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# SUPPLEMENTATION EFFECTS OF TANNIN AND SAPONIN EXTRACTS TO DIETS WITH DIFFERENT FORAGE TO CONCENTRATE RATIO ON *In vitro* RUMEN FERMENTATION AND METHANOGENESIS

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## ABSTRAK

Penelitian ini bertujuan untuk menginvestigasi pengaruh penambahan kombinasi ekstrak tanin dan saponin terhadap emisi gas metana dalam pakan dengan proporsi hijauan:konsentrat yang berbeda dalam fermentasi rumen secara *in vitro*. Rancangan percobaan menggunakan rancangan acak kelompok (RAK) pola factorial. Faktor pertama adalah proporsi hijauan:konsentrat dalam pakan (70:30 and 30:70) dan faktor kedua adalah penambahan ekstrak tanin dan saponin (kontrol, tanin, saponin, tanin+saponin) pada dosis 2 mg/ml. Peubah yang diamati meliputi produksi gas, produksi gas metana, kecernaan bahan kering (KBK) dan bahan organik (KBO), dan konsentrasi amonia. Hasil menunjukkan bahwa penambahan tanin, saponin, dan kombinasinya secara umum menurunkan produksi gas dan metana pada kedua tipe pakan selama waktu inkubasi 24 dan 48 jam (P<0,05), namun kombinasi tanin dan saponin dibandingkan dengan penggunaan secara terpisah tidak menunjukkan perbedaan yang nyata. Penambahan kombinasi tanin dan saponin berpengaruh menurunkan KBK, KBO, dan ammonia secara nyata (P<0,05). Dapat disimpulkan bahwa penambahan ekstrak tanin, saponin dan kombinasi keduanya pada dosis 2mg/ml dapat menurunkan emisi gas metana tetapi diikuti dengan menurunnya KBK, KBO dan ammonia.

*Kata kunci: tanin, saponin, hijauan, konsentrat, metana, in vitro*

## ABSTRACT

This experiment was aimed to investigate the effect of combining tannin and saponin extracts on ruminal methane emission of diets with different proportion of forage to concentrate in the *in vitro* fermentation. The experiment was conducted in a factorial block design. The first factor was the proportion of forage:concentate in diets (70:30 and 30:70) and the second was addition of tannin and saponin extracts (control, tannins, saponins, tannins + saponins) in the dose of 2 mg/ml. Variables observed were gas production kinetics, methane production, dry matter digestibility (DMD), organic matter digestibility (OMD) and ammonia concentration. Results revealed that addition of tannins, saponins and their combination generally lowered total gas and methane production during 24 and 48 h of incubation period in both types of diets (P<0.05), but combination of tannins and saponins compared with their separated forms did not show any significant differences. The addition of tannins, saponins and their combination reduced DMD, OMD and ammonia significantly (P<0.05). It can be concluded that the addition of tannin, saponin and their combination at a dose of 2 mg/ml could reduce methane emission but followed by a decline in the DMD, OMD and ammonia.

*Keywords: tannin, saponin, forage, concentrate, methane, in vitro*

## INTRODUCTION

Global warming is a major environmental problem faced by mankind, especially in the last century. Intergovernmental Panel on Climate Change (IPCC) in 2007 reported that the average temperature of earth's surface has increased by 0.74 ± 0.18ºC in the 20th century and such fact is the largest temperature rise within the last few thousand years. Furthermore, modeling scenarios developed by IPCC also suggest that the earth's surface temperature could increase by 2.4 to 6.4ºC by the year of 2090 to 2099. If this is the case in the future, it will greatly impact on various aspects of human life. Main causes of global warming have been known to be associated with avery high rate of accumulation of greenhouse gases in theupper atmosphere such as carbon dioxide ($CO_2$), methane ($CH_4$), nitrous oxide ($N_2O$) and chlorofluoro carbon (CFC) as a result of the increasing intensity of various human activity (Thorpe, 2009). Methane is the second largest contributor to greenhouse gas (16% of total) after $CO_2$, but, its ability to retain heat (global warming potential) is 21 times higher than that of $CO_2$ (Iqbal, 2008).

