# Phenolic fraction-specific effects of tropical plants on *in vitro* ruminal methane production and fermentation characteristics

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**Abstract.** This study evaluated how plants with different dominant phenolic fractions affect in vitro ruminal fermentation characteristics and methane production. Three phenolic-rich species were tested: *Swietenia mahagoni* (high condensed tannins), *Clidemia hirta* (high hydrolysable tannins), and *Eugenia aquea* (high non-tannin phenolics). Each was incubated alone or mixed with *Carica papaya* (CP), a low-phenolic substrate. A 24 h in vitro rumen fermentation assay was conducted to measure total gas production, organic matter digestibility, methane output, microbial population, volatile fatty acid (VFA) profiles, and ammonia concentration. Data were analyzed using two-way ANOVA followed by Duncan's test (n = 4 per treatment). Mixing phenolic-rich plants with CP significantly reduced methane emissions ($P < 0.05$), with CP + S. mahagoni showing the greatest reduction (18.6%). This effect was likely associated with tannin–nutrient complex formation, which modulated rumen microbial activity, supported fibre fermentation and VFA production, and limited protein degradation for ammonia synthesis ($P < 0.05$). The strong negative relationship between total phenols or tannins and methane output highlights the important role of phenolic compounds in regulating rumen fermentation. These findings suggest that tannin-rich plants are promising natural feed additives for mitigating enteric methane emissions in ruminants.

## 1 Introduction

Modern livestock production objectives have shifted beyond productivity alone toward a more system-based perspective that also considers environmental sustainability, product quality, and animal welfare. From an environmental standpoint, livestock (particularly ruminants) are recognized as a substantial source of the greenhouse gas methane, thereby contributing to global warming [1]. Methane emissions are not only an environmental concern; they also reflect a measurable loss of dietary energy, estimated up to a maximum of

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6–10% of gross energy intake, which equals 8–14% of digestible energy intake [1, 2]. Consequently, numerous methods have been developed to lower methane production in ruminant systems [3].

Ruminal methane emissions could be decreased by using plants rich in phenolic compounds as a feed additive in diets, as reported in many both *in vitro* and *in vivo* evaluations [4-6]. Nevertheless, it remains unclear whether these methane-reducing effects persist when phenolic-rich plants are combined with forages that contain negligible amounts of phenolics. In addition, because phenolics comprise chemically diverse groups of compounds, evidence comparing methane mitigation across plants with different phenolic fractions remains limited. Therefore, this study investigated these issues using *Swietenia mahagoni* (SM; containing high condensed tannins), *Clidemia hirta* (CH; containing high hydrolysable tannins), and *Eugenia aquea* (EA; containing high non-tannin phenolics), tested in mixtures with *Carica papaya* (CP), a low-phenolic forage.

## 2 Materials and methods

### 2.1 Plant materials

All plant materials used in this experiment, including *Swietenia mahagoni* (SM), *Clidemia hirta* (CH), *Eugenia aquea* (EA), and *Carica papaya* (CP), were obtained from Bogor, West Java, Indonesia, where it is common for rural communities to utilize as ruminant feed resources and traditional veterinary medicinal plants in various parts of the country.

### 2.2 Sample preparation

Approximately 3 kg of fresh material from each species was harvested. Soon after collection, samples were air-dried in a greenhouse for 2 days, followed by oven-drying overnight at 50°C. Dried materials were then ground to pass through a 1-mm sieve. The samples were subsequently analyzed for their chemical composition, including fraction of phenolic, proximate components, also fiber fractions by Van Soest's.

### 2.3 *In vitro* fermentation procedure

*In vitro* incubation was carried out using a rumen-buffer mixture according to the procedure described by Menke and Steingass [7]. Each plant was incubated either as a single substrate or in a 1:1 (w/w) mixture with a control substrate containing negligible phenolics. Incubated fluid was prepared in a Hohenheim gas test syringe from 200 mg dry matter of sample, which was poured in mixture of 10 mL rumen fluid and 20 mL buffer solution. Soon, each mix-fluid were incubated for 24h at 39°C. Two ports of the syringes were used to fill and remove the liquid phase and to collect the gas samples from other port (i.e., methane).

