Potential of *Citrullus colocynthis* as herbal feed additive for ruminants

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ABSTRACT

To assess the potential of *Citrullus colocynthis* as herbal feed additive for ruminants, colocynth fruit as a whole, its seeds and peel-pulp were evaluated at various levels (0, 0.5, 1.0, 1.5, 2.0, 2.5, 4.0%) with total mixed ration (roughage to concentrate ratio: 65:35) as substrate in 3×7 factorial design by *in vitro* gas production technique. Phytochemical analysis revealed significant variation (P<0.01) in total phenols, non-tannin phenols, true tannins, saponins, flavonoids and vitamin C content among different parts of colocynth fruit. Irrespective of the part used, varying level of *Citrullus colocynthis* had significant effect on net gas production (NGP; ml/24/g), metabolizable energy (ME) availability, methanogenesis, digestibility of neutral detergent fiber (NDF) and true organic matter (TOM) in comparison to control; however the effect was pronounced at 1% level of supplementation. No significant difference in total volatile fatty acids (TVFA) production and acetate production was observed at varying levels of *Citrullus colocynthis* except at 4% level when total mixed ration (TMR) was used as substrate. Irrespective of its level, supplementation of *Citrullus colocynthis* fruit as a whole, seeds and peel-pulp significantly improved NGP (P<0.001) and ME (P<0.01) availability in comparison to control group whereas partitioning factor (PF; P>0.578), digestibility of NDF (P=0.905) and true OM (P=0.228) remained unaffected between different test groups. However, TVFA production, acetate, propionate and butyrate concentration was observed lower (P<0.001) in peel-pulp and seed supplemented group. From above results it can be concluded that supplementation of *Citrullus colocynthis* fruit at 1% level of substrate had potential to improve rumen fermentation characteristics and reduce methanogenesis, however *in vivo* assessment on ruminants need to be conducted to evaluate the persistency of effects along with health concerns.

Key words: *Citrullus colocynthis*, Feed additive, Methanogenesis, Rumen fermentation

Designing of nutritive strategies to modulate ruminal fermentation, to improve fibre utilization, to reduce methane emission and nitrogen excretion by the potential addition of distinct plants or extracts rich in secondary compounds to animal feeds (Rira et al. 2015, Hundal et al. 2019 a & b) is a hot topic among researchers. But effects of plant secondary metabolites (tannins, saponins, essential oils, flavonoids etc.) on methanogenesis and rumen function are variable and source dependent (Patra et al. 2011, Hundal et al. 2019a). Therefore, there is need to explore new herbal feed additives (HFA) to assess their potential.

The *Citrullus colocynthis*, which is commonly known as colocynth/bitter apple, belonging to the *Cucurbitaceae* family, is widely distributed in the Sahara and Arabian deserts, Sudan and Southern part of Asia including all over India, Pakistan and Southern Islands (Hussain et al. 2014).

On cultivation, air dried fruit yield of 120 to 150 q/ha and a seed yield of 450 kg/ha can be obtained by better management practices (Yadav ND and Singh PM 1992). *Citrullus colocynthis* traditionally used as medicinal plant because of presence of a variety of bioactive components, grouped as glycosides, flavonoids, alkaloids, carbohydrates, fatty acids and essential oils (Sagar R and Dumka VK 2018) but its potential to be used as herbal feed additive in ruminants still remained un-explored. Therefore, keeping in view the above background, the present study was designed to assess the potential of *Citrullus colocynthis* fruit as a whole, its seeds and peel-pulp supplementation on rumen fermentation characteristics at different levels (0%, 0.5%, 1%, 1.5%, 2%, 2.5%, 4%) using conventional total mixed ration as substrate in *in vitro*.

MATERIALS AND METHODS

The study was carried out at Department of Animal Nutrition, Guru Angad Dev Veterinary and Animal Science University, Ludhiana (30°5′42″ 62 2 N, 75°48′2 162 2 E, 240 m above sea level) in 2019.

Sample preparation and analysis: Unripen but fully developed fruits of *Citrullus colocynthis* were hand-picked...
and divided into three parts. One part manually cut into small pieces and placed in a hot air oven (Narang Scientific works, New Delhi) at 65°C for drying. Seeds of fruits were embedded in white spongy pulp. After removal of pericarp by peeling from the second part, seeds were removed manually from pulp and similarly dried in the oven. From the third part of fruits, after removal of seed and juice, remained leftover (peel and pulp) was dried in oven. The dried and finely grounded feed and Citrullus colocynthis samples were analysed for bioactive components, proximate principles DM, CP, EE and total ash, N (AOAC 2007) and fibre fractions (Van Soest et al. 1991).

