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In vitro Assessment of the Effect of Plant Extracts on Digestibility, Estimated Energy Value, Microbial Mass and Rumen Fermentation Kinetics

Research Article

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ABSTRACT

Three ethanol extracts, chamomile (CHA), clove (CLO) and tarragon (TAR), were tested at five doses (0, 250, 500, 750 and 1000 µL/L) to determine their effects on *in vitro* organic matter digestibility (IVOMD), metabolizable energy (ME), net energy of lactation (NEL), short-chain fatty acids (SCFA), microbial mass (MM) and rumen fermentation kinetics of a 40:60 forage: concentrate diet using *in vitro* gas production. These three extracts had significant effects on gas production kinetics. CHA (at 500 µL/L dose) and CLO (at 1000 µL/L dose) decreased ($P<0.05$) potential gas production. The initial gas production rate constants (*c*) was increased ($P<0.05$). However, CHA, CLO and TAR ethanol decreased ($P<0.05$) later gas production rate constants (*d*). Lag time (h) was decreased ($P<0.05$) due to addition of CHA and TAR ethanol (at 750 µL/L dose), and CLO (at 500 and 1000 µL/L doses). TAR and CLO ethanol did not affect fermentation rate (h^{-1}), but CHA at 1000 µL/L increased it. The TAR and CLO ethanol did not affect IVOMD, ME, NEL, SCFA and microbial mass. However, organic matter digestibility, ME, NEL, SCFA and microbial mass were increased by addition of CHA ethanol at 750 and 1000 µL/L doses. Results suggest that CHA, CLO and TAR ethanol extracts at appropriate doses may have potential to improve the rumen fermentation kinetics and nutritive value of ruminant diets due to secondary metabolites contents.

KEY WORDS *in vitro* gas production, plant ethanol extract, rumen fermentation kinetics.

INTRODUCTION

In the recent years, the use of plant extracts in dairy cattle rations have been considered worldwide by ruminant nutritionists especially after the prohibition of growth promoting antibiotics by the (EC number 1831/2003; European Union, 2003), because plant extracts were believed to be natural, safe and efficient without negative side effects. Secondary metabolites present in the natural plant extracts can modify rumen fermentation kinetics and improve milk production in dairy cattle (Alexander *et al*. 2007; Benchaar *et al*. 2008; Hart *et al*. 2008; Naseri *et al*. 2012; Naseri *et al*. 2015). It has also been observed that secondary metabolites suppressed protozoal populations, increased bacterial and fungal populations, propionate production, microbial yield and efficiency of microbial protein synthesis (EMPS), increased dietary dry matter (DM), organic matter (OM) and neutral detergent fibre (NDF) degradation and reduced dietary crude protein (CP) degradation and methanogenesis. A number of fast and cost-effective *in vitro* gas measurement methods have been used by several groups to evaluate the nutritional value of feedstuffs and kinetics of rumen fermentation (Getachew *et al.* 1998; Getachew *et al*. 2004; Makkar, 2005; Mirzaei-Aghsaghali *et al*. 2011a; Naseri *et*

al. 2015). These methods can provide useful data on fermentation kinetics of feedstuffs, prediction of feed intake (Khazaal *et al*. 1995; Mirzaei-Aghsaghali *et al*. 2011b), digestibility, and microbial nitrogen supply, amount of short-chain fatty acids, carbon dioxides and metabolizable energy of feeds for ruminants (Menke and Steingass, 1988; Babayemi, 2007; Mirzaei-Aghsaghali *et al*. 2008b; Mirzaei-Aghsaghali *et al*. 2008a; Maheri-Sis *et al*. 2008; Maheri-Sis *et al*. 2007). The ease of measuring fermentation end-products makes these methods more preferable (Makkar, 2005).