Livestock, especially ruminants like cattle, goats, and sheep contribute to accumulation of methane emmision in the atmosfer due to methanogenesis by archeametanogen in the rumen (Cottle *et al.*, 2011). Such emission does not only affect the global warming, but it also represents energy loss from the animals, in which the lost can be between, 8 to 14% from total digestible energy. Nutritional strategies to mitigate methane emission based on natural substances are preferred over the synthetic ones (Jayanegara *et al.*, 2009a). Accordingly, secondary plant metabolites such as tannins and saponins are potential to be used in mitigating methane emissions from ruminants. Tannins can reduce methane emissions through a reduction in methanogen population (Bhatta *et al.*, 2009) whereas saponins work through a reduction in protozoa population (Hess *et al.*, 2003) in which part of the methanogen is living symbiotically (Finlay *et al.*, 1994). If these two compounds are used simultaneously, is could be expected to decrease in methane emission further.

Therefore, the purpose of this study was to investigate the effect of combining tannin and saponin extracts when added into two types of rations with different forage to concentrate ratio on ruminal methane emission, gas production, digestibility of dry matter and organic matter, and ammonia concentration through an *in vitro* assay.

## MATERIALS AND METHODS

### Extraction of Tannins and Saponins

Tannin extract was taken from the leaves of mahogany (*Swieteniamahagoni*) while the saponin extract was taken from the lerak fruit (*Sapindusrarak*) in which both of them were collected from Bogor area. Mahogany leaves and lerak fruits were oven-dried at 60ºC to obtain approximately 90% dry matter and then, ground immediately to obtain powdered forms. Mahogany leaves powder extracted with a combination of 70% methanol:30% water, while the lerak fruits powder was extracted with 100% methanol solvent by using an ultrasonic water bath for 30 min (Yuliana *et al.*, 2014). Subsequently, the solid and liquid fractions were filtered using a Whatman paper. The liquid fraction was then evaporated in a rotary evaporator to evaporate the organic solvents, freeze dried and kept in air tight bags at freezer (-4ºC). These procedures produced dried tannin and saponin extracts.

### *In Vitro* Fermentation

The substrate used in the *in vitro* test was consisted of two types of diet withdifferent forage to concentrate proportion, i.e.70:30 and 30:70, respectively. Forage used was elephant grass (*Pennisetumpurpureum*) with nutrient content (dry matter basis) of crude protein (CP): 8.96%, neutral detergent fibre (NDF): 65.61%, and acid detergent fibre (ADF): 44.72%. The concentrate was a commercial concentrate of dairy cows with trademark Lactofeed produced from CV. Tani Mulya, Bogor, Indonesia, contained of 11.45% CP. The grass was dried in an oven at 50ºC until themoisture content was around 10%. The substrates were ground using a grinder to pass a 1 mm sieve size

The *in vitro* fermentation technique was according to the method of Theodorou (1990). A total of 100 mg of substrate treatment was inserted into a 100 ml bottle size and buffered rumen fluid as the incubation medium. The incubation medium was consisted bicarbonate buffer solution: (24.1%), macro-mineral solution: (12.1%), micro-mineral solution: (0.00613%), resazurin: (0.0612%), distilled water: (36.2%), reducing solution: (2.3%) and rumen fluid: (25.3%). Rumen fluid was collected just before

morning feeding from a rumen fistulated Friesian Holstein cow in Balai PenelitianTernak, Ciawi, Bogor; the cow was fed with elephant grass and commercial concentrate at a ratio of 60:40, respectively. The rumen fluid was filtered through a nylon cloth and, then inserted into a container and immediately brought to the laboratory. Incubation was carried out in a water bath maintained at 39-42ºC for 48 h. During the incubation, the bottles were shaked.

