The effect of methanol extract of *Viscum album* on fermentation pattern and digestibility of cottonseed meal and alfalfa hay using gas test technique.

H.R. Mohammadian Tabrizi*, Z. Mosanezhad, M.H. Palizdar.

Department of Animal Science, Islamic Azad University, Chalous Branch. Mazandaran-Iran.
* Corresponding author email: hr_mohamadian@yahoo.com (+989111421238)

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Abstract

The objective of this study was to determine the effect of different levels of methanolic extract of *Viscum album* on fermentation pattern of cottonseed meal in *vitro* gas production technique. Completely randomized experimental design with four treatments namely 0, 0.1, 0.2 and 0.3 ml of *Viscum album* and three replicates for each treatment were used. Gas production was measured at 2, 4, 6, 8, 12, 24, 48, 72 and 96 h. The results showed that methanol extract of *Viscum album* had significant effect on the amount of gas production from cottonseed meal at 2, 24, 48, 72 and 96 of incubation ($P < 0.05$), however at 4, 6, 8 and 12 hour of incubation had no significant effect. The addition of *Viscum album* extract to cottonseed meal reduced gas production at all hours of incubation, except for treatment 2, that gas production increased at 12 hour of incubation. With the addition of 0.1, 0.2 and 0.3 ml of *Viscum album* the potential of gas production (a+b) for cottonseed meal was reduced in all treatments compared to the control. The addition of 0.1, 0.2 and 0.3 of *Viscum album* extract decreased gas from the insoluble fraction of cottonseed meal in all treatments compared to the control. The rate of gas production (c) of cottonseed meal increased by the addition of *Viscum album* extract. The methanol extract of *Viscum album* was significant for cottonseed meal, which the amount of organic matter digestibility, metabolizable energy and net energy for lactation for cottonseed meal by adding levels of 0.1, 0.2 and 0.3 ml of *Viscum album* extracts decreased in all compared to control. In this study we find finally that by adding *viscum album* extract to fermentation syringes, the ruminal digestibility of cottonseed meal decreased and it may provide more bypass protein for intestine.

Keywords: *Viscum album;* alfalfa; Cottonseed meal; Gas Test Technique

Introduction

With increasing role of nutrition in animal husbandry economy, from middle of the last century, experts in animal nutrition and even some international organization consider to identifying food value for feeding, identifying food needs for farm land animal and physiological aspects and biochemical nutrition and especially, other relation of food with producing energy in animal body. This caring led to study a lot of research in this filed. Ruminants make symbiotic relation with rumen micro-organism so that animal provides nutrient and suitable situation for food fermentation and microorganisms decomposite raw fiber and synthesize microbial protein. In this process, some energy is lost in form of gas (Halimi Shabestari et al., 2011). Gas production has a close relationship with fermentation and digestibility of organic substances in rumen (Moss et al., 2000). Antibiotics and ionophores were used previously as rumen fermentation modulators in order to decrease ammonia and methane production in rumen but because of some complications their usage has been stopped in Europe since 2006, consequently today researchers pay their attention to plant extracts as an alternative (Castillejos et al., 2006). It is common in tropical regions of Iran as well as north and it can be found on trees crown such as carpinus, betulus, ulmus minor and parrotia persica (Kartoolinejad et al., 2007). Anti-nutrient factors extracted from *viscum album* as tannins, lectin, thionine and viscotoxins (Pryme et al., 2006) may have great impact on rumen fermentation. Flavonoids, polysaccharids, phytosterol, hydrocarbons, vitamins and long chain fatty acid are the important constituents of *viscum album* which have medical benifits (Delior man et al., 2001). *viscum album* can affect rumen microorganisms Regarding the fact that it has high tannin, lectin and other compositions. It may be able to decrease fermentation of protein meal with high value of in rumen and more value passes through rumen (McSweeney et al., 1999). Many researchers have been done about plant Extracts. It has been shown that methanolic extract of thyme (0.15 ml) decreases gas production of soybean meal in *in vitro* up to 8 h of incubation (Salamatazar et al., 2012a). It was also reported that adding 0.5 ml of thyme extract to soybean meal reduces gas production at all incubation times in comparison to the control (Rezaei et al, 2011). The purpose of this study was to evaluate the addition of *viscum album* extract in different levels to rumen fluid containing cottonseed 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 in Azad Islamic University of Chalous. Experimental treatments were 1) cottonseed meal without adding *viscum album* extract to the rumen fluid, 2) addition of 0.1 ml of *viscum album* extract to rumen fluid which contained cottonseed meal, 3) addition of 0.2 ml of *viscum album* extract to rumen fluid which contained cottonseed meal 4) addition of 0.3 ml of *viscum album* extract to rumen fluid which contained cottonseed meal. To produce *viscum album* extract, approximately 100 g of *viscum album* (leaf and stem) was dried at 60°C. Dried *viscum album* 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 (Niasati et al., 2014).