This work aimed to evaluate the *in vitro* gas production kinetics and estimate the *in vitro* organic matter digestibility (IVOMD), metabolizable energy (ME), short-chain fatty acids (SCFA), net energy of lactation (NEl) and microbial protein production of high-concentrate diet for dairy cattle after supplementing the feed material with ethanol extract of chamomilla (*Matricaria chamomilla*), clove (*Syzygium aromaticum*) and tarragon (*Artemisia dracunculus*).

MATERIALS AND METHODS

Selection of plants

Three medicinal plants: chamomile, clove and tarragon were selected on the basis of their traditional usage for the various digestive ailments, and in the light of recent literature (Patra, 2011).

Preparation of plant extract

Chamomile and tarragon leaves used in this study were collected at vegetative stage from Abidar Mountains and clove buds were purchased from local markets in Sanandaj (longitude 46.99 ˚E, latitude 35.32 ˚N and Köppen-Geiger climate), Iran. Approximately 100 g of fresh chamomile and tarragon leaves were cut into small pieces, placed into a blender (Saya Quick, QMC-20) and added 80 mL 70% ethanol then they were well blended three times for 5 minutes per time. The blended material was squeezed through four layers of muslin cloth into the labeled beaker and fibrous materials discarded. The combined filtrate was filtered using Whatman No.1 filter paper, and then transferred to a round-bottom Buchi flask. Also, the clove buds crushed into small pieces, oven-dried at 39 ˚C and ground to pass a 1mm screen. Fifty of ground sample was weighed into a 250 mL conical flask and added 200 mL 70% ethanol. The extraction was completed by placing the flasks in a shaker at 22 ˚C and 200 rpm for 24 h. Contents of the flask were squeezed through four layers of muslin cloth into the labeled beaker and fibrous materials discarded. The combined liquid phase was filtered using Whatman No.1 filter paper and then transferred to a round-bottom Buchi flask. Finally, ethanol was evaporated by using a vacuum evaporator (Heidolph Laborota 4011 digital) at 40-50 ˚C until the ethanol-streak stopped on the side of the bottle. The remaining concentrate was resuspended in 10 mL water, transferred into 10 mL sterile anaerobic crimped serum vials, and stored at -20 ˚C.

Inoculum and substrate

The inoculum was prepared according to the method of Tilley and Terry (1963). Briefly, rumen fluid was obtained from three rumen cannulated rams before the morning feeding. The rumen fluid was mixed on volume basis then it was bubbled with CO_2 for approximately 2 min and strained through four layers of cheese cloth. The incubation inoculum was prepared by diluting the fluid inoculum with the buffer (Tilley and Terry, 1963) in a 1:4 (V/V) ratio and stirring in a water bath at 39 ˚C with purging CO_2 until its use. The ration of the rams consisted of 40% alfalfa, 35% barley grain, 15% corn grain, 9% soybean meal, 0.5% salt and 0.5% vitamin-mineral premix. The substrate used in the *in vitro* ruminal fermentation was at 40:60 forage:concentrate ratio, formulated for dairy cattle (Table 1), oven dried (at 39 ˚C for 72 h) and finely ground to pass through a 1 mm screen.

In vitro gas production

The method used for gas production measurements was as described by Theodorou *et al*. (1994). Approximately 250 mg dry matter (DM) of substrate was weighed into 100 mL sterile tubes, kept at 39 ˚C. Plant extracts were added at different volumes (0, 250, 500, 750 and 1000 µL/L). Each sample was incubated in three replicates. Thirty milliliters of incubation inoculum (in the proportion of 20% rumen fluid+80% buffer) prepared (as described in the inoculum and substrate) and by flushing CO_2 before was anaerobically dispensed in each tube at 39 ˚C. The samples were swirled to mix the contents and placed in ashaker incubator (Thermoshaker Gerhardt) at 39 ˚C (Blümmel and Ørskov, 1993). The pressure of gas produced in each tube was recorded using a pressure transducer (Testo 512; Testo Inc., Germany) at 0, 2, 4, 8, 16, 24, 48 and 72nd h of incubation. To estimate the kinetics of gas production, data on cumulative gas volume produced were fitted using the generalized Mitscherlich model, proposed by France *et al*. (1993):

$$G = A\left(1 - e^{-c(t-L)-d\left(\sqrt{t}-\sqrt{L}\right)}\right)$$

Where:
G (mL): denotes cumulative gas production at time t.
A (mL): asymptotic gas production.
c (h^{-1}): initial gas production rate constant.
d ($h^{-1/2}$): later gas production rate constant rate constants.
L (h): lag time.