Factorial (2 × 4) randomized complete block was used in this study. Factor A: Different forage to concentrate ratio:

A1: 70% forage:30% concentrate

A2: 30% forage:70% concentrate

Factor B : Addition of tannin and or saponin extracts at (a dose of 2mg/ml rumen fluid):

B1: Control

B2: B1 + 2 mg/ml tannin extract

B3: B1 + 2 mg/ml saponin extract

B4: B1 + 1 mg/ml tannin extract + 1 mg/mL saponin extract

**Variable Measurements**

Variables observed in this study were gas production kinetics, methane production, *in vitro* dry matter digestibility (DMD), *in vitro* organic matter digestibility (OMD) and ammonia concentration. Gas production was observed at 1, 3, 6, 10, 12, 14, 21, 24, 30, 36 and 48 h after incubation. Methane production was measured by using $CO_2$ trapping method with NaOH at the interval when te residue was filtered and dried in an oven at 105 ºC for 24 h. Dry matter and organic matter residue were determined to calculate the DMD and OMD. Ammonia concentration was measured with the Conway micro-diffusion technique.

**Statistical Analysis**

Data obtained were analyzed by the factorial analysis of variance (ANOVA). When a particular variable showed significantly different at $P<0.05$ in the ANOVA result, a post-hoc test namely Duncan's multiple range test was employed to compare among different treatment means. All statistical analyses were performed by using SPSS software version 17.

## RESULTS AND DISCUSSION

**Total Gas and Methane Production**

Total gas production *in vitro* increased at higher incubation period but with a declining rate. This is due to the decreasing quantity of fermentable substrates (Jayanegara *et al.*, 2006). At high forage ration (HFR), addition of tannin and/or saponin extracts did not affect total gas production up to 6 h of *in vitro* incubation as compared to control; the change was begun later (Table 1). After 24 h incubation, the addition of tannins, saponins and their combination at 2mg/ml in HFR significantly reduced the total gas production by 18.3, 16.9, and 11.2% from control, respectively ($P<0.05$). However, such additions to HCR did not decrease total gas production at 24 h. Different pattern was observed at 48 h of incubation; addition of tannin and saponin extracts in combination increased total gas production especially in HCR as compared to control ($P<0.05$).

Total gas in the *in vitro* rumen fermentation is produced from the fermentation of substrates, primarily composed of $CO_2$ and $CH_4$ (Getachew *et al.*, 1998). The reduction of gas production at 24 h fermentation due to addition of tannin extract was in agreement with Jayanegara *et al.* (2009a) although the level tested was different. While the decline in gas production due to the addition of saponin extract was also observed by Makkar *et al.* (1995). Mechanism of tannins in reducing gas production is through their ability to interact withfeed components mainly protein and fiber which have a major contribution in generating gas (Makkar, 2003; Makkar *et al.*, 2007), whereas the mechanism of saponins is more ability to inhibit the activity of enzymes that degrade the fiber components (Hristov *et al.*, 2003). Interestingly, when tannins and saponins were combined, the addition did not decrease gas production especially at longer incubation period. Apparently they interacts each other and alleviate the negative impact on the *in vitro* rumen fermentation activity. In relation to starting from 3 h of incubation until the end (48 h), HCR produced lower methane concentration than that of HFR ($P<0.05$; Table 2). Additions of tannins, saponins and the combination of tannins+saponins generally decreased methane concentration as compared to control both in HFR and HCR ($P<0.05$). The response was consistent until 48 h of incubation. No significant interaction was found between different forage to concentrate diet and tannin/saponin additions. Simultaneous addition of tannins+saponins lowered methane concentration than their individual addition especially during early incubation period and in HCR.