In vitro evaluation: The effect of supplementing whole colocynthis fruit, its seed or peel-pulp at 0.5, 1.0, 1.5, 2.0, 2.5 and 4.0% of the diet having roughage to concentrate ratio 65:35 were carried out by in vitro gas production technique (Menke and Steingass 1988). Rumen fistulated male buffaloes were maintained on standard diet (65 parts roughage: 35 parts concentrate) as per ICAR (2013) was used as a donor for rumen liquor. The roughage portion was made up of wheat straw and green berseem in 70:30 ratio, while the conventional concentrate was made up of maize (15), wheat (15), de-oiled mustard cake (15), mustard cake (10), soybean meal (10), rice bran (15), de-oiled rice bran (16), urea (1), salt (1) and mineral mixture 2% each.

Rumen liquor was collected from fistulated animals maintained on conventional diet at the farm before feeding at 0900 h in a thermost flask flushed with CO₂ and maintained at 39°C. The rumen contents were blended for 2–3 min in a blender and strained through four-layers of muslin cloth. The solution, containing 960 ml distilled water, 0.16 ml micro-mineral solution, 660 ml bicarbonate buffer, 330 ml macro-mineral solution and 1.6 ml resazurin (0.1%) were mixed in a Woulff flask with magnetic stirrer in a water bath at 39°C. The mixture was continuously flushed with CO₂. Then strained rumen liquor was added to the buffer media in the ratio of 1:2. Different herbs were added to 100 ml calibrated glass syringes (Haberle Labortecnich, Germany) containing 375 mg complete feed (as percent over 375 mg) with buffered rumen fluid. Blank and sample of standard hay were run in triplicate with each set. If the volume of gas in the syringe exceeded 70 ml after 8 h the volume was recorded and the gas was expelled (Menke et al. 1979; Menke KH and Steingass H 1988). After 24 h, the volume of gas produced in each syringe was recorded and the content of syringes were transferred to spout-less beaker, boiled with neutral detergent solution for assessing the true OM and NDF digestibility. For methane estimation, 200 mg of substrate was incubated for 24 h with buffered rumen liquor in duplicate. After the stipulated period, total gas production was measured. A 50/50 mixture of CH₄ and CO₂ (Spancan; Spantech Products Ltd., England) was used as a standard for estimation of methane with Netchrom 9100 gas chromatograph. The VFAs (Cottyn BG and Boucque CV 1968) and ammonia (AOAC 2007) were estimated and ME (Menke et al. 1979) value be worked out.

Statistical analysis: The data were subjected to analysis of variance (ANOVA) 3×7 factorial design by using SPSS (2012) software version 20.0, taking different part of Citrullus colocynthis as one factor and their level as second factor. The means were tested for significant difference by using Tukey’s b test. The statistical model used was:

\[ Y_{ijk} = \mu + P_i + L_j + IP\times L + e_{ijk} \]

where, \( Y_{ijk} \) the kth observation on the parameter; \( \mu \), population mean; \( P_i \), Effect of ith part of Citrullus colocynthis (whole fruit, seed or peel-pulp); \( L_j \), Effect of jth level (0.5%, 1%, 1.5%, 2%, 2.5%, 4%); IP×L, Effect of ith part of Citrullus colocynthis at jth level; \( e_{ijk} \), Error.

RESULTS AND DISCUSSION

Chemical composition of different parts of Colocyn and substrate: The Citrullus colocynthis fruit contained 8.75% total ash, 17.15% crude protein (CP), 12.15% ether extract (EE), 29.4% cellulose, 55.3% neutral detergent fiber (NDF), 45.8% acid detergent fibre (ADF) and 15.7% acid detergent lignin (ADL) on dry matter basis whereas its peel-pulp and seed contained 16.0 and 2.35% total ash, 12.9 and 21.95% CP, 8.0 and 14.8% EE, 23.8 and 30.4% cellulose, 38.4 and 63.7% NDF and 32.2 and 45.3% ADF, respectively. Earlier studies observed that protein, fat and ash content varied from 13.19 to 26.86%, 14.48 to 24.62% and 2.00 to 4.46%, respectively in seeds of Citrullus colocynthis (Sadou et al. 2007) whereas, as per NRC (2006), the seed kernel contained about 50% oil, 30% protein, 10% carbohydrate, 4% ash and 3% fiber. The chemical analysis of total mixed ration (% DM) which was used as substrate for in vitro analysis had shown 21.45% CP, 3.35% EE, 20.5% cellulose, 57.4% NDF and 26.1% ADF content.