### 2.4 Measured variables

Variables that were evaluated at the end of incubation included total gas production, organic matter digestibility (OMD), and methane output. The method is determined as described in Anuraga *et al.* [4]. The volatile fatty acids (VFA) and ammonia ($NH_3$) concentrations were analyzed from the liquid residue of fermentation, and it could be used to quantify bacterial and protozoal populations by using Fuchs-Rosenthal counting chamber as explained in Tilley and Terry [8].

### 2.5 Data analysis

Treatments were assigned to experimental units using a randomized complete block design and performed in 4 replicates per treatment represented in one syringe each run (n = 4 per treatment). The rumen inoculum used in each run served as the blocking factor. Data were analyzed using two-way analysis of variance (ANOVA). Duncan's multiple range test was used to evaluated differences among means that show a significant effect ($P<0.05$) for the given variable.

## 3 Result and discussion

In terms of phenolic composition, SM was predominantly characterized by condensed tannins (41.6% of TP), CH was dominated by hydrolysable tannins (93.5% of TP), and EA contained mainly non-tannin phenolics (60.4% of TP). The compounds of each plant are shown in Table 1.

**Table 1.** Total phenolic and total tannin compounds of *Carica papaya, Swietenia mahagoni, Clidemia hirta,* and *Eugenia aquea*.

| Phenolic Fraction | CP | SM | CH | EA |
|---|---|---|---|---|
| Total phenolic (% dry matter) | 2.5 | 20.7 | 21.6 | 16.9 |
| Total tannin (% dry matter) | 0.8 | 13.8 | 21.2 | 6.7 |

CP: *Carica papaya*; SM: *Swietenia mahagoni*; CH: *Clidemia hirta*; EA: *Eugenia aquea*.

Mixing each phenolic-containing plant with CP significantly reduced 24.7% (SM), 23.1% (CH), and 13.4% (EA) methane output compared with CP alone ($P<0.05$; Table 2). These results are consistent with earlier reports demonstrating the potential of plant phenolics to mitigate methane [4, 9]. Such reductions may arise from decreased nutrient digestibility ($P<0.05$), direct suppression of methanogenic archaea, and defaunation effects on protozoa that host symbiotic methanogens [10]. The addition of phenolic-rich plants likewise reduced ruminal digestibility due to complexes between tannin and fiber or protein that could pass the rumen leading to addition of nutrient digestibility in intestine. Many studies indicate that high tannin in ruminant diets may be toxic and it should be used in levels up to 2-3% depending on the plant compounds [5, 11]. In line with this, gas production declined significantly ($P<0.05$) when CP was mixed with SM, CH, or EA than CP individually. The findings further indicate that plants dominated by condensed or hydrolysable tannins exerted stronger methane suppression than those rich in non-tannin phenolics [12]. The associations between plant total phenol (TP) or total tannin (TT) concentrations and *in vitro* ruminal $CH_4$ production are shown in Figure 1.

**Table 2.** The output of total gas, OMD (organic matter digestibility), methane emissions, and number of rumen microbial by *in vitro* incubated evaluation for each treatments.