Table 1.Gas Production Kinetics (in ml) of High Forage Ration (HFR) and High Concentrate Ration (HCR) on Addition of tannin and Saponin Extracts

| Time (h) | HFR (70%F:30%C) | | | | HCR (30%F:70%C) | | | | Significancy | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Ctl | T | S | T+S | Ctl | T | S | T+S | FC | TS | INT |
| 1 | $15.7^{a}$ | $16.2^{ab}$ | $16.2^{ab}$ | $20.8^{abc}$ | $17.8^{ab}$ | $19.8^{ab}$ | $20.9^{bc}$ | $24.8^{c}$ | ** | ** | ns |
| 3 | $30.6^{ab}$ | $28.6^{a}$ | $31.7^{ab}$ | $33.8^{ab}$ | $34.6^{ab}$ | $35.3^{bc}$ | $40.5^{cd}$ | $41.3^{d}$ | ** | ** | ns |
| 6 | $46.3^{a}$ | $42.7^{a}$ | $47.8^{ab}$ | $51.4^{abc}$ | $59.7^{cde}$ | $56.8^{bcd}$ | $62.8^{de}$ | $68.9^{e}$ | ** | * | ns |
| 10 | $77.9^{bc}$ | $58.2^{a}$ | $69^{ab}$ | $72.1^{b}$ | $99.1^{de}$ | $87.7^{cd}$ | $96.7^{de}$ | $104^{e}$ | ** | * | ns |
| 12 | $94.9^{bc}$ | $67.7^{a}$ | $81.5^{b}$ | $83.2^{b}$ | $116.9^{de}$ | $104.7^{cd}$ | $119.9^{e}$ | $123.5^{e}$ | ** | ** | ns |
| 14 | $109.6^{c}$ | $77.1^{a}$ | $91.6^{b}$ | $93.1^{b}$ | $131.9^{de}$ | $119.4^{cd}$ | $136.6^{e}$ | $139.5^{e}$ | ** | ** | ns |
| 21 | $140.2^{b}$ | $110.4^{a}$ | $113.6^{a}$ | $121^{a}$ | $158.9^{c}$ | $154.1^{c}$ | $160.5^{c}$ | $164.3^{c}$ | ** | ** | * |
| 24 | $151^{b}$ | $123.4^{a}$ | $125.5^{a}$ | $134.1^{a}$ | $167.5^{c}$ | $165.8^{c}$ | $169.3^{c}$ | $174^{c}$ | ** | * | * |
| 30 | $164.9^{b}$ | $147.7^{a}$ | $141.7^{a}$ | $152.2^{ab}$ | $178^{c}$ | $180.4^{c}$ | $180.9^{c}$ | $185.8^{c}$ | ** | ns | * |
| 36 | $173.2^{bc}$ | $162.1^{ab}$ | $156.4^{a}$ | $168.9^{a}$ | $183.7^{cd}$ | $188.6^{d}$ | $190.3^{d}$ | $195.5^{d}$ | ** | ns | ns |
| 48 | $184.8^{bc}$ | $174.3^{ab}$ | $169.5^{a}$ | $187.4^{c}$ | $190.1^{cd}$ | $199.2^{d}$ | $203.2^{e}$ | $207.5^{e}$ | ** | * | ** |

Different superscripts within the same row are significantly different at P<0.05

F= forage; C = concentrate; Ctl= control; T = tannin; S = saponin; T+S = tannin + saponin; FC= factor forage to concentrate ratio; TS = factor addition of tannin and saponin; INT = interaction between FC and TS; ** = highly significant (P <0.01); * = Significant (P<0.05); ns = non-significant

Table 2.Methane Production (in % Total Gas) of High Forage Ration (HFR) and High Concentrate Ration (HCR) on Addition of Tannin and Saponin Extracts