Calculation

The half-life ($t_{1/2}$, h) of the degradable fraction of substrate was calculated as the time taken for gas accumulation to reach 50% of its asymptotic value. The fractional degradation rate at $t_{1/2}$ ($\mu_{1/2}$, h^{-1}) was calculated as:

$$\mu_{1/2} = c + \frac{d}{2\sqrt{t_{1/2}}}$$

The metabolizable energy (MJ/kg DM) content of the substrate and *in vitro* organic matter digestibility were calculated using the equations below (Menke *et al*. 1979) as:

ME (MJ/Kg DM)= 2.20 + 0.136 GP + 0.0057 CP + 0.00029 EE^2

IVOMD (%)= 14.88 + 0.889 GP + 0.45 CP + 0.0651 XA

Where:
GP: 24 h net gas production (mL/250 mg^{-1}).
CP: crude protein (%).
EE: ether extract (%).
XA: ash content (%).

Short-chain fatty acid (SCFA) content was calculated using the equation of Makkar (2005); Maheri-Sis *et al*. (2007) and Maheri-Sis *et al*. (2008):

SCFA (mmol)= 0.0222 × GP – 0.00425 (Makkar, 2005).

Where:
GP: 24 h net gas production (mL/250 mg^{-1}).

Net energy for lactation (NEL) was calculated using the equation of Abas *et al*. (2005) as follows:

NEL (MJ/kg DM)= 0.115 GP + 0.0054 CP + 0.014 EE - 0.0054 CA - 0.36

Microbial mass (mg) was estimated using equation of Blummel *et al*. (1997):

Microbial mass (mg)= mg substrate truly degraded (OMD) - (GP×stoichiometrical factor)

The stoichiometrical factor was 2.20.

Chemical analysis

The substrate was analysed for DM (24 h at 103 ˚C), ash and organic matter (OM) (4 h at 550 ˚C), CP content was adapted for an automatic distiller Kjeldahl apparatus (Kjeltec Auto 1030 Analyser; Tecator, Höganäs, Sweden) and using $CuSO_4$/Se as catalyst instead of $CuSO_4/TiO_2$, ether extract using petroleum ether for distillation instead of diethyl ether (AOAC, 1990). The neutral detergent fibre (NDF) contents were determined as described (Van Soest *et al*. 1991).

Statistical analysis

Data were subjected to analysis of variance (ANOVA) using the general linear model (GLM). Significant differences between individual means were identified using Duncan's test (all pairwise multiple comparison procedures). All statements of significance were based on a probability of ($P<0.05$) (SAS, 1996).

RESULTS AND DISCUSSION

Chemical composition

The chemical composition of diet which used as fermentation substrate is shown in Table 1.

Table 1 Chemical composition (g/kg DM) of substrate used for *in vitro* gas production

Parameters	Substrate
Dry matter	945
Organic matter	900
Ash	100
Crude protein	160
Ether extract	40
Neutral detergent fibre (NDF)	300

Effect of plant ethanol extracts on *in vitro* rumen fermentation kinetics

Effect of ethanol extracts of chamomille, clove and tarragon on *in vitro* fermentation kinetics is presented in Tables 2, 3 and 4, respectively. Potential gas production (A) decreased (by 7%) significantly ($P<0.05$) by the addition of chamomile and clove extracts at 500 and 1000 (µl/L) doses, respectively.

