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Effect of methanolic extract of nettle (*Urtica dioica*) on *in vitro* fermentation and gas production of canola meal

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Abstract

A study was conducted to evaluate the effect of methanolic extract of nettle on fermentation pattern of canola meal. The experiment was conducted in a completely randomized design using four levels of nettle extract (zero, 0.2, 0.4 and 0.6 ml). The amount of gas produced during different hours (2 to 96 h) of incubation was measured. The chemical composition of canola meal regarding crude protein, crude fat and ash was 36.05, 0.84 and 7.35% respectively. The results showed that in all hours of incubation effect of treatment on gas production was significant ($P<0.05$). After 2, 4, 6, 8 and 12 h of incubation, *in vitro* gas production of canola meal significantly decreased compared to control treatment in addition to increased gas production in the other hours of incubation. Rate of gas production for canola meal significantly declined relative to the control. Potential of gas production and gas production from the insoluble fraction increased with increasing doses of nettle extract ($P<0.05$). The amount of organic matter digestibility, metabolizable energy and net energy for lactation increased with addition of increasing doses of nettle extract ($P<0.05$). In conclusion, the nettle extract in 0.2 ml level could successfully reduce gas production and degradability of canola meal *in vitro*.

Keywords: Nettle extract; canola meal; *in vitro* gas production

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Introduction

Structural carbohydrates are essential for proper rumen microbial activity and synthesis of microbial protein in ruminants. However, microbial fermentation may result in considerable energy and protein losses in the form of methane and ammonia (Cardozo et al., 2012). About 8 to 12 percent of the digestible energy may be lost in the rumen as methane (Salamatazar et al., 2011a). Currently, to meet protein requirements of lactating dairy cows and finishing steer, animal nutritionists are focussing on rumen un-degradable protein in the diet which could be digested in the small intestine efficiently (Van Soest, 1994).

Additives such as antibiotics and growth promoters have been used to reduce methane and ammonia emission from ruminants. Because of the risk of the presence of antibiotic residues in milk and meat and its effects on human health, the use of these additives was prohibited by the European Union in 2006 (Sallam et al., 2009). Since natural plant extracts have antimicrobial properties (Kamel, 2001) and according to the relative safety of these extracts, nowadays, most researchers focused on plant extracts to alter protein fermentation in the rumen (Salamatazar et al., 2011b). Many plants produce secondary metabolites such as phenolic compounds, essential oils, and sarsaponins that affect microbial activity (Kamel, 2001). It has been shown that methanolic extract of thyme (0.15 ml) decreased gas production of soybean meal *in vitro* up to 8 h of incubation (Salamatazar et al., 2012). It was also reported that adding 0.5 ml of thyme extract to soybean meal reduced gas production at all incubation times in comparison to the control (Rezaei, 2011). Many studies also showed that nettle extract has antibacterial effects (Obertreis et al., 1996). Nettle leaves contain chlorophyll, carotene, xanthophylls, and flavonoid compounds (Iranian Herbal Pharmacopoea Committee,

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2003). The root of nettle contains tannins, coumarin (scopoletin), triterpens, lignans, lectins, sterols (sitosterol, campesterol, andstigmasterol), and flavonoids (Bisser, 1994). Nettle phenolic compounds such as thymol and carvacrol have been shown to possess antimicrobial and antifungal activity (Palic et al., 2002). This herb also contains Neophytadiene and Neophytadiene as antibacterial compounds (Palic et al., 2002; Modupe et al., 2010).

The purpose of this study was to evaluate the addition of nettle extract in different levels to rumen fluid containing canola meal to study *in vitro* fermentation kinetics and gas production.

Materials and Methods

The present experiment was conducted in a completely randomized design with 4 treatments and 3 replicates for each treatment. Experimental treatments were 1) canola meal without adding nettle extract to the rumen fluid, 2) addition of 0.2 ml of nettle extract to rumen fluid which contained canola meal, 3) addition of 0.4 ml of nettle extract to rumen fluid which contained canola meal 4) addition of 0.6 ml of nettle extract to rumen fluid which contained canola meal. To produce nettle extract, approximately 100 g of nettle (leaf, stem and root) was dried at 60°C. Dried nettle was added to 1000 ml methanol and mixed gently for 24 h at room temperature. Afterward the mixture of solvent and extract was filtered to obtain the initial extracts. The initial extract was distilled off and the solvent was evaporated at 60°C for an hour.

