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# Research Article
# Study of *Hibiscus tiliaceus* Leaf Extract Carrier as Additive in the Diets for Fattening of Local Cattle (*in vitro*)

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## Abstract

**Objective:** The purpose of this study was to find the type of *Hibiscus tiliaceus* leaf extract carrier for improving the fermentation products and ruminal metabolism efficiency. **Methodology:** This study was designed according to completely randomized design with 3×2 factorial patterned and repeated three times. The first factor was the type of feed ingredients as carrier such as cassava waste meal, rice bran and rice straw ammoniated and the second factor was the doses of *Hibiscus tiliaceus* extract i.e., 0 and 200 ppm. **Results:** Analysis of variance showed that additional leaf extract of *H. tiliaceus* with various carrier materials (cassava waste meal, rice bran and rice straw ammoniated) did not affect ($p>0.05$) on the total protozoa, microbial protein synthesis, fermentation product and activity of hydrolytic enzymes in rumen liquid. However, additional *H. tiliaceus* leaf extract at 200 ppm tended to reduce total protozoa, but total VFA and the activity of rumen amylase, protease and cellulase increased. Feedstuff with higher capacity to reduce protozoa population and to increase VFA and enzyme activity was rice bran. Cassava waste meal, rice bran and ammoniated wheat straw can be used as carrier of ethanolic extracts of *H. tiliaceus* leaf. **Conclusion:** This study recommended that rice bran as carrier for *in vivo* application due to the defaunation effects of rumen protozoa and high hydrolytic enzymes activity in the rumen.

Key words: *Hibiscus tilaceus*, saponin, enzymes, beef cattle-rumen

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

Several types of organic acid and saponin are abundant in extract leaves or flowers as part of bioactive component (phytogenic component). Acamovic and Brooker[1] and Hashemi and Davoodi[2] declared that phytogenic is the result of secondary metabolite plants containing compounds with nutrition, without nutrition or antinutrition). Phytogenic or phytobiotic feed additive are products derived from plants for feed to improve performance. According to Karaskova *et al*.[3] phytogenic feed additives can be classified into several groups viz., sensory additives, technological additives, zootechnical additives and nutritional additives.

Bioactive components as fumaric acid and saponin found in *Hibiscus* sp. Olaleye[4] reported aquoeus-methanolic extract of *H. sabdariffa* containing flavonoid, saponin and alkaloids that potential to exhibited bacterial activity. Istiqomah *et al*.[5] informed that *H. rosanensis* are served defaunation agent in beef cattle and sheep. Our previous study found phytogenic components in the leaves of *Hibiscus tiliaceus* analysed by GC-MS informed that the main organic compounds are fatty acids and ester (31%), nitogenous compounds (18.28%) and quinoline (23%). Quinoline is an alkaloids and it has antiprotozoa and antioxidant activity. Oktora[6] reported that there were interaction ($p<0.01$) between forage: Concentrate ratio and supplemented *Hibiscus tiliaceus* leaf extract on total protozoa and rumen fermentation product *in vitro*. Decreasing of protozoa population, methane gas and total gas production achieved at dry matter ratio of rice straw ammoniation and concentrates of 55:45% for 58.21, 36.64 and 22.34%, respectively, while, propionic proportion was increased. Concentration of rumen N-NH3 increased due to the decreasing rumen microbial protein synthesis. However, utilizing *Hibiscus tiliaceus* leaf extract as commercial additive feed will meet obstacle in the mixing process with other feed component. Alternative carrier is therefor important for utilizable additive feed. Several prevalent concentrates for beef cattle are cassava waste meal and rice bran as energy source with different fermentability and degradability level in rumen. Accordingly, further investigation on its utilization as additive feed carrier to limit methane production is essential to improve the efficient rumen metabolism and beef cattle performance.

The objective of this study was to investigate the carrier of *Hibiscus tiliaceus* leaf extract in beef cattle feed (*in vitro*) to find the best feed material that improved the efficient rumen metabolism regarding the amount of protozoa, microbial protein synthesis, rumen fermentation product (VFA and $N\text{-}NH_3$) and the activity of rumen hydrolytic enzymes activity.

