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Effects of Anthelmintic Plant Extracts on Ruminal Fermentation Characteristics, Bacterial Diversity and Methane Production *in vitro*

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ABSTRACT

The objective of this study was to know the *in vitro* effects of supplemental anthelmintic plant extracts on the inhibition of protozoa for reducing methane production in the rumen. A fistulated Holstein cow was used as a donor of rumen fluid. The plant extracts (*Lonicera japonica, Zanthoxylum piperitum, Pyrethrum, Torreya nucifera, Ruta graveolens*) known to have anthelmintic effect were added to the *in vitro* fermentation bottles containing the rumen fluid and medium. The rumen protozoal population was depressed by the addition of *Pyrethrum, Torreya nucifera* and *Ruta graveolens*. The methane production was also significantly ($p<0.05$) reduced by addition of *Pyrethrum* (2.20 ml/g DM), *Torreya nucifera* (2.36 ml/g DM) and *Ruta graveolens* (2.20 ml/g DM). The microbial growth in the treatments of *Ruta graveolens* or *Zanthoxylum piperitum* was the greatest after 12 h and 24 h incubations, respectively. The results of this study indicated that anthelmintic plant extracts appeared to reduce methane production by inhibition of ruminal protozoa related with the methanogens living endosymbiotic in protozoal cells.

Key words - Anthelmintic plant extract, Fungi, Methane, Microbial growth, Protozoa

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I. Introduction

Global warming due to an increase in the atmospheric concentration of greenhouse gas such as methane is major of interest in the recent time. The methane emission from livestock industries, in particular from ruminants, is a significant contribution to the problem. Methane has a 23 times greater global warming potential than carbon dioxide. Methane emission related to enteric fermentation from ruminants are due to the rumen microbial ecosystem present as the anaerobic environment within provided excellent conditions for the growth of bacteria, protozoa and fungi. Methane emission in the rumen is an energetically wasteful process from the ruminants as it causes a substantial loss of 12% of the dietary energy intake (Johnson *et al*., 1995). Many chemical dietary additives have been examined to reduce methane emission in the rumen, but chemical additives were either toxic to host animals or cause side effects on ruminal microbes. The selection of bio-active compounds for the elimination of protozoa has been an approach studied with the aim of reducing methane emission from digestive origin. The control of methanogenesis in the rumen has been achieved with a range of chemical inhibitors (Van Nevel & Demeyer, 1995; McCrabb *et al*., 1997). However, the risk of using chemicals in animals destined for human consumption is of growing public concern. Anthelmintic plant extracts are good alternative candidates in manipulating the rumen microbial ecosystem and inhibition of protozoa for reducing methane emission from ruminants. This approach has been set as one of the most important goals for animal nutritionists (Teferedegne, 2000). Plant extracts are not only known for its medicinal and anthelmintic properties, in treatments and food preparations, it is also known to have antimicrobial compounds. These anthelmintic plants containing secondary metabolites have been found to inhibit or eliminate protozoa from the rumen (*Lonicera japonica* (Houghton *et al*., 1993; Kim *et al*., 1994), *Zanthoxylum piperitum* (Kim *et al*., 1993), *Pyrethrum*, *Torreya nucifera* (Lee, 1966; Chung & Ko, 1978; Lee, 1993), *Ruta graveolens* (Ahn *et al*., 2000)).

The objective of this study was to know the effects of anthelmintic plant extracts on the ruminal fermentation characteristics, microbial diversity and methane production *in vitro*.

II. Materials and Methods

2.1 Rumen fluid and anthelmintic plant extracts

A fistulated Holstein cow was used as a donor of rumen fluid. Italian ryegrass and concentrate in the ratio of 60 to 40 were fed at 2% of body weight twice a day (06:00 and 16:00 h). Water and mineral-vitamin block were allowed *ad libitum*. Rumen fluid was collected via rumen cannula before the morning feeding, and squeezed through four layers of cheese cloths into a flask. Anthelmintic plant extracts (*Lonicera japonica, Zanthoxylum piperitum, Pyrethrum, Torreya nucifera, Ruta graveolens*) were obtained from the company (EuroCostech Co., Ltd) in Korea.