in vitro gas production

GP was determined by the procedure of Menke and Steingass (1988). Samples (200 mg) were weighed into 100 ml calibrated glass syringes with pistons lubricated with Vaseline. Buffered mineral solution (Menke and Steingass, 1988) was prepared and placed in a water bath at 39°C under continuous flushing with CO2. Rumen fluid was collected after the morning feeding from three ruminally fistulated Taleshi that were fed diet containing alfalfa hay (600 g/kg) plus a concentrate mixture (400 g/kg) at 9:00 and 18:00 h. Rumen fluid was pumped from the rumen with a manually operated vacuum pump and transferred into two pre-warmed thermos flasks, transported to the laboratory, combined, filtered through eight layers of cheesecloth and flushed with CO2. 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. All handling was under continuous flushing with CO2. 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 rotaryshaker 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 for fat coated and uncoated samples. Kinetics of total GP was calculated (Ørskov and McDonald., 1979) for fat coated and uncoated barley grain. Differences in the composition and activity of rumen fluid inoculum were controlled by parallel measurements in incubation of buffered ruminal fluid without substrate (Blank test).Cumulative GP data were fitted to the exponential equation: $Y = a+b\ (1 - e-ct)$ where Y is the gas produced at t time, a the GP from the immediately soluble fraction (ml), b the GP from the insoluble fraction (ml), a+b potential of GP (after 96 h) from fermentable fraction (ml/200g DM), c the GP rate constant for b, and t is the time of incubation (h). The metabolizable energy (ME) contents and organic matter digestibility (OMD) were calculated using equations of Menke and Steingass (1988) as: ME (MJ/kg DM) = 0.157 × Gp + 0.0084 × CP + 0.022 × EE – 0.0081 × XA + 1.06, NEL(MJ/KgDM)= 0.115×GP + 0.0054×CP + 0.014×EE – 0.0054×XA – 0.36 ,OMD (g/100 g DM) = 0.9991 × Gp + 0.0595 × CP + 0.0181 × XA+ 9, Where CP is crude protein in g/100 g DM, XA ash in g/100 g DM and Gp is the net gas production (ml) from 200 mg after 24 h of incubation. Statistical analysis Data on *in vitro* GP were subjected to Analysis of Variance (ANOVA) in a completely randomized design using the SAS program General Linear Model (GLM) procedure (SAS, 9.1). Significant means were compared using the least square means method. Mean differences were considered significant at $P<0.05$ (5% test level of significance).Standard errors of means were calculated from the residual mean square in the analysis of variance.

Results and Discussion

Effect of *Viscum album* extracts on gas production was significant during whole incubation time ($P<0.05$) except at 4, 6, 8 and 12h (Table 1). Decreased gas production at mentioned hours of incubation may also be due to phenolic substances and anti-nutrients such as tannin and lectin in *Viscum album* extracts that can affect rumen microorganism functions (McSweeney et al., 1999). Furthermore, tannin could make complex with proteins preventing the enzyme activity (Frutos et al., 2004). The tannin phenolic group is a strong hydrogen donor to form hydrogen bond with the carboxyl group of the protein (Daneshmesgaran et al., 2009). It has been shown that tannin can affect gas production in the rumen (Makkar et al., 1997b). Salamatazar et al., 2012a reported that addition of 0.1, 0.2 and 0.3 ml of salvia extract decreased gas production from canola meal in 2, 4, 6, 8, 12 and 24h after incubation and addition of 0.3 ml of salvia extract reduced gas production from canola meal in 12 and 24h after incubation compared to the control which is in agreement with current study. At all levels increased gas production from canola meal at 72 and 96h after incubation that was not in agreement with our results (Niasati et al., 2014) reported that addition of 0.15 and 0.3ml of thyme extract increased gas production from sunflower meal in all of the hours of incubation that was not in agreement with our result (Salamatazar et al., 2012b). The researchers reported that the active compounds thymol and carvacrol in thyme extract improve protein metabolism in the rumen and increased gas production could be due to intake and thyme extract the inefficacy of the level of bacteria in rumen proteolytic especially a certain type of bacteria known as Hapbacteria and decreased gas production could be due to the effect of the active compounds in thyme, especially thymol the bacteria responsible for protein degradation in the rumen.