## MATERIALS AND METHODS

**Statistical analysis:** This experimental factorial-patterned research was subject to completely randomized design with three replicates. The obtained data were subject to analysis of variance, and in case of treatment effect on the measured variables, polynomial orthogonal test ensued.

**Ruminal fluid[7] and treatments:** Ruminal fluid for *in vitro* test was from three beef cattles in slaughter house soon after the beef cattle were slaughtered. The first factor was three kinds of energy feed (cassava waste meal, rice bran and ammoniated wheat straw) and the second was ethanol level of *Hibiscus tiliaceus* leaf extract, 0 ppm ($W_0$) and 200 ppm ($W_1$). Table 1 presents feed composition of the total six treatment combinations as follows:

$R_{1,2,3}W_0$ = (Cassava waste meal, rice bran and ammoniated wheat straw) without extract

$R_{1,2,3}W_1$ = $R_{1,2,3}W_0$+200 ppm ethanol extract of *Hibiscus tiliaceus* leaf

**Extract preparation:** *Hibiscus tiliaceus* leaf sample was obtained from coastal area in Cilacap and *Hibiscus tiliaceus* leaf extract was derived from extraction using ethanol solvent according to Wettasinghe *et al*.[8].

The measured and observed variables were microbial protein synthesis under modified gradual centrifugation by Makkar *et al*.[9]. The VFA product was measured with steam extraction technique and N-NH3 with Conway's micro diffusion method by Conway[10]. Total protozoa was calculated using Sedgewick Rafter Counting Chamber according to Ogimoto and Imai[11]. Walter method[12] with casein substrate was used to measure protease activity in rumen fluid. Amylase and cellulase activity used DNS solvent according to Miller[13] with starch substrate according to Bernfeld[14] and Whatman filter paper No. 1 according to Camassola *et al*.[15].

Table 1: Feed composition

| | Feed (%) | | |
|---|---|---|---|
| Feed material | A | B | C |
| Cassava waste meal | 45 | 45 | 45 |
| Coconut waste | 13 | 13 | 13 |
| Soybean waste | 10.5 | 10.5 | 10.5 |
| Rice bran | 20 | 20 | 20 |
| Pollard | 15 | 15 | 15 |
| Mineral mix | 1.5 | 1.5 | 1.5 |
| Salt | 1 | 1 | 1 |
| *Hibiscus tiliaceus* leaf extract (ppm) in carrier | 0 | 200 | 400 |

## RESULTS AND DISCUSSION

**Rumen protozoa:** Supplementing 0-220 ppm ethanol extract of *Hibiscus tiliaceus* leaf to cassava waste meal, rice bran and ammoniated wheat straw (JPA) did not have statistically significant affect (p>0.05) but tended to decrease rumen protozoa. The most protozoa decreased or 57.69% was in rice bran carrier, followed by cassava waste meal and JPA or 35.29 and 32%, respectively. Analysis of variance result showed that no interaction was found between extract dose with carrier (Table 2). The different decreasing rate of protozoa was assumedly due to the different fermentation characteristics of carrier feed in rumen although cassava waste meal and rice bran were energy feed. Cassava waste meal was more fermentable than rice bran but rice bran contained higher protein (3 vs 10%). Protein is macromolecule made of several types of amino acid, among which has positive and negative side-chain residue that was expected to bind bioactive component. The charged amino acid residue was assumed to bind bioactive in *Hibiscus tiliaceus* leaf extract to keep the bioactive components from rumen microbe degradation and to function more durably. Nevertheless, the detail mechanism remains unknown.