2.2 *In vitro* batch incubation

The rumen liquor was mixed with McDougall buffer (pH 6.8) maintaining at 39℃. The 15 ml of rumen fluid-buffer mixture, comprising McDougall buffer and rumen liquor in the ratio of 4 to 1, was dispensed anaerobically into 50 ml serum bottles, filled with O_2-free N_2 gas, containing 0.3 g DM of timothy hay substrate and anthelmintic plant extracts (0.5% of total volume (v/v), respectively), and then

capped with a butyl rubber stopper. The serum bottles were held in a shaking incubator (120 rpm, SI-900R, JEIO TECH, Korea) at 39℃ for 12 and 24 h.

2.3 Estimation of the fermentation characteristics

After each sampling time, gas production was determined using a water displacement apparatus (Fedorah & Hrudey, 1983) and The pH was immediately determined after opening the bottles by pH meter (Mettle-Toledo, MP230). Methane was analyzed by the gas chromatography (GC-2010, Shimadzu, Japan) equipped with Porapak NQ column (Q 80-100 mesh, Waters Corporation, USA). Helium and hydrogen were used as carrier gases. For analysis of volatile fatty acids (VFA), 1 ml of 25% meta-phosphoric acid was added to 5 ml of incubated mixture, centrifuged (10,000×g for 10 min at 4°C) and the supernatant was collected for analysis. VFA concentration of rumen fluid was analyzed by gas chromatography (GC-2010, Shimadzu, Japan) according to the method of Erwin *et al*. (1961). *In vitro* dry matter disappearance was measured by filteration process. After incubation, the solution cultured was filtered with filter paper (Whatman No. 1). The filtered pellets were dried to a constant weight at 60℃. DM disappearance rate was determined the differences between filter paper weight and filter paper weight with pellet after drying.

2.4 Rumen microbial assay

Microbial growth rate: Incubated samples taken from each fermentation period were centrifuged at 3,000 rpm for 3 min to remove feed particles, and the supernatants were re-centrifuged at 14,000 rpm for 3 min to settle the pellets down. After that, sodium phosphate buffer (pH 6.5) was added to these precipitates and vortexed. The growth rates of microbes were estimated as optical density (OD) values using spectrophotometer (Model 680, BIO-RAD, Japan) at 550 nm (Lee *et al*., 2011). This process was repeated three times and means were determined.

Microbial count: Microbial populations were enumerated using a roll tube and microscopy. Viable cells were counted by the cell- or thallus forming unit method for bacteria, protozoa and fungi, respectively. Anaerobic bacterial population was determined using modified Dehority's artificial medium (MDAM) agar by the method of Dehority (1965). The MDAM agar was added to a 2% Bacto agar at Dehority's artificial medium agar. The anaerobic mold population was determined using Modified Lowe's agar Medium according to the method of Lowe *et al*. (1985). Modified Lowe's agar medium has an addition of 2% Bacto agar to the original medium. One milliliter of sample (diluted 10^{-3}to10^{-6}) was added to 9 ml of modified Lowe's agar with antibiotic (2×10^4 IU/ml benzylpenicillin G with 2 mg/ml of streptomycin sulfate) and was then incubated in anaerobic condition at 38.5°C for 5 days. TBFS buffer (trypanblue-formalin-salin; 900 ml of distilled water, 100 ml of 35% formaldehyde, 2 g of trypanblue, 8 g of NaCl) was mixed with samples to determine the living cell of protozoa. Protozoa were counted microscopically with using plankton counter glass following the procedure described by Abe & Kumeno (1972).

2.5 Statistical analysis

Data were analyzed as repeated measures using the General Linear Model (GLM) procedure of SAS (2002). Duncan's Multiple Range Test was used to test the significance ($P<0.05$) of differences among means.