Potential of gas production (a + b), gas production from the insoluble fraction (b) and Gas production rate (c) for cottonseed meal significantly ($P<0.05$) decreased compared to the control (Table 2). Addition of 0.1, 0.2 and 0.3 ml of *viscum album* extract decreased Potential of gas production (a + b) from cottonseed meal in containing treatments compared to the control treatment. The least reduced amount of gas potential of cottonseed meal was observed in treatment 2(the use 0.1 ml of *viscum album* extract) in which the addition of 0.1 ml of *viscum album* extract caused a reduction from 40.87 ml to 26.36 ml. Niasati et al., 2014 reported that addition of 0.2, 0.4 and 0.6 ml of *viscum album* extract increased gas production potential of canola meal and soybean meal that was not in agreement with our result. Also Chalandari et al., 2014 reported that addition of 0.2 and 0.4 ml of nettle extract increased gas production potential of soybean meal that was not in agreement with our result. Salamatazar et al., 2012a reported that addition of 0.3ml of thyme extract decreased gas production potential from canola meal compared to control which was in agreement with current study. Also addition of 0.1, 0.2 and 0.3 ml of *viscum album* extract decreased gas production from the insoluble fraction of cottonseed meal compared to the control. The least reduced amount of gas production from the insoluble fraction of cottonseed meal was observed in treatment 2 (the use 0.1 ml of *viscum album* extract) in which addition

0.1 ml of *viscum album* extract caused a reduction from 38.73 ml to 27.37 ml. Addition of *viscum album* extract increased gas production rate (c) for cottonseed meal. The most increased amount of gas production rate (c) of cottonseed meal was observed in treatment 2(the use 0.1 ml of *viscum album* extract) in which the addition of 0.1 ml of *viscum album* extract caused an increase from 0.07 ml/h to 0.19 ml/h. Niasati et al., 2014 reported that addition of 0.2, 0.4 and 0.6 ml of *viscum album* extract decreased gas production rate (c) of canola meal and soybean meal that was not in agreement with our result.

Table 1: Effect of *vicum album* extract on gas production during incubation of cottonseed meal

	Incubation time (h)								
Treatment	2	4	6	8	12	24	48	72	96
1	6.74^{a}	13.95	17.56^{a}	21.78	25.39	30.41^{a}	36.83^{a}	37.14^{a}	48.59^{a}
2	5.90^{ab}	13.84	16.92^{ab}	21.11	25.43	26.30^{b}	27.29^{b}	24.02^{c}	26.29^{c}
3	5.28^{b}	13.41	16.84^{ab}	17.73	25.36	26.08^{b}	26.29^{b}	25.33^{bc}	29.17^{bc}
4	5.07^{b}	13.17	16.41^{b}	20.74	25.38	26.10^{b}	26.15^{b}	27.04^{b}	31.99^{b}
SEM	0.26	0.26	0.24	0.94	0.37	0.43	0.61	0.70	0.90
P- value	0.0221	0.4012	0.0859	0.3698	0.9997	0.0006	<0.0001	<0.001	<0.0001

1)Cottonseed meal without adding any extract to the rumen fluid, 2) addition of 0.1 ml of *viscum album* extract to rumen fluid which contained Cottonseed meal 3) addition of 0.2 ml of *viscum album* extract to rumen fluid including Cottonseed meal 4) addition of 0.3 ml of *viscum album* extract to rumen fluid which contained Cottonseed meal

Table 2: Effect of different levels of *Viscum album* extract on cottonseed meal gas production