Protozoa decrease due to supplementing saponin or saponin-bearing feed was also reported in previous researches. Wang *et al.*[16] reported that tea saponins (TS) extracted from seeds, leaves or roots of tea plant have a lasting antiprotozoal effect. The TS decreased methanogen activity, it seems influenced the activity of the methanogens indirectly via the depressed ciliate protozoal population. Saponins or saponin like substances have been reported to suppress methane production, reduce rumen protozoa counts and modulate rumen fermentation patterns[17,18]. Wallace *et al.*[19] showed that saponin might kill or damage protozoa by forming sterol complex on the surface of protozoa membrane, causing disorder in membrane function and eventually perished. Wina *et al.*[20] stated that several reports however indicated no saponin effect on protozoa, while the others reported an increase effect. Teferedegne *et al.*[21] and Ivan *et al.*[22] informed that several types of protozoa negatively affected protozoa but not persistent after few days feeding. This condition was absent in bacteria cell. Klita *et al.*[23] explained that the susceptibility of rumen protozoa and insusceptibility of rumen bacteria against protozoa was due to cholesterol in eukaryotic membrane (including protozoa) but not found in prokaryotic of bacteria cells.

**Rumaen fermentation product:** Total VFA yield was not affected (p>0.05) by supplementation of *Hibiscus tiliaceus* leaf extract but tended to increase on rice bran carrier from 118-142.67 mM (Table 2). There are varying reports on the effects of saponins on ruminal Volatile Fatty Acid (VFA) concentrations, as digestibility of feeds varied among the studies. Guo *et al.*[24] and Pen *et al.*[25] reported that no difference in total VFA concentrations with the supplementation of saponins. Contrastively, Hess *et al.*[26] stated that saponin-enriched fruit plant additive to plant would lower VFA.

The $N\text{-}NH_3$ was not affected (p>0.05) by the supplemented *Hibiscus tiliaceus* leaf extract and carrier feed. Muetzel *et al.*[27] stated that additional saponin oftentimes failed to lower ammonia concentration because saponin did not obstruct protein feed degradation *in vitro*. On the other hand, several researchers reported that *Yucca schidigera* extract lowered 48% $N\text{-}NH_3$ while *Qulla jasaponaria* was only 21% compared to control[25]. It was further reported that *Yucca schidigera* contained two fractions, gliko and saponin. The $N\text{-}NH_3$ decrease might due to bondable gliko-fraction to ammonia, while saponin fraction indirectly affected toxin through ciliate protozoa[19]. Ammonia concentrate decreased in rumen particularly when protozoa growth was disrupted, might due to the decreasing bacteria lysis[25].

This result showed that saponin effect on fermentation product such as VFA and $N\text{-}NH_3$ was inconsistent with the previous findings, probably because the type of saponinin *Hibiscus tiliaceus* leaf extract was different from that in other plant extract. Patra and Saxena[28] reported that the inconsistent effect of saponin on rumen fermentation was related to saponin chemical structure and dose, feed compoisition, microbe community and microbiotic adaptation with saponin.

Table 2: Parameter of *in vitro* fermentation on the level of *Hibiscus tiliaceus* leaf extract with different carrier

| | Cassava wheat meal (ppm) | | Rice bran (ppm) | | JPA (ppm) | | Significance | | |
|---|---|---|---|---|---|---|---|---|---|
| Variables | 0 | 200 | 0 | 200 | 0 | 200 | BPP | DES | INT |
| Protozoa (cell $mL^{-1}$)×$10^4$ | 34±1.98 | 22±1.31 | 26±1.47 | 11±0.91 | 25±1.94 | 17±0.99 | TS | TS | TS |
| VFA (mM) | 148.67±17.93 | 149.33±8.33 | 118.00±5.29 | 142.67±13.01 | 139.33±11.72 | 126.67±8.33 | TS | TS | TS |
| $N\text{-}NH_3$ (mM) | 9.130±0.42 | 9.33±0.83 | 9.60±0.92 | 7.80±0.87 | 8.43±1.32 | 9.90±1.31 | TS | TS | TS |
| SPM (μg $mL^{-1}$) | 8.5133±1.58 | 9.3533±0.7223 | 9.2800±0.8416 | 9.3733±1.9335 | 3.9167±0.3691 | 5.0067±0.5972 | TS | TS | TS |
| Protein (mg $mL^{-1}$) | 0.073±0.0056 | 0.063±0.0091 | 0.097±0.0060 | 0.0700±0.0143 | 0.0810±0.0089 | 0.0680±0.0121 | TS | TS | TS |