Approximately 200 mg of sample was weighed and placed in 100 ml graduated glass syringes. Buffer mineral solution was prepared and placed in a water bath at 39°C under continuous flushing with CO_2. Rumen fluid was collected after the morning feeding from two ruminally fistulated, Iranian Taleshi steers fed a total mixed ration consisting of approximately 40 percent chopped alfalfa hay and 60 percent concentrate ingredients. Rumen fluid was pumped with a manually operated vacuum pump from the rumen into pre-warmed thermos flasks. The rumen fluid from the two cows was mixed and filtered through four layers of cheese cloth and flushed with CO_2. The well mixed and CO_2 flushed rumen fluid was added to the buffered mineral solution (1:2 v/v), which was maintained in a water bath at 39°C, and mixed. Rumen fluid was added to the buffered mineral solution with constant stirring, while maintained in a water bath at 39°C. About 30 ml of buffered rumen fluid was dispensed into syringes containing the samples. Nettle extract (0, 0.2, 0.4 and 0.6 ml) was injected into the experimental syringes using insulin syringes. All handling was conducted under continuous flushing with CO_2. After closing the clips on the silicon tube at the syringe tip, syringes were gently shaken and the clips were opened to remove gas by pushing the piston upwards to achieve complete gas removal. The clip was closed, the initial volume recorded and the syringes were affixed to a rotary shaker platform (Lab-line Instruments Inc Melors dark, USA) set at 120 rpm housed in an incubator at 39°C. Incubation was completed in triplicate with readings of GP after incubation for 0, 2, 4, 6, 8, 12, 24, 48, 72 and 96 h. Differences in the composition and activity of rumen fluid inoculum were controlled by parallel measurements with incubation of buffered ruminal fluid without substrate (Blank test). Cumulative GP data were fitted to the exponential equation:

$$Y = a + b\ (1 - \exp^{-ct})$$

Where, Y is the gas produced at "t" time, "a" is the GP from the immediately soluble fraction (ml), "b" is the GP from the insoluble fraction (ml), "c" is the GP rate constant for "b" and "t" is the time of incubation (h). The metabolizable energy (ME), net energy for lactation (NEL) contents and organic matter digestibility (OMD) were calculated using equations of Menke and Steingass (1988) as:

$$ME\ (MJ/kg\ DM) = 0.157\times GP+0.0084\times CP+0.022\times EE-0.0081\times XA+1.06$$

$$NEL\ (MJ/kg\ DM) = 0.115 \times GP + 0.0054 \times CP + 0.014 \times EE - 0.0054 \times XA - 0.36$$

$$OMD\ (g/100\ g\ DM) = 0.9991 \times GP + 0.0595 \times CP + 0.0181 \times XA + 9$$

Where, CP is crude protein in g/100 g DM, XA ash in g/100 g DM, EE is ether extract in g/100 g DM and GP is the net gas production (ml) from 200 mg after 24 h of incubation.

Statistical analysis

Data were subjected to analysis of variance in a completely randomized design using the SAS program General Linear Model (GLM) procedure (SAS, 9.1, 2005). Significant means were compared using the least square means method. Mean differences were considered significant at $P<0.05$. Standard errors of means were calculated from the residual mean square in the analysis of variance.

Results and Discussion

Effect of treatment on gas production was significant during whole incubation period ($P<0.05$). After 2, 4, 6, 8 and 12 h incubation, gas production of canola meal significantly reduced compared to control treatment. Consistent to our results, it has been reported that the addition of 0.15 ml thyme extract in syringes containing 200 mg of canola meal with 30 ml of mixed rumen-buffer decreased gas production in 2, 4, 6, 8 and

Table 1: Effect of treatments on gas production of canola meal during incubation

Treatments	incubation time (hours)								
	2	4	6	8	12	24	48	72	96
1	9.33[a]	24.02[a]	30.93[a]	38.36[a]	43.88[a]	53.04[c]	63.37[b]	65.13[c]	67.03[c]
2	5.04[c]	17.71[c]	22.57[c]	30.04[c]	37.51[b]	54.34[bc]	69.60[a]	80.90[b]	87.31[ab]
3	5.54[bc]	19.07[bc]	24.44[bc]	32.23[bc]	40.04[ab]	57.01[ab]	71.90[a]	79.19[b]	85.45[b]
4	6.41[b]	20.98[b]	26.69[b]	35.36[ab]	43.16[a]	59.44[a]	71.91[a]	88.21[a]	93.44[a]
SEM	0.583	1.32	1.79	1.71	2.19	1.46	2.87	2.36	3.65
P-value	<0.01	<0.01	<0.01	<0.01	0.025	<0.01	0.011	<0.01	<0.01

1) Canola meal without adding nettle extract to the rumen fluid, 2) addition of 0.2 ml of nettle extract to rumen fluid which contained canola meal, 3) addition of 0.4 ml of nettle extract to rumen fluid which contained canola meal 4) addition of 0.6 ml of nettle extract to rumen fluid which contained canola meal. SEM: Standard error of means.