Treatment	a+b	c	b
	ml	Ml/h	ml
1	40.87^{a}	0.07^{b}	38.73^{a}
2	26.36^{b}	0.19^{a}	27.37^{b}
3	27.14^{b}	0.16^{a}	27.92^{b}
4	28.26^{b}	0.16^{a}	29.27^{b}
SEM	0.62	0.08	0.40
P-Value	<0.0001	0.0003	<0.0001

1) Cottonseed meal without adding any extract to the rumen fluid, 2) addition of 0.1 ml of *viscum album* extract to rumen fluid which contained cottonseed meal 3) addition of 0.2 ml of *viscum album* extract to rumen fluid including cottonseed meal 4) addition of 0.3 ml of *viscum album* extract to rumen fluid which contained cottonseed meal. a+b : potential of gas production, c: rate of gas production and b: gas production from insoluble but fermentable fraction.

Table 3: Effect of different levels of *viscum album* extracts on organic matter digestibility, net energy lactation and metabolizable energy

Treatment	OMD	NEL	ME
	%	Mj/Kg DM	Mj/Kg DM
1	41.06^{a}	3.36^{a}	6.19^{a}
2	36.96^{b}	2.89^{b}	5.54^{b}
3	36.74^{b}	2.86^{b}	5.50^{b}
4	36.76^{b}	2.87^{b}	5.51^{b}
SEM	0.42	0.05	0.07
P-Value	0.0006	0.0006	0.0006

1)Cottonseed meal without adding any extract to the rumen fluid, 2) addition of 0.1 ml of *viscum album* extract to rumen fluid which contained cottonseed meal, 3) addition of 0.2 ml of *viscum album* extract to rumen fluid including cottonseed meal, 4) addition of 0.3 ml of *viscum album* extract to rumen fluid which contained cottonseed meal. OMD: organic matter digestibility, ME: metabolizable energy and NEL: net energy for lactation.

Also Chalandari et al., 2014 reported that addition of 0.2 and 0.4 ml of nettle extract decreased gas production rate (c) of soybean meal and canola meal that was not in agreement with our result. The most important property of tannin is protein binding capacity that prevents the effect of enzymes. The tannins are as inhibitors of microbial growth that can react with the cell wall and extra cellular enzymes that this process inhibits the transport of nutrients into cells and slows bacterial growth (Daneshmesgaran et al., 2009).

The amount of organic matter digestibility, metabolizable energy and net energy for lactation was significant (P<0.05) in *viscum album* (Table 3) that the addition of 0.1, 0.2 and 0.3 ml of *viscum album* extract decreased the amount of organic matter digestibility, metabolizable energy and net energy for lactation of cottonseed meal in all treatments compared to the control that *viscum album* extract is effects of microbial fermentation on rumen. The least reduced amount of organic matter digestibility,

metabolizable energy and net energy for lactation of cottonseed meal was observed in treatment 3(the use 0.2 ml of *viscum album* extract) in which addition of 0.2 ml of *viscum album* extract decreased the amount of organic matter digestibility from 41.06 (%) to 37.74 (%), the amount of metabolizable energy from 6.19 Mj/kg DM to 5.50 Mj/kg DM and the amount of net energy for lactation from 3.36 Mj/kg DM to 2.86 Mj/kg DM of cottonseed meal. 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). Salamatazar et al., 2012a reported that addition of 0.2 ml of salvia extract decreased organic matter digestibility, metabolizable energy and net energy for lactation of canola meal in compared to the control which is in agreement with current study. Niasati et al., 2014 reported that addition of 0.2 ml of *viscum album* extract decreased organic matter digestibility, metabolizable energy and net energy for lactation of canola meal and soybean meal in compared to the control which is in agreement with current study. Chalandari et al., 2014 reported that addition of 0.2 ml of nettle extract increased organic matter digestibility, metabolizable energy and net energy for lactation of canola meal and soybean meal in comparison to the control that was not in agreement with our result. Zarei et al., 2014 reported that addition of 0.15 ml of thyme extract increased organic matter digestibility, metabolizable energy and net energy for lactation of soybean meal in comparison to the control that was not in agreement with our result.

Conclusion In this study we find finally that by adding *viscum album* extract to fermentation syringes, the ruminal digestibility of cottonseed meal decreased and it may provide more bypass protein for intestine.

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