III. Results and Discussion

3.1 Effects of anthelmintic plant extracts on the ruminal fermentation *in vitro*

The total gas and methane production, and dry matter disappearance are shown in Table 1. Total gas production as well as methane production and protozoal population were affected by the addition of anthelmintic plant extracts. The methane production in the *Pyrethrum, Torreya nucifera* and *Ruta graveolens* was significantly lower ($p<0.05$) than that of the control. Total VFAs and the ratio of acetate to propionate in addition of all plant extracts were significantly lower ($p<0.05$) than that of the control (Table 1). In particular, methane emission in the rumen is closely related to the acetate:propionate (C2:C3) ratio, and the decreased methane emission led to a higher molar proportion of propionate and low C2:C3 ratio (Nellet *et al*., 1997; Mitsumori & Sun, 2008). This study indicates that the plant with anthelmintic property may potentially affect to the ruminal methanogenesis, resulting in a reduction of methane emission. Although dry matter disappearance was not significantly different, DM disappearance rate was inclined to increase by the addition of *Torreya nucifera* extract (30.76%), while DM disappearance in the *Lonicera japonica* (24.23%), *Zanthoxylum piperitum* (24.88%) and *Pyrethrum* (23.13%) was inclined to decrease compared to the control. The secondary compounds of anthelmintic plant extracts might be associated with adverse effects such as anti-nutritional factor, causing lower DM disappearance and reduced digestion of protein and fiber. Another possible explanation is the increased bacterial counts resultant from a reduction in bacteria-degrading activity of the protozoa (Jouany, 1994).

3.2 Effects of anthelmintic plant extracts on microbial diversity

The plant extracts (*Lonicera japonica, Zanthoxylum piperitum, Pyrethrum, Torreya nucifera, Ruta graveolens*) used in this experiment is known to have anthelmintic, antibacterial and antiviral effects (Houghton *et al*., 1993), and has been used as oriental medicine materials. The secondary compounds, like flavonoid in *Torreya nucifera* (Jeon *et al*., 2009), *Lonicera japonica* (Shin & Yoo, 2012)

Table 1. Effects of addition of anthelmintic plant extracts on ruminal fermentation characteristics and methane production after 24 hr incubation *in vitro*

Item	Control	*Lonicera japonica*	*Zanthoxylum piperitum*	*Pyrethrum*	*Torreya nucifera*	*Ruta graveolens*	SEM[1)]
pH	6.48[a]	6.45[a]	6.35[b]	6.44[a]	6.44[a]	6.47[a]	0.05
Total gas (ml/g DM)	71.1[a]	69.9[a]	75.0[a]	56.7[b]	56.9[b]	56.4[b]	8.59
CH_4 (ml/g DM)	3.64[a]	3.49[a]	4.09[a]	2.20[b]	2.36[b]	2.20[b]	0.86
tVFAs (mM)	39.71[a]	29.02[d]	26.09[e]	36.43[b]	32.00[c]	37.07[b]	5.09
Acetate:propionate ratio	2.64[a]	2.12[e]	2.10[e]	2.45[c]	2.30[d]	2.54[b]	0.21
Disappearance of DM (%)	25.69	24.23	24.88	23.13	30.76	27.01	4.75

[ab]Means within a row with different superscripts differ significantly ($p<0.05$).
[1)]Standard error of the mean.