| Time (h) | HFR (70%F:30%C) | | | | HCR(30%F:70%C) | | | | Significancy | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Ctl | T | S | T+S | Ctl | T | S | T+S | FC | TS | INT |
| 1 | $34.14^{c}$ | $26.002^{ab}$ | $27.55^{ab}$ | $23.099^{ab}$ | $29.03^{bc}$ | $26.21^{ab}$ | $25.02^{ab}$ | $22.06^{a}$ | ns | ** | ns |
| 3 | $36.20^{d}$ | $26.83^{bc}$ | $25.34^{bc}$ | $23.094^{ab}$ | $28.33^{c}$ | $27.58^{c}$ | $22.95^{ab}$ | $20.91^{a}$ | * | ** | ns |
| 6 | $34.48^{d}$ | $28.65^{c}$ | $26.61^{abc}$ | $24.38^{ab}$ | $28.11^{bc}$ | $27.42^{bc}$ | $24.11^{ab}$ | $22.85^{a}$ | ** | ** | ns |
| 10 | $33.81^{d}$ | $28.82^{c}$ | $28.11^{bc}$ | $24.51^{ab}$ | $27.90^{bc}$ | $26.45^{bc}$ | $24.45^{ab}$ | $22.19^{a}$ | ** | ** | ns |
| 12 | $32.87^{d}$ | $27.77^{c}$ | $27.30^{bc}$ | $24.73^{abc}$ | $27.35^{bc}$ | $25.59^{abc}$ | $24.12^{ab}$ | $22.27^{a}$ | ** | ** | ns |
| 14 | $32.08^{d}$ | $27.67^{c}$ | $26.63^{bc}$ | $24.68^{ab}$ | $26.53^{bc}$ | $25.29^{bc}$ | $23.88^{ab}$ | $22.14^{a}$ | ** | ** | ns |
| 21 | $31.21^{d}$ | $26.85^{bc}$ | $26.50^{bc}$ | $24.68^{abc}$ | $26.99^{c}$ | $22.66^{abc}$ | $24.03^{ab}$ | $22.66^{a}$ | ** | ** | ns |
| 24 | $31.23^{d}$ | $26.60^{c}$ | $26.22^{bc}$ | $24.78^{abc}$ | $27.07^{c}$ | $25.12^{abc}$ | $23.91^{ab}$ | $22.75^{a}$ | ** | ** | ns |
| 30 | $30.99^{d}$ | $24.70^{abc}$ | $26.30^{bc}$ | $24.56^{bc}$ | $27.12^{c}$ | $25.14^{abc}$ | $24.05^{ab}$ | $22.47^{a}$ | ** | ** | ns |
| 36 | $30.95^{d}$ | $24.32^{ab}$ | $25.96^{bc}$ | $24.21^{bc}$ | $27.22^{c}$ | $25.08^{abc}$ | $24.22^{ab}$ | $22.54^{a}$ | * | ** | ns |
| 48 | $31.13^{c}$ | $23.90^{a}$ | $25.37^{ab}$ | $24.46^{a}$ | $27.31^{b}$ | $24.96^{ab}$ | $24.24^{a}$ | $22.68^{a}$ | * | ** | ns |

Explanation of Ctl, T, S, FC, TS, INT, * and **: see Table 1

Effect of tannins in reducing methane emission on high forage ration was in line with that reported by Jayanegara *et al.* (2010); addition of purified tannins from chestnut and Sumach at 1 mg/ml into hay:concentrate (70:30) diet decreased methane concentration by 6.5 and 7.2%, respectively. With regard to saponins, in contrast to the present study, saponins from lerak fruits did not decrease rumen methanogens which can be correlated with the methane emissions. Such difference may occur because of the differences in the dose of saponin extracts, duration of incubation, and the incubation medium. Other reports have shown the methane mitigation effect of saponins from various sources, such as from *Camellia sinensis* (Guo *et al.*, 2008) and *Knautiaarvensis* (Goel *et al.*, 2008). The ability of tannins and saponins to reduce ruminal methane emissions has different mechanisms. Tannins, including hydrolysable and condensed tannins reduce methane through a direct inhibition on archea metanogen population in the rumen (Bhatta *et al.*, 2009). On the other hand, saponins decrease methane through a reduction in ruminal protozoal population (Hess *et al.*, 2003) in which part of the methanogens are symbiotically living together with the fauna and contribute up to 37% of the total methane emissions from the rumen (Finlay *et al.*,1994).