| Plant | Total gas ($mL\ g^{-1}$) | OMD ($mg\ g^{-1}$) | $CH_4$ ($mL\ L^{-1}$ gas) | $CH_4$/OMD ($mL\ g^{-1}$ DM) | Bacteria ($cell\ mL^{-1}$) | Protozoa ($cell\ mL^{-1}$) |
|---|---|---|---|---|---|---|
| CP | $220^{e}$ | $769^{f}$ | $186^{e}$ | $53.5^{e}$ | $2.8 \times 10^{9}$ | $25,100^{bc}$ |
| SM | $111^{b}$ | $435^{c}$ | $94^{a}$ | $24.2^{a}$ | $2.5 \times 10^{9}$ | $15,800^{a}$ |
| CH | $106^{b}$ | $417^{b}$ | $115^{b}$ | $29.1^{b}$ | $2.2 \times 10^{9}$ | $19,100^{ab}$ |
| EA | $54^{a}$ | $296^{a}$ | $117^{b}$ | $21.8^{a}$ | $2.0 \times 10^{9}$ | $31,600^{c}$ |
| CP + SM | $162^{d}$ | $595^{e}$ | $140^{c}$ | $38.3^{c}$ | $3.0 \times 10^{9}$ | $25,700^{bc}$ |
| CP + CH | $165^{d}$ | $596^{e}$ | $143^{c}$ | $39.7^{cd}$ | $2.5 \times 10^{9}$ | $17,000^{ab}$ |
| CP + EA | $138^{c}$ | $532^{d}$ | $161^{d}$ | $41.9^{d}$ | $2.6 \times 10^{9}$ | $17,800^{ab}$ |
| P-value | <0.001 | <0.001 | <0.001 | <0.001 | 0.338 | 0.008 |

OMD: organic dry mattter; CP: *Carica papaya*; SM: *Swietenia mahagoni*; CH: *Clidemia hirta*; EA: *Eugenia aquea*.

Bacterial populations were no different significantly (P>0.05) among plant treatments and remained within normal safe range (9-10 log CFU/mL [13]). However, SM and CH significantly reduced (P<0.05) protozoal populations due to the formation of protective feed particles, particularly tannin-fiber complexes which limit the availability of degradable substrates for protozoa [6]. The suppression in protozoa abundance subsequently decrease the supply of $H_2$ and $CO_2$ required for methanogenesis [10, 14], thereby contributing to methane mitigation via hydrogenases in bacteria, protozoa, and fungi which linked with ruminal VFA metabolism. It also decreased risk of ruminal acidosis. It proves by decreasing number of volatile fatty acid (VFA) due to tannin-fiber complexes compared between all combination of phenolic plants, with only CP (P<0.05; Table 3). Consequently, only CP maintained total VFA production within the recommended physiological range (70–130 mM) [14].

**Table 3.** Total VFA, acetate, propionate, butyrate, ratio of acetate and propionate, and ammonia production of the incubated experimental plants.

| Plant | Total VFA (mM) | $C_2$ (mM) | $C_3$ (mM) | $C_4$ (mM) | $C_2$:$C_3$ | $NH_3$ (mM) |
|---|---|---|---|---|---|---|
| CP | $73.9^{e}$ | $51.6^{e}$ | $12.7^{d}$ | $6.7^{d}$ | $4.15^{a}$ | $28.0^{d}$ |
| SM | $50.8^{b}$ | $38.3^{b}$ | $8.1^{b}$ | $3.4^{a}$ | $4.73^{b}$ | $8.3^{a}$ |
| CH | $49.7^{b}$ | $36.4^{b}$ | $7.8^{b}$ | $4.5^{b}$ | $4.71^{b}$ | $8.9^{a}$ |
| EA | $42.7^{a}$ | $32.0^{a}$ | $6.1^{a}$ | $3.8^{a}$ | $5.24^{c}$ | $11.0^{ab}$ |
| CP + SM | $62.3^{d}$ | $45.5^{d}$ | $10.1^{c}$ | $5.3^{c}$ | $4.49^{ab}$ | $13.5^{bc}$ |
| CP + CH | $59.8^{cd}$ | $43.2^{cd}$ | $10.0^{c}$ | $5.2^{c}$ | $4.34^{ab}$ | $14.0^{bc}$ |
| CP + EA | $57.9^{c}$ | $41.9^{c}$ | $9.3^{c}$ | $5.1^{c}$ | $4.51^{ab}$ | $15.8^{c}$ |
| P-value | <0.001 | 0.001 | <0.001 | <0.001 | 0.003 | <0.001 |

$C_2$: acetate ;$C_3$: propionate; $C_4$: butyrate; VFA: volatile fatty acid; CP: *Carica papaya*; SM: *Swietenia mahagoni*; CH: *Clidemia hirta*; EA: *Eugenia aquea*.