In addition, 500 and 750 µL/L doses of tarragon extract were also found to be effective in decreasing potential gas production (A) by 8% ($P=0.07$).

The main active compounds of chamomile, clove and tarragon extract were terpenoids α-bisabolol and chamazulene, eugenol (phenylpropanoid) and methyleugenol, respectively (Janmejai *et al*. 2010; Jamalian *et al*. 2012; Renata and Grażyna, 2014).

These active compounds are known as of plant secondary metabolites, which include terpenoids, alkaloids and phenolics present in the essential oil fraction of many plants (Sallam *et al*. 2011).

Essential oils have antimicrobial activities against both gram-negative and gram-positive bacteria, a property that has been attributed to the presence of terpenoid and phenolic compounds (Conner, 1993; Dorman and Deans, 2000; Calsamiglia *et al*. 2007).

Table 2 Parameters estimated by fitting generalized mitscherlich model to gas production values, recorded for a high-concentrate diet for dairy cattle treated with different levels (0, 250, 500, 750 and 1000 µL/L) of ethanol Chamomile (*Matricaria chamomilla*) extract

Different levels of ethanol Chamomilla extract (µL/L)	Kinetics parameters					
	A (mL/250 mg DM)	*c* (h^{-1})	*d* ($h^{-1/2}$)	L (h)	Half-life (h)	Fermentation rate (h^{-1})
0	68.34^{ab}	0.017^{c}	0.330^{a}	0.740^{b}	7.24^{d}	0.080^{bc}
250	65.18^{bc}	0.043^{b}	0.193^{b}	0.667^{b}	7.84^{ab}	0.077^{c}
500	64.46^{c}	0.023^{c}	0.333^{a}	1.010^{a}	7.38^{cd}	0.080^{bc}
750	66.36^{abc}	0.077^{a}	0.060^{c}	0.417^{c}	8.06^{a}	0.087^{ab}
1000	69.35^{a}	0.063^{a}	0.143^{b}	0.757^{b}	7.65^{bc}	0.093^{a}
SEM	0.63	0.01	0.03	0.06	0.09	0.01
P-value	0.0324	< 0.0001	< 0.0001	0.0031	0.0004	0.0070

A: asymptotic gas production; *c* (h^{-1}): initial gas production rate constant; *d* ($h^{-1/2}$): later gas production rate constant rate constants and L (h): lag time.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Table 3 Parameters estimated by fitting generalized mitscherlich model to gas production values, recorded for a high-concentrate diet for dairy cattle treated with different levels (0, 250, 500, 750 and 1000 µL/L) of ethanol Clove (*Syzygium aromaticum*) extract

Different levels of ethanol Clove extract (µL/L)	Kinetics parameters					
	A (mL/250 mg DM)	*c* (h^{-1})	*d* ($h^{-1/2}$)	L (h)	Half-life (h)	Fermentation rate (h^{-1})
0	68.34^{a}	0.017^{b}	0.330^{a}	0.740^{a}	7.24^{c}	0.080
250	65.48^{ab}	0.073^{a}	0.073^{c}	0.503^{ab}	7.91^{a}	0.083
500	66.58^{ab}	0.057^{a}	0.120^{bc}	0.380^{b}	7.91^{a}	0.080
750	66.42^{ab}	0.053^{a}	0.177^{b}	0.570^{ab}	7.61^{b}	0.083
1000	63.80^{c}	0.073^{a}	0.073^{c}	0.283^{b}	7.79^{ab}	0.087
SEM	0.62	0.01	0.03	0.05	0.07	0.01
P-value	0.2275	0.0004	0.0002	0.0311	0.0005	0.3818

A: asymptotic gas production; *c* (h^{-1}): initial gas production rate constant; *d* ($h^{-1/2}$): later gas production rate constant rate constants and L (h): lag time.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Table 4 Parameters estimated by fitting generalized mitscherlich model to gas production values, recorded for a high-concentrate diet for dairy cattle treated with different levels (0, 250, 500, 750 and 1000 µL/L) of ethanol Tarragon (*Artemisia dracunculus*) extract