**Feed Digestibility and Fermentation**

Data on feeddry matter digestibility (DMD) and organic matter digestibility (OMD) are presented in Figure 1 and Figure 2, respectively. The addition of tannins, saponins and their combination at 2 mg/ml during the 48 h incubation period significantly decreased DMD and OMD ($P<0.05$). TheDMD decrease on the addition of tannins, saponins and the combination in HFR 20.7%, 20.5% and 24.7%, respectively, in HCR were 19.0%, 12.2% and, 13.3%, respectively. Similarly, OMD decrease due to addition of tannins, saponins and tannins+saponins were 35.0%, 27.2% and 30.7%, respectively in HFR and 19.9%, 16.2% and 16.1% in HCR. The decline of ruminal digestibility due to addition of tannins and/or saponins has also been reported by some other authors (Makkar *et al.*, 1995; Wina *et al.*, 2005; Jayanegara *et al.*, 2009b). The mechanisms of tannins and saponins in reducing ruminal digestibility of dry matter and organic matter are similar as in the reduction of gas production; tannins inhibit feed degradation process through their interactions with protein and fiber components (Makkar, 2003; Makkar *et al.*, 2007) while saponins inhibit the activity of enzymes that degrade fiber components (Hristov *et al.*, 2003).

Addition of saponins and the combination resulted in the decrease of rumen ammonia concentration both in HFR and HCR ($P<0.05$; Figure 3). No difference was observed between HFR and HCR with regard to ammonia concentration. Combination of tannins+saponins decreased ammonia further than those of their individuals ($P<0.05$). This may indicate the

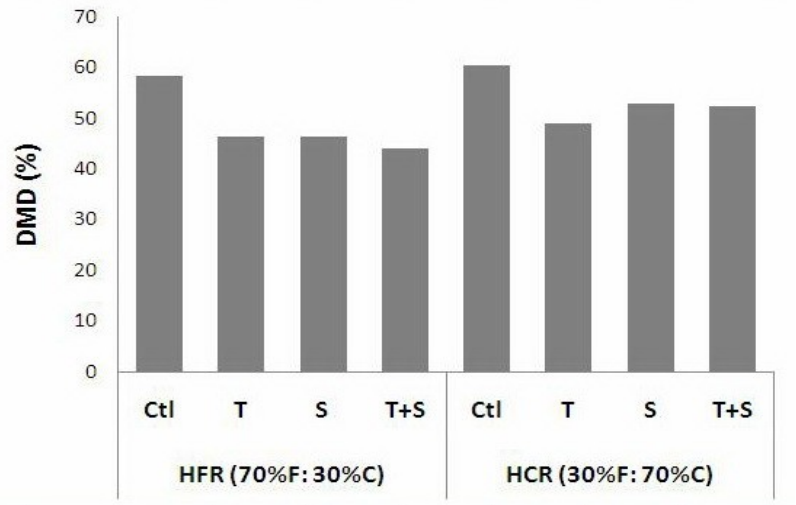


Figure 1. Dry Matter Digestibility (DMD) of High Forage Ration (HFR) and High Concentrate Ration (HCR) on Addition of Tannin and Saponin Extracts. Ctl = control; T = tannin; S = saponin; T+S = tannin + saponin; HFR= high forage ration; HCR= high concentrate ration; F= forage; C= concentrate.