JPA: Ammoniated wheat straw, BPP: Carrier feed, DES: Saponin extract dose, INT: BPP×DES interaction

Table 3: Parameter of *in vitro* fermentation (enzyme activity) on the level of *Hibiscus tiliaceus* leaf carrier with different feed carrier

| | Cassava waste meal (ppm) | | Rice bran (ppm) | | Ammoniated wheat straw (ppm) | | Significance | | |
|---|---|---|---|---|---|---|---|---|---|
| Variables | 0 | 200 | 0 | 200 | 0 | 200 | BPP | DES | INT |
| Cellulase (U mg$^{-1}$) | 14.02±1.06 | 15.77±2.54 | 10.51±0.63 | 17.35±3.98 | 12.41±1.19 | 15.17±3.51 | TS | TS | TS |
| Amylase (U mg$^{-1}$) | 2.92±0.78 | 4.92±0.99 | 2.38±0.17 | 4.28±0.77 | 3.12±0.51 | 4.82±1.15 | TS | TS | TS |
| Protease (U mg$^{-1}$) | 1.87±0.13 | 2.36±0.34 | 1.67±0.13 | 2.47±0.51 | 1.89±0.18 | 2.41±0.42 | TS | TS | TS |

JPA: Ammoniated wheat straw, BPP: Carrier feed, DES: Saponin extract dose, INT: BPP×DES interaction

Owens dan Bergen[29] stated that ammonia in rumen is derived from protein degradation, Non-Protein Nitrogen (NPN) and microbe lysis and tended to deplete after feeding due to the decreasing substrate and ammonia used by rumen microorganism. Table 2 shows that N-$NH_3$ level and Microbial Protein Synthesis (MPS) was not affected ($p>0.05$) by extract dose or the carrier. The MPS however tended to increase on ammoniated wheat straw as the extract dose increased, in contrast with protozoa decrease. If saponin killed bacteria, predator of bacteria would be depleted by protozoa so as increasing bacteria population and slowing down protein turnover[20]. Makkar and Becker[30] found the efficient *in vitro* microbial protein synthesis increased linear to *Quillaja* saponin supplementation (0.4-1.2 mg mL$^{-1}$) in hay substrate. Similar finding was reported by Makkar *et al.*[31] that saponin from *Quillaja*, *Yucca* and *Acacia auriculiformis* increased microbial biomass and efficient microbial protein synthesis.

Protein in ruminal fluid tended to decrease as the dose of *Hibiscus tiliaceus* leaf extract increased in several carriers, although not statistically significant ($p>0.05$). The decrease was due to the diminished protozoa population. Van Soest *et al.*[32] reported that protozoa contributed 10- 40% of total nitrogen in rumen. Williams and Coleman[33] found that protozoa weighed a half of microorganism biomass in rumen.

**Hydrolytic enzyme activity in rumen:** Analysis of variance showed that supplementing *Hibiscus tiliaceus* leaf extract and carrier did not significantly affect ($p>0.05$) enzyme activity. However, the activity of cellulase, amylase and protease tended to increase as the saponin dose increased, as observed in rice bran as carrier with the highest increase (Table 3). The increasing enzyme activity as the dose raised from 0-200 ppm was detailed as follows. Cellulase activity increased by 39% in rice bran carrier, followed by ammoniated wheat straw and cassava waste meal, 41 and 35%, respectively. Similarly, the highest protease increase was in rice bran carrier or 32% then in ammoniated wheat straw and cassava waste meal, 18 and 11%, respectively. Amylase activity increased by 44% in rice bran carrier, 41% in cassava waste meal and 35% in ammoniated wheat straw. Protease activity also increased, the highest was 31% in rice bran, followed by 22 and 20% in ammoniated wheat straw and cassava waste meal, respectively (Fig. 1). The difference was assumedly due