Different levels of ethanol Tarragon extract (µL/L)	Kinetics parameters					
	A (mL/250 mg DM)	*c* (h^{-1})	*d* ($h^{-1/2}$)	L (h)	Half-life (h)	Fermentation rate (h^{-1})
0	68.34^{ab}	0.017^{c}	0.330^{ab}	0.740^{b}	7.24^{b}	0.080
250	69.19^{a}	0.013^{c}	0.380^{a}	0.920^{a}	7.12^{b}	0.087
500	63.29^{b}	0.030^{b}	0.297^{b}	0.713^{b}	7.11^{b}	0.087
750	63.49^{b}	0.053^{a}	0.190^{c}	0.527^{c}	7.30^{ab}	0.087
1000	67.09^{ab}	0.027^{b}	0.273^{b}	0.673^{b}	7.49^{a}	0.080
SEM	0.89	0.01	0.02	0.04	0.05	0.01
P-value	0.0743	< 0.0001	0.0002	0.0015	0.0201	0.1705

A: asymptotic gas production; *c* (h^{-1}): initial gas production rate constant; *d* ($h^{-1/2}$): later gas production rate constant rate constants and L (h): lag time.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Debashis-Roy *et al*. (2015) have reported that eugenol hadamore effective antimicrobial potential in comparison with other non phenolic plant secondary metabolites because of the presence of a hydroxyl group in its phenolic structure and resulted in the loss of integrity of bacterial cell membrane and ultimately in reduction in glucose-uptake of bacteria. It has also been demonstrated that α-bisabolol and Chamazulene had the strongest activity against both gram-positive and gram-negative bacteria (Janmejai *et al*. 2010). However, decrease in potential gas production may be due to their secondary metabolites. In the present study, it was evidenced that other kinetic parameters of fermentation also affected.

Overall, initial gas production rate constant (*c*) increased (P<0.05) due to addition of plant ethanol extracts to medium.

But, ethanol extracts decreased (P<0.05) later gas production rate constant (*d*). Chamomile extract at 750 µL/L, clove extract at 1000 µL/L and tarragon extract at 750 µL/L had the lowest lag time, resulting in a faster rate of fermentation.

Effect of plant ethanol extracts on *in vitro* OM digestibility, estimated energy value and microbial mass

In vitro OM digestibility, estimated energy value and microbial mass results were presented (Tables 5, 6 and 7).

Table 5 Predictions of *in vitro* organic matter digestibility (IVOMD), metabolizable energy (ME), short-chain fatty acids (SCFA), net energy lactation (NEL) and microbial mass estimation (MM) for a high-concentrate diet for dairy cattle treated with different levels (0, 250, 500, 750 and 1000 µl/L) of ethanol Chamomile (*Matricaria chamomilla*) extract

Different levels of ethanol Chamomile extract (µL/L)	IVOMD (%)	ME (MJ/Kg DM)	SCFA (mmol)	NEL (MJ/Kg DM)	Microbial mass (mg)
0	73.14^{bc}	10.87^{bc}	1.26^{bc}	6.25^{bc}	58.10^{b}
250	71.04^{bc}	10.55^{bc}	1.20^{bc}	5.98^{bc}	58.05^{b}
500	70.85^{c}	10.52^{c}	1.19^{c}	5.95^{c}	58.04^{b}
750	74.05^{ab}	11.01^{ab}	1.28^{ab}	6.36^{ab}	58.13^{ab}
1000	76.75^{a}	11.42^{a}	1.34^{a}	6.72^{a}	58.19^{a}
SEM	0.68	0.10	0.02	0.09	0.02
P-value	0.0072	0.0073	0.0069	0.0070	0.0094

