        |
| Ca (g/kg DM)                    | 7.8         | 13.4        | 11.9            | 5.5         |
| P (g/kg DM)                     | 3.3         | 1.2         | 2.0             | 1.4         |

Cu = copper, Zn = zinc, K = potassium, Na = sodium, Ca = calcium, P = phosphorus.
Table 3. Comparative in vitro evaluation of the experimental plants on ruminal fermentation

| Items    | P americanum | A saligna | L leucocephala | K indica |
|----------|--------------|-----------|----------------|----------|
| pH       | 6.05±0.13    | 6.08±0.04 | 6.06±0.01      | 6.59±0.04|
| NH3-N    | 16.7±0.5     | 19.1±1.0  | 19.3±0.9       | 20.8±0.3 |
| Total SCFA's | 82.7±1.7    | 78.5±1.74 | 78.4±0.5      | 83.9±1.7 |
| Acetic   | 52.1±1.7     | 42.2±1.9  | 45.8±1.0      | 48.7±0.6 |
| Propionic| 19.0±0.6     | 19.4±0.5  | 19.4±0.6      | 19.7±0.3 |
| But.     | 9.9±0.7      | 10.8±0.23 | 10.0±0.4      | 11.2±0.4 |
| Iso- But.| 1.5±0.1      | 1.6±0.14  | 1.4±0.02      | 1.4±0.03 |
| Val.     | 1.2±0.03     | 1.3±0.03  | 1.2±0.01      | 1.3±0.04 |
| Iso-Val. | 0.8±0.07     | 0.7±0.03  | 0.7±0.04      | 0.6±0.04 |
| AC: Pr   | 2.7±0.1      | 2.2±0.14  | 2.4±0.13      | 2.5±0.1  |
| TGP      | 175.1±18.3   | 121.7±3.9 | 122.1±8.8     | 159.2±4.2|
| CH4      | 10.54±0.22   | 6.41±0.35 | 7.97±0.17     | 6.81±0.32|
| Bact *10^6 | 7.5±0.7     | 8.8±0.5   | 8.6±0.4       | 9.7±0.8 |
| Prot *10^6 | 4.7±0.4      | 4.9±0.4   | 4.2±0.8       | 5.1±0.1 |
| MP       | 11.0±1.6     | 12.5±1.1  | 13.5±1.7      | 12.6±0.8 |

a,b,c Means having different superscripts within the same row differed significantly (P < 0.05), otherwise no significant differences were detected. Total SCFA’s = total short chain fatty acid, But. = Butyric acid, Val. = Valeric acid, AC = acetic acid, TGP = total gas production, CH4 = methane, Bact. = Bacteria, Prot. = protozoa, MP = microbial Protein.
Table 4. In vitro nutrients digestibility of the experimental plants

| Items       | P americanum | A saligna | K indica |
|-------------|--------------|-----------|----------|
| IVDMD       | 488.3 ±7.4   | 408.6 ±6.6 | 366.7 ±5.9 | 453.5 ±5.6 |
| IVOMD       | 493.7 ±6.9   | 397.3 ±6.2 | 355.5 ±5.6 | 459.1 ±6.9 |
| IVNDFD      | 443.7 ±13.8  | 363.3 ±10.5 | 329.6 ±11.0 | 426.5 ±5.9 |
| IVCPD       | 109.8 ±12.6  | 116.6 ±6.0  | 117.6 ±9.5  | 127.9 ±10.5 |
| b           | 135.4 ±4.4   | 118.5 ±4.0   | 116.8 ±2.3  | 164.5 ±8.1  |
| C           | 0.09 ±0.01   | 0.11 ±0.02   | 0.11 ±0.01  | 0.09 ±0.01  |

IVDMD = in vitro dry matter digestibility, IVOMD = in vitro organic matter digestibility, IVNDFD = in vitro nutrient detergent fiber digestibility, IVCPD = in vitro crude protein digestibility, b, c = fermentation kinetics.
Conclusion

In conclusion, the results indicated that the salt-tolerant plants used in the experiment could be promising feed resources to decrease energy loss as methane in ruminant diets. However, the presence of secondary metabolites and protein nitrogen (NPN) should be taken into consideration when formulating diets containing salt-tolerant forages for feeding small ruminants.

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