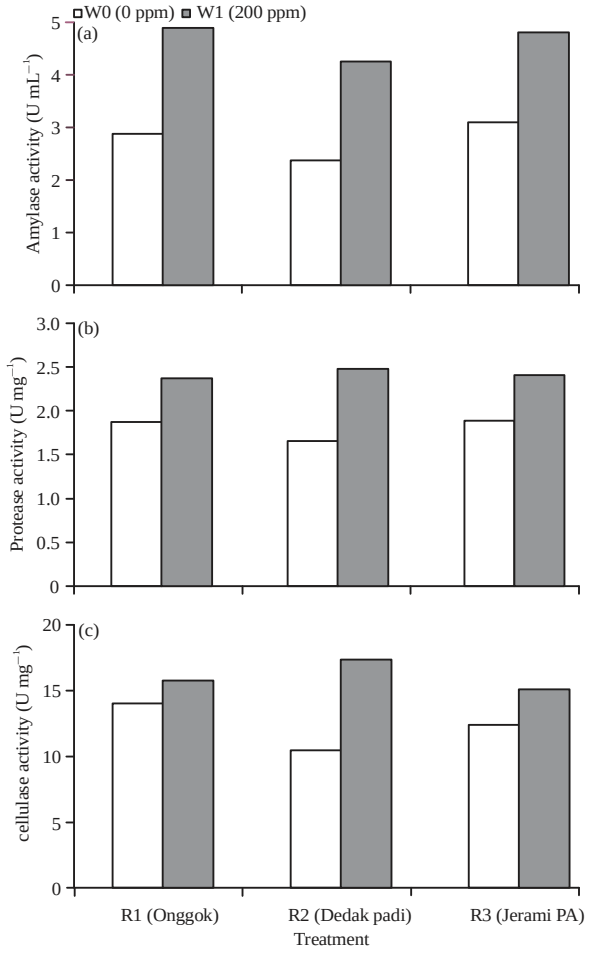


Fig. 1(a-c): Activity of (a) Amylase, (b) Protease and (c) Cellulase at 0 ppm and 200 *Hibiscus tiliaceus* leaf extract with different carrier feed

to chemical components and characteristics of degradable or fermentable feed in rumen.

The tendency of protease activity to increase was in line with Wina *et al.*[20] that while protease activity increased, deaminase and peptidase activity was not affected by the addition of *Yucca* extract in feed (alfalfa hay:barley was 1:1 b/b) in Rusitec system. *Yucca* extract did not limit deaminase activity or proteolytic activity in free cell ruminal fluid of beef cattle. Supplementing saponin from *Sesbani sesban* leaves did not affect the activity of proteolytic,

peptodolytic or deaminase in rumen. The ineffective saponin in proteolytic activity indicated that saponin was assumedly not affecting feed protein degradation in rumen.

The tendency of cellulase activity to increase also occurred (Fig. 1c) in several carrier feeds despite the absence of statistically significant effect ($p>0.05$). Muetzel[34] investigated saponin in *S. pachycarpa* leaves, its supplementation did not affect the activity of Carboxy methyl cellulase (CMCase) in Rusitec, meanwhile. Hristov *et al.*[35] reported yucca extract lowered CMCase, xylanase and amylase in Rusitec. The declining activity of xylanase or CMCase in rumen seemed to closely relate with the decreasing protozoa population rather than the decreasing fibrolytic microbe population There was significant correlation between protozoa amount with xylanase activity[20] which supported by Williams and Withers[36] that protozoa also excreted fibrolytic enzyme. Measuring cellulase activity in this research was using substrate filter paper Whatman No.1 containing cellulase. The different substrate and bioactive components in plant is assumed to cause different research result.

## CONCLUSION

Rice bran and cassava waste meal were viable saponin extract carrier from *Hibiscus tiliaceus* leaf powder. It is however recommended to use rice bran because of the better defaunation effect and enzyme activity than cassava waste meal. Further *in vivo* test is essential to investigate the effect on ruminant productivity.

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