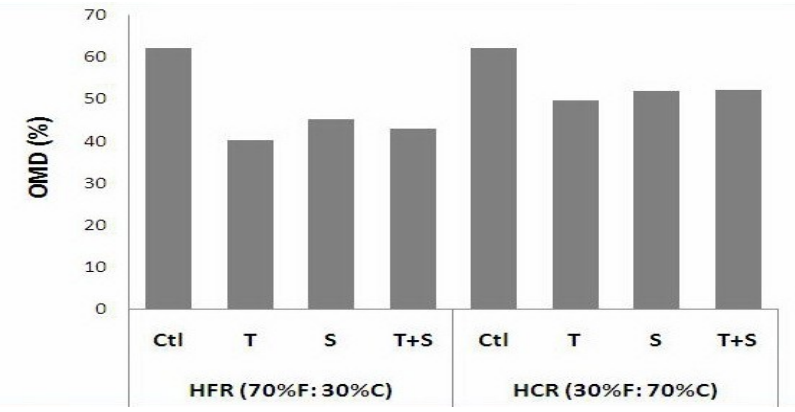


Figure 2. Organic Matter Digestibility (OMD) of High Forage Ration (HFR) and High Concentrate Ration (HCR) on Addition of Tannin and Saponin Extracts. Ctl : control; T : tannin; S : saponin; T+S : tannin + saponin; HFR : high forage ration; HCR : high concentrate ration; F : forage; C : concentrate.

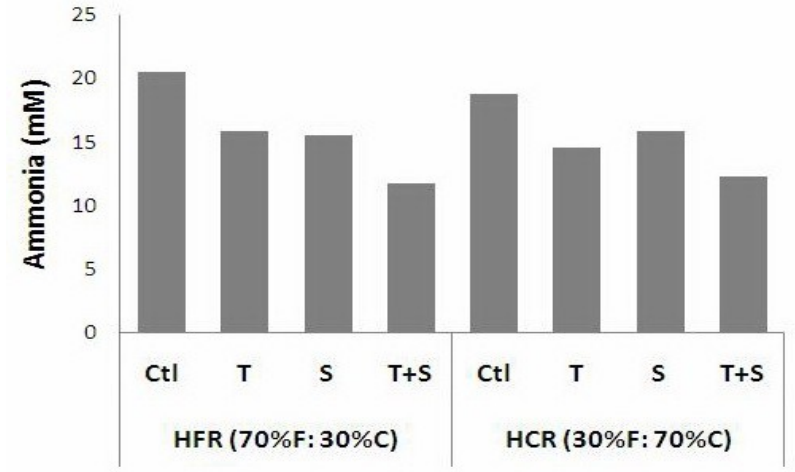


Figure 3. Ammonia Concentration of High Forage Ration (HFR) and High Concentrate Ration (HCR) on Addition of Tannin and Saponin Extracts. Ctl : control; T : tannin; S : saponin; T+S : tannin + saponin; HFR : high forage ration; HCR : high concentrate ration; F : forage; C : concentrate.

presence of associative effect between tannins and saponins in decreasing rumen ammonia concentration. A number of studies have reported that tannins and saponins reduced ammonia concentration in the rumen, both *in vitro* and *in vivo* (Makkar *et al.*, 1998; Wina *et al.*, 2005).

Concentration of ammonia in the rumen is derived from the lysis of microbes and degradation of feed protein. Most of ammonia is absorbed through the rumen wall and the rest is used directly by rumen microbes to meet the needs of nitrogen; about 50-80% requirements for microbial nitrogen is derived from ammonia (Leng, 1984). Tannins decrease ammonia concentrations of ammonia by binding with feed protein and, hence, prevent its degradation by proteolytic microbes (Tanner *et al.*, 1994). The decrease in ammonia due to the addition of saponins occurred by an indirect mechanism through a reduced protozoal population (VanSoest, 1994). When both tannins and saponins were added simultaneously, apparently both mechanisms occur and lead a synergistic effect for further reduction of rumen ammonia

concentration.

## CONCLUSION

Addition of tannins, saponins and their combination were able to reduce ruminal methane emissions *in vitro* when added both in high fiber and high concentrate rations at 2 mg/ml. Although the additions also decreased DMD, OMD and ammonia concentration in the rumen, it does not always mean that a negative effect on animal performance will occur. It has to be noted that the depression of digestibility is taken place in the rumen, not in the total digestive tract. Further *in vivo* study is therefore needed to confirm the present *in vitro* results and to investigate their effects on animal performance.

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