Phytochemical screening of different parts of Colocyn for bioactive components (Table 1) revealed that total phenols, non-tannin phenols, true tannins and aqueous saponin content were highest (\( P<0.001 \)) in Citrullus colocynthis fruit as a whole in comparison to peel-pulp and seed whereas peel-pulp of Citrullus colocynthis was rich (\( P<0.01 \)) in total flavonoids and vitamin C in comparison to seed however values remained at par with whole fruit. Methanolic extraction of whole fruit, peel-pulp and seed of Citrullus colocynthis revealed that methanol soluble saponin content remained comparable (\( P=0.05 \)) among different

| Parameter         | Whole (%) | Peel-pulp (%) | Seed (%) | P value |
|-------------------|-----------|---------------|----------|---------|
| Total phenolics *** | 1.15a     | 1.91b         | 1.27b    | 0.000   |
| Non tannin phenols *** | 0.35b   | 0.53b         | 0.16b    | 0.000   |
| True tannins ***   | 0.79a     | 1.37c         | 1.11b    | 0.000   |
| Vitamin C ***      | 0.076b    | 0.078b        | 0.014b   | 0.005   |
| Flavonoids **      | 0.15b     | 0.14b         | 0.11a    | 0.003   |
| Aq. saponin***     | 0.604b    | 0.861c        | 0.373a   | 0.000   |
| Meth. Saponin      | 5.20      | 5.63          | 5.33     | 0.292   |
| Sugars***          | 3.49b     | 5.40b         | 1.17a    | 0.000   |

Figures with different superscripts in a row differ significantly, ***\( (P<0.001) \), **\( (P<0.01) \).
parts. Earlier, Kumar et al. (2008) reported that seeds of *Citrus colocynthis* contained 0.74% total phenols and 0.13% flavonoids on DM basis.

**Level of Citrus colocynthis supplementation and rumen fermentation characteristics, irrespective of its part:** Results of the *in vitro* studies indicated that varying level of *Citrus colocynthis* had significant effect on rumen fermentation characteristics when TMR was used as substrate (Table 2). The NGP (mL/24/g) was significantly (P<0.001) higher at 1% level of supplementation as compared to control whereas it remained comparable with 0.5 and 1.5% supplementation levels. The findings are in line with Singh et al. (2018) who reported higher (P<0.05) NGP on supplementation of tannin and saponin containing herbs to total mixed ration at increasing levels. No difference in the digestibility of NDFD and true OM was observed at varying levels of *Citrus colocynthis*, however depression in digestibility was observed at 4.0% level, which might be due to the cumulative effect of tannins, saponins and flavonoids or their interaction with rumen microbes at comparatively higher concentration. Contrary to present findings, Hundal et al. (2016a) observed reduced digestibility of NDF and true OM significantly (P<0.01) in the tannin supplemented groups as compared to control groups at all levels, but remained comparable within the tannin supplemented groups. Similarly, Patra et al. (2006) noted that addition of extract of plants containing tannin reduced DM and true OM digestibility by about 7% in

Table 2. Effect of supplementation of different levels of *Citrus colocynthis* fruit on *in-vitro* fermentation characteristics, irrespective of its part