and rutin in *Ruta graveolens* (Cho *et al*., 2005), might manipulate ruminal fermentation and inhibit the microbes, especially protozoa, in the rumen. Addition of anthelmintic plant extracts significantly affected to rumen microbial growth (Table 2). The microbial growth in added *Ruta graveolens* and *Zanthoxylum piperitum* was significantly higher ($p<0.05$) at 12 h incubation and at 24 h incubation, respectively. Hungate (1968) reported that the microbial growth was closely related to gas production. This finding is in agreement with the result of total gas production in added *Zanthoxylum piperitum* in this study (Table 1). *Zanthoxylum piperitum* has been shown to possess strong antibiotic characteristics against various bacteria (Kim *et al*., 2007), and *Pyrethrum* is well known as a natural insecticide perhaps have anthelmintic, antibacterial and antiviral properties like previous secondary metabolites. This *in vitro* experiment determined that how much the rumen micro-organisms were affected by anthelmintic plant extracts during the *in vitro* fermentation incubated with rumen fluid. In particular, this study was expected to depress the rumen protozoa related to methanogens by addition of anthelmintic plant extracts, their effectiveness for decreasing methane emission. Population of ruminal bacteria, protozoa and fungi is shown in Table 3. The bacteria populations in added *Zanthoxylum piperitum* and *Torreya nucifera* were higher, while the bacteria populations in added *Lonicera japonica* and *Ruta graveolens* was significantly ($p<0.05$) lower than that of the control. The population of fungi in all treatments was significantly ($p<0.05$) higher than that of the control. The population of live protozoa cell was not affected by addition of anthelmintic plant extracts, while dead protozoa cell was significantly ($p<0.05$) reduced in added the *Pyrethrum, the Torreya nucifera* and the *Ruta graveolens* compared to the control. In the absence of a mixed protozoal community, the bacterial population in the rumen always increases (Lowe *et al*., 1985) while the total methanogens decreases (Takenaka & Itabashi, 1995) in absolute numbers as well as decreasing as a proportion of the total bacteria diversity. This finding is in agreement with the result of the methane production in Table 1. Previous findings found that methanogens attached to the surface of rumen protozoa may generate 37% of the rumen methane emission (Finlay *et al*., 1994). Defaunation of protozoa usually leads to reduce methane emission in the rumen (Nagaraja, 1995) as methanogens lose symbiotic partner and in turn reduce their biological activity. Stumm *et al*. (1982) reported that 20% of methanogens might inhabit within and on the outer surface of the protozoa. However, the attachment of methanogen is not permanent. In the elimination of protozoa, methane emission in the rumen is reduced on average by 20%. A decreased methanogens might be observed in a protozoa-free rumen thus decreases methane emission. Methane emission in ruminants resulting

Table 2. Effects of anthelmintic plant extracts on rumen microbial growth (OD) by incubation time *in vitro*

	Control	*Lonicera japonica*	*Zanthoxylum piperitum*	*Pyrethrum*	*Torreya nucifera*	*Ruta graveolens*	SEM[1)]
12 h	0.39^{b}	0.38^{b}	0.45^{ab}	0.49^{ab}	0.53^{ab}	0.58^{a}	0.03
24 h	0.58^{bc}	0.63^{ab}	0.68^{a}	0.59^{abc}	0.52^{c}	0.55^{bc}	0.02

^{abc}Means within a row with different superscripts differ significantly ($p<0.05$).
[1)]Standard error of the mean.

Table 3. Effects of anthelmintic plant extracts on microbial diversity after 12 h incubation *in vitro*

Item	Microbes			
	Bacteria	Protozoa		Fungi
		Live cell	Dead cell	
	cfu[1)]×10^7	cell×10^3	cell×10^3	tfu[2)]×10^2
Control	11.33±0.33[ab]	10.20±3.34	4.00±0.71[a]	1.67±0.33[d]
Lonicera japonica	5.00±1.00[c]	11.40±1.72	4.80±1.07[a]	7.33±2.73[cd]
Zanthoxylum piperitum	16.33±0.33[ab]	11.20±0.86	3.80±0.37[ab]	18.00±5.00[ab]
Pyrethrum	6.33±2.85[bc]	8.40±0.40	2.00±0.32[bc]	16.00±3.00[abc]
Torreya nucifera	17.67±7.36[a]	8.00±0.70	1.41±0.68[c]	24.50±4.50[a]
Ruta graveolens	1.67±0.33[c]	8.50±1.48	1.50±0.50[c]	11.67±0.88[bc]

Mean±standard error
[1)]Colony formation unit/ml.
[2)]Thallus formation unit/ml.
[abcd]Means with different superscripts in the same column differ significantly ($p<0.05$).

from relationship between protozoa and methanogens depends on the rate of relationship between methanogens and protozoa and rate of methane emission per methanogenic cell (Machmüller *et al*., 2003). In current study, anthelmintic plant extracts had an effect on protozoa population.

In conclusion, there were unexpected results that anthelmintic plant extracts inhibited the bacteria population and total gas production. In addition, the increased population of fungi might have a potential influence on methane production. However, anthelmintic plant extracts might influence ruminal methanogenesis activity by affecting the methanogens resulting from inhibition of the surface of rumen protozoa according to the protozoan population.

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