The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Table 6 Predictions of *in vitro* organic matter digestibility (IVOMD), metabolizable energy (ME), short-chain fatty acids (SCFA), net energy lactation (NEL) and microbial mass estimation (MM) for a high-concentrate diet for dairy cattle treated with different levels (0, 250, 500, 750 and 1000 µl/L) of ethanol Clove (*Syzygium aromaticum*) extract

Different levels of ethanol Clove extract (µL/L)	IVOMD (%)	ME (MJ/Kg DM)	SCFA (mmol)	NEL (MJ/Kg DM)	Microbial mass (mg)
0	73.14	10.87	1.26	6.25	58.10
250	73.67	10.95	1.27	6.32	58.12
500	73.33	10.90	1.26	6.27	58.11
750	73.27	10.89	1.26	6.27	58.11
1000	72.20	10.73	1.23	6.13	58.08
SEM	0.34	0.05	0.01	0.04	0.01
P-value	0.7805	0.7842	0.7134	0.7904	0.7561

The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Table 7 Predictions of *in vitro* organic matter digestibility (IVOMD), metabolizable energy (ME), short-chain fatty acids (SCFA), net energy lactation (NEL), and microbial massestimation (MM) for a high-concentrate diet for dairy cattle treated with different levels (0, 250, 500, 750 and 1000 µl/L) of ethanol Tarragon (*Artemisia dracunculus*) extract

Different levels of ethanol Tarragon extract (µL/L)	IVOMD (%)	ME (MJ/Kg DM)	SCFA (mmol)	NEL (MJ/Kg DM)	Microbial mass (mg)
0	73.14	10.87	1.26	6.25	58.10
250	71.39	10.60	1.21	6.02	58.06
500	70.52	10.47	1.19	5.91	58.04
750	72.09	10.71	1.23	6.11	58.08
1000	72.76	10.81	1.25	6.20	58.09
SEM	0.43	0.06	0.01	0.05	0.01
P-value	0.3194	0.3178	0.3083	0.3189	0.3793

The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Chamomile extract at 1000 µL/L increased (approximately 5 to 8%) significantly (P<0.05) IVOMD, metabolizable energy, SCFA, net energy lactation and microbial mass. Ethanol extracts of clove and tarragon did not affect *in vitro* OM digestibility of substrate, estimated energy value and microbial mass.

The results of GP measurement revealed that chamomile ethanol extract at 1000 µL/L resulted in an increase in GP compared with the control, which was consistent with an increase in IVOMD, metabolizable energy, SCFA and NEL. However, an increase in OM digestibility because of the addition of chamomile ethanol extract at high dose could also be attributed to stimulated bacterial activity (Naseri *et al*. 2012), which results in an increase in potential gas production. Generally, medicinal plants or their extracts usually yield complex mixtures of biochemical so that identification of the phytochemical fractions that might be involved in the effects observed was not possible (Scehovic, 1999).

However, three explanations can be made as follows: (1) the inhibitory or stimulatory action of plant secondary metabolites (PSM) on some rumen microorganisms; (2) the effect of the degradation products of PSM and (3) direct action of other secondary metabolites. Therefore, in the current study, our observations possibly might have resulted from the inhibitory or stimulatory action of PSM, especially from the presence of essential oils (EOs) on some rumen microorganisms.

CONCLUSION

In vitro effect of ethanol extracts of chamomilla (*Matricaria chamomilla*), clove (*Syzygium aromaticum*) and tarragon (*Artemisia dracunculus*) at differing concentrations on organic matter digestibility, estimated energy value, microbial mass, and rumen fermentation kinetics of a high-concentrate diet for dairy cattle, suggested that chamomile, clove and tarragon extracts have potential to alter rumen fermentation kinetics. However, these findings should be considered preliminary and further investigation should be undertaken which also use *in vivo* methods in order to better assess the value of these plant extracts as feed additives to improve the yield of dairy products.

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