| Parameter | Control | Level of *Citrus colocynthis* fruit (% DMB) | P value |
|-----------|---------|-------------------------------------------|---------|
| **Fermentation parameters** | 0.5 | 1.0 | 1.5 | 2.0 | 2.5 | 4.0 |
| NGP, mL/24 h/g*** | 125.7a | 136.8ab | 142.9b | 138.6ab | 135.7a | 135.4a | 132.3a | 0.000 |
| NDFD, %** | 39.02ab | 38.6ab | 43.8b | 42.8ab | 40.91ab | 40.62ab | 37.72a | 0.005 |
| TOMD, %** | 63.2ab | 60.6a | 64.8b | 64.0ab | 62.8ab | 62.5ab | 60.4a | 0.006 |
| PF, mg/ml | 1.50 | 1.31 | 1.35 | 1.78 | 1.89 | 1.43 | 2.79 | 0.377 |
| ME, MJ/kg DM*** | 7.53a | 7.81bc | 7.97c | 7.85bc | 7.86bc | 7.80bc | 7.68bc | 0.000 |
| CH₄, % of NGP*** | 35.5c | 33.4b | 31.7a | 31.5a | 31.5a | 31.2a | 30.5a | 0.000 |
| CH₄, mL/g DM*** | 44.5bc | 45.7d | 45.4cd | 43.7bc | 42.6b | 42.2b | 40.1b | 0.000 |
| NH₃-N, mg/dl * | 0.025b | 0.021a | 0.022a | 0.021a | 0.022a | 0.023ab | 0.0212a | 0.044 |
| **Volatile fatty acid production, mM/dl** | | | | | | | | |
| TVFA* | 6.77b | 6.31ab | 6.56ab | 6.30ab | 6.12b | 6.10ab | 5.91a | 0.016 |
| Acetate* | 4.48b | 4.12ab | 4.31ab | 4.18ab | 4.06ab | 4.11ab | 3.80a | 0.046 |
| Propionate* | 1.81b | 1.61a | 1.65a | 1.60a | 1.53a | 1.54a | 1.52a | 0.013 |
| Butyrate | 0.286 | 0.272 | 0.266 | 0.258 | 0.252 | 0.242 | 0.237 | 0.088 |
| A:P | 2.47 | 2.56 | 2.62 | 2.61 | 2.66 | 2.67 | 2.50 | 0.052 |

Figures with different superscripts in a row differ significantly, *** (P<0.001), **(P<0.01), * (P<0.05).

Table 3. Effect of supplementation of different parts of *Citrus colocynthis* fruit on *in vitro* fermentation characteristics, irrespective of its level and their interaction with level

| Parameters | Control | Part of *Citrus colocynthis* fruit (% DM) | P value | Parts of *Citrus colocynthis* × Level |
|------------|---------|------------------------------------------|---------|-------------------------------------|
| **Fermentation parameters** | Whole | Peel-pulp | Seed | | |
| NGP, mL/24 h/g** | 125.7a | 133.3b | 135.8b | 139.6b | 0.001 | * |
| NDFD, % | 39.02 | 40.8 | 41.5 | 40.5 | 0.905 | NS |
| TOMD, % | 63.1 | 63.1 | 63.2 | 61.3 | 0.228 | NS |
| PF, mg/ml | 1.50 | 2.24 | 1.60 | 1.54 | 0.578 | NS |
| ME, MJ/kg/DM*** | 7.53a | 7.78b | 7.83b | 7.88b | 0.005 | NS |
| CH₄, % of NGP*** | 35.5c | 31.6a | 32.2a | 31.0a | 0.000 | NS |
| CH₄, mL/g DM*** | 44.5 | 43.8 | 43.4 | 43.06 | 0.616 | NS |
| NH₃-N, mg/dl ** | 0.025b | 0.022a | 0.021a | 0.022b | 0.008 | *** |
| **Volatile fatty acid production, mM/dl** | | | | | | |
| TVFA*** | 6.77b | 6.59b | 5.97a | 6.09b | 0.000 | * |
| Acetate*** | 4.48b | 4.42b | 3.94a | 3.93 | 0.000 | NS |
| Propionate*** | 1.81b | 1.67b | 1.52a | 1.54a | 0.000 | NS |
| Butyrate*** | 0.285b | 0.274b | 0.234a | 2.55ab | 0.000 | NS |
| A:P | 2.47 | 2.65 | 2.60 | 2.56 | 0.092 | NS |

Figures with different superscripts in a row differ significantly, *** (P<0.001), **(P<0.01), * (P<0.05).
comparison to control in in vitro. The difference may be attributed to the variability in type, dose and source of plant secondary metabolites used in two studies.

The availability of ME (MJ/Kg DM) followed the trend of NGP and reported (P<0.001) higher at 1.0% level of Citrullus colocynthis supplementation in TMR. In present study, the partitioning factor—an index for efficiency of utilization of organic matter remained statistically (P>0.377) comparable at all inclusion levels of herb and is supported by findings of Hundal et al. (2016 a&b).