Consistent decreases were observed in $C_2$, $C_3$, and $C_4$ concentrations (P<0.05), which are mainly produced by fibrolytic, amylolytic, and saccharolytic bacteria. Tannins inhibit these microbial groups by restricting fiber accessibility as a fermentable substrate [4, 6]. Acetate and butyrate serve as key precursors for VFA synthesis, whereas propionate is the principal substrate for gluconeogenesis.

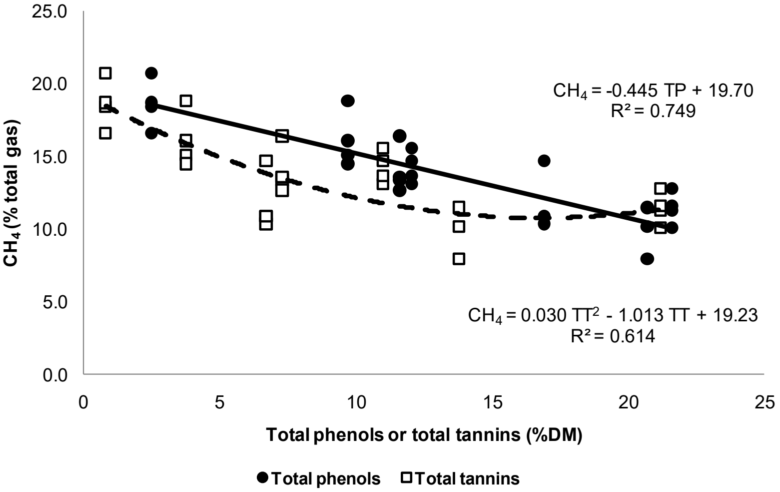

**Fig. 1.** Relation of Total Phenol (TP) and Total Tannin (TT) contents in plants with ruminal CH4 emissions *in vitro* (% total gas)

Ruminal $NH_3$ concentration declined significantly ($P<0.05$; Table 3), reflecting enhanced microbial utilization of ammonia for protein synthesis and limiting ruminal proteolysis. The standard safe range ammonia concentration in rumen around 7.7 to 17.3 mM [15]. This suggests adequate rumen protein protection and improved bypass protein supply, due to tannin strong affinity for dietary protein and fiber [11]. Tannin supplementation appears to sort rumen-undegradable protein, thereby reducing the activity of ammonia- producing microorganisms.

Figure 1 demonstrates a negative relationship between phenolic compounds and *in vitro* methane ($CH_4$) production. Total phenol content exhibited a stronger and linear negative correlation ($R^2= 0.749$) with $CH_4$ production compared with total tannin content, indicating that phenolic fractions consistently suppress methanogenesis, potentially through direct inhibition of methanogenic microorganisms, reduced hydrogen availability, or shifts in rumen fermentation pathways [9]. In contrast, total tannin content showed a quadratic relationship ($R^2= 0.614$) with $CH_4$ production, suggesting a level-dependent effect. Low to moderate tannin levels effectively reduced $CH_4$ emissions, whereas higher concentrations resulted in a plateau effect. This response may be influenced by tannin concentration, chemical composition, molecular structure, or the presence of non-tannin phenolic compounds during rumen fermentation [12].

## 4 Conclusion

Phenolic-rich plants, either condensed or hydrolysable tannins, decreased *in vitro* ruminal methane production, followed by lower nutrient digestibility, ruminal microbe population, VFA, and ammonia concentration compared than plant rich in non-tannin phenolics. Tannin could bind protein and fiber that reduce supply of substrates for microbes. It affected to ruminal nutrient digestibility and ability of microbial function to produces VFA which produce $H_2$ for methanogenesis. The tannin-protein complex also increases protein bypass thereby reducing ammonia production in rumen. The strong negative association between total phenol or total tannin and methane output further underscores the key role of phenolic compounds in modulating rumen microbial activity. These results indicate that targeted inclusion of phenolic-rich plants in diets of ruminant could be an effective strategy with nutrition-based to mitigate methane emissions while enhancing ruminal protein protection.

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