Table 2: Effect of treatments on gas production parameters of canola meal

Treatment	a+b	c	b
1	64.74[c]	0.010[a]	63.47[c]
2	79.67[b]	0.052[b]	78.87[b]
3	81.64[b]	0.053[b]	80.87[ab]
4	89.40[a]	0.048[b]	86.51[a]
SEM	6.11	0.0102	3.68
P-value	<0.01	<0.01	<0.01

1) Canola meal without adding nettle extract to the rumen fluid, 2) addition of 0.2 ml of nettle extract to rumen fluid which contained canola meal, 3) addition of 0.4 ml of nettle extract to rumen fluid which contained canola meal 4) addition of 0.6 ml of nettle extract to rumen fluid which contained canola meal. SEM: Standard error of means. b: gas production from the insoluble fraction (ml), c: rate of gas production (ml/h), a + b: a potential gas production (ml)

Table 3: Effect of treatments on metabolizable energy (ME), net energy of lactation (NEL), organic matter digestibility (OMD) of canola meal

Treatment	OMD (%)	NEL (MJ/ kg DM)	ME (MJ/ kg DM)
1	64.27[c]	5.91[c]	9.65[c]
2	65.56[bc]	6.06[bc]	9.85[bc]
3	68.25[ab]	6.36[ab]	10.27[ab]
4	70.67[a]	6.64[a]	10.65[a]
SEM	1.46	0.169	0.197
P-value	<0.01	<0.01	<0.01

1) Canola meal without adding nettle extract to the rumen fluid, 2) addition of 0.2 ml of nettle extract to rumen fluid which contained canola meal, 3) addition of 0.4 ml of nettle extract to rumen fluid which contained canola meal 4) addition of 0.6 ml of nettle extract to rumen fluid which contained canola meal. SEM: Standard error of means.

12h after incubation. However, the result of 0.3 ml of the extract was not in agreement to our results (Palic et al., 2002; Salamatazar et al., 2011a). It has been suggested that decreasing gas production is related to thymol and carvacrol in thyme extract (Salamatazar et al., 2011a). It is believed that most of plant extracts have the antimicrobial activity; they could interact with bacterial cell membrane and involve in electron transfer, ion gradients, displacement of protein phosphorylation and other reactions of enzyme dependent (Dorman and Deans, 2000). Accordingly it is likely accepted that thymol and carvacrol may create complex with proteins of bacterial membrane and exert their antibacterial activity (Castillejos et al., 2006). Additions of thymol and carvacrol, Neophytadiene and fatty acids such as phthalic acid and malate in nettle (Modupe et al., 2010) have antimicrobial effects that may reduce degradation of food protein in the rumen.

The reduction of gas production for canola meal in this study may be related to phenolic compounds in nettle (like Caffeic acid and Ferulic acid). These compounds have great impact on some bacteria species such as *Escherichia coli*, *Proteus* and *Klebsiella* and could disrupt their activities (Scalbert, 1991). Nettle root also contains tannins which can interact with the extracellular enzymes of bacteria as well as nutrient transformation into bacterium cell. Consequently, tannins have particular effects on cell membranes and ultimately reduce bacteria access to the nutrients (Scalbert, 1991).

Gas production rate (c) for canola meal significantly ($P<0.05$) decreased compared to the control (Table 2). Potential of gas production (a + b) and gas production from the insoluble fraction (b) of canola meal increased significantly in comparison to the control ($P<0.05$).

Consistent to our results, it has been reported that thyme extract (0.15 ml) reduced rate of gas production and increased potential of gas production and gas production from the insoluble fraction of canola meal (Salamatazar et al., 2011b). It is possible that the observed reduction in the rate of gas production is related to the tannins present in nettle extract which could reduce microorganism contact to the food particles. Tannin compounds are toxic to the bacteria as well and they can reduce particular groups of the microorganism population in the rumen (Danesh Mesgaran, 2009).

The amount of organic matter digestibility, metabolizable energy and net energy for lactation increased significantly ($P<0.05$) in nettle containing treatments compared to the control treatment (Table 3). It has been reported that the addition of 0.3 and 0.15 ml of thyme extract could decrease the amount of organic matter digestibility, metabolizable energy and net energy for lactation of canola meal compared to the control treatment (Salamatazar et al., 2011b).

Gas volume at 24 h of incubation is the most important criteria to estimate the metabolizable energy, net energy for lactation and dry matter digestibility since it has a high positive correlation with gas production and nutrients digestibility (Menke and Steingass, 1988). It may also be a determinant to predict nutrient digestibility, by-products of fermentation and microbial protein synthesis.

Conclusions

In conclusion, the nettle extract at 0.2 ml level could successfully reduce gas production and degradability of canola meal *in vitro*.

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