Methane production (CH₄ % NGP) which indicates loss of energy, reduced significantly (P<0.001) on supplementation of colocynth at all levels in comparison to control but remained at par among different treatment groups. Hundal et al. (2016a) had reported similar effects with increasing tannin level supplementation on methane production. Hariadi BT and Santoso B (2010) also reported that CH₄ production decreased with increasing concentration of total tannin in plants. The positive or negative effects of tannin on CH₄ production may vary depending on the amount of tannin. The condensed tannins reduce methane production from ruminants either indirectly through a reduction in fibre digestion, which decreases H₂ production or directly through inhibition of the growth of methanogens (Hundal et al. 2019a).

The NH₃-N production was reduced (P<0.05) on inclusion of herbs (0.5–4%) comparable to control and the findings are in agreement with Hundal et al. (2016a) who also reported depressed NH₃-N at 3 to 5% level of supplementation of tannins to the substrate. Irrespective of the part of colocynth supplementation, total VFA production and acetate were found at par among control and test levels however significant depression in both (P<0.05) was observed at 4.0% supplementation level.

Supplementation of Citrullus colocynthis fruit, seeds or peel-pulp and rumen fermentation characteristics, irrespective of its level: The supplementation of Citrullus colocynthis fruit or its parts led to significant increase in NGP (P<0.001) and ME (P<0.01) availability in comparison to control group. No significant effect of inclusion of colocynth with respect to PF (P>0.578), digestibility of NDF (P>0.905) and total OM (P>0.228) were observed among different treatment groups (Table 3). Supplementation of different parts of colocynth resulted in significant (P<0.001) depression in methane production from 9% (peel-pulp group) to 12.7% (seed group) as compared to control group. Earlier researchers opined that tannins and saponins content in plants inhibited ruminal protozoa and methanogens which might be related with decrease in methanogenesis in in vitro studies (Makkar et al. 1995, Hristov et al. 2003, Patra AK and Saxena J 2011). Moreover, Oskoueian et al. (2013) observed the inhibitory effect of flavonoids on total protozoa and methanogens as well as ruminal methane production. Citrullus colocynthis fruit, seeds and peel-pulp used in treatment groups contained varying concentration of tannins, saponins, flavonoids and vitamin C, which individually or interactively led to reduction in methanogenesis in the present study.

Ruminal NH₃-N concentration was reported lower (P<0.01) in Citrullus colocynthis fruit or peel-pulp supplemented group whereas values remained statistically analogous in control and seed supplemented group which may resulted from increase in incorporation of ammonia for synthesis of microbial protein. The microbial protein synthesis efficiency or better synchronization of nutrients in the presence of tannins improved microbial protein synthesis in rumen (Makkar et al. 1995).

TVFA production, acetate, propionate and butyrate concentration was observed lower (P<0.001) in peel-pulp and seed supplemented group in comparison to control and whole fruit supplemented group, however acetate to propionate ratio remained unaffected between different test groups (Table 3). The lower levels of TVFA and acetate in groups supplemented with peel-pulp and seed might be due to inhibitory effect on rumen microbes as colocynth has cucurbitacins which exhibited potential antibacterial activity (Hussain et al. 2014).

Interaction between level and different parts of Citrullus colocynthis fruit: Interactions between level × different parts of Citrullus colocynthis fruit w.r.t. fermentation characteristics are presented in Table 3. It was observed that interactions between different parts of Citrullus colocynthis fruit (fruit as a whole, seeds or peel-pulp) at different levels (0%, 0.5%, 1%, 1.5%, 2%, 2.5% or 4%) of supplementation had significant influence on NGP (ml/24 h/g), NH₃-N (mg/dl) and TVFA (mM/dl) in in vitro evaluation. The findings are in line with Singh et al. (2018) who reported strong interaction between type of herb and its level w.r.t. to various fermentation characteristics, however contrary to the results of Singh et al. (2018), the interaction between herb and its level didn’t influence ME availability, methane production, fermentation efficiency, digestibility of NDF and TOM in our study. The difference observed may be attributed to the type of herb, concentration of bioactive components and their interaction with each other or with rumen microbes during in vitro incubation.

From this study it is concluded that the addition of Citrullus colocynthis fruit had potential to improve rumen fermentation characteristics and in vitro methanogenesis however the best response with respect to the net gas production, digestibility of nutrients, methane production and ME availability from TMRs was observed at 1% level of supplementation but in vivo assessment on ruminants need to be conducted to evaluate the persistency of effects along with health concerns.

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