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Investigation of potential new opportunities for plant extracts on rumen microbial fermentation *in vitro*

S.M.A. Sallam*, I.C.S. Bueno, P. Brigide**, P.B. Godoy**, D.M.S.S. Vitti** and A.L. Abdalla*,****

*Animal Production Department, Faculty of Agriculture, University of Alexandria, El-Shatby, Alexandria (Egypt)
**Centre for Nuclear Energy in Agriculture, University of São Paulo,
Av. Centenário 303, 13400-970 Piracicaba, São Paulo (Brazil)

Abstract. The fermentation characteristics of adding different levels (0, 0.5, 1.0 and 1.5 ml/75 ml buffered rumen fluid) of plant extracts to a basal substrate (50% roughage:50% concentrate) were evaluated *in vitro* by a semi automatic gas production (GP) technique. The investigated plant extracts were thyme (*Thymes capitus* – $T_{0.5}$, $T_{1.0}$, $T_{1.5}$), fennel (*Foenicum vulgary* – $F_{0.5}$, $F_{1.0}$, $F_{1.5}$), ginger (*Zingiber officinale* – $G_{0.5}$, $G_{1.0}$, $G_{1.5}$) and black seed (*Nigila sativa* – $BS_{0.5}$, $BS_{1.0}$, $BS_{1.5}$). The plant extracts linearly increased GP by 11.7 and 59.7% for $T_{1.5}$ and $BS_{1.0}$, respectively except $G_{1.5}$ and $BS_{1.5}$ which the GP decreased by 4.6 and 22.5%, respectively. All the plant extracts increased the methane concentration from 53 to 166% except $BS_{1.5}$ that decreased methane by 64.1%. Efficiency of microbial protein production was estimated *in vitro* by the partitioning factor (PF). All the extracts, except $BS_{1.5}$, decreased the PF value. Although inclusion of thyme extract increased the GP, it decreased significantly the dry and organic matter degradation (DMD and OMD). On the other hand, the $F_{1.5}$ improved significantly ($P < 0.05$) DMD and OMD. The black seed and fennel extract significantly decreased the NH_3-N concentration, while $T_{0.5}$, $T_{1.0}$, $G_{1.0}$ and $G_{1.5}$ increased it. The $G_{0.5}$, $BS_{1.5}$, $G_{1.0}$, and $G_{1.5}$ extracts decreased the protozoa number by 25.4, 25.4, 30.5 and 37.3%, respectively, while the thyme and fennel extract increased the protozoa number. This study suggested that the plant extracts have the potential to affect fermentation efficiency, and black seed extract could be a promising methane mitigating agent.

Keywords. Herbal plant extract – Gas production – Methane – Protozoa – Degradation.

***Identification de nouvelles opportunités à partir d'extraits de plantes pour la manipulation de la fermentation ruminale* in vitro**

Résumé. *Les caractéristiques de fermentation des extraits végétaux apportés à des doses croissantes (0, 0,5, 1,0 et 1,5 ml/75 ml de jus de rumen tamponné) à un substrat de base (50% fourrage:50% concentré) ont été évaluées* in vitro *par la technique semi-automatique de production du gaz (PG). Les extraits végétaux étudiés ont été : thym (*Thymes capitus – *$T_{0,5}$, $T_{1,0}$, $T_{1,5}$), fenouil (*Foenicum vulgare – *$F_{0,5}$, $F_{1,0}$, $F_{1,5}$), gingembre (*Zingiber officinale – *$G_{0,5}$, $G_{1,0}$, $G_{1,5}$) et la graine noire (*Nigila sativa – *$GN_{0,5}$, $GN_{1,0}$, $GN_{1,5}$). Les extraits végétaux ont augmenté linéairement la PG de 11,7 et 59,7% pour le $T_{1,5}$ et le $GN_{1,0}$, respectivement, à l'exception du $G_{1,5}$ et $GN_{1,5}$ qui diminuaient la PG de 4,6 et 22,5%, respectivement. Tous les extraits végétaux étudiés ont augmenté la production de méthane de 53 à 166% à l'exception du $GN_{1,5}$ qui l'a diminué de 64,1%. L'efficacité de la production de protéine microbienne* in vitro *a été estimée par le facteur de division (FD). Tous les extraits, sauf le $GN_{1,5}$, ont diminué le FD. Bien que l'ajout de l'extrait de thym ait augmenté la PG, elle a diminué significativement la dégradation de la matière sèche et de la matière organique (DMS et DMO). D'autre part, le $F_{1,5}$ a amélioré significativement la DMS et DMO. Les extraits de la graine noire et du fenouil ont diminué significativement la concentration de NH_3-N, tandis que $T_{0,5}$, $T_{1,0}$, $G_{1,0}$, et $G_{1,5}$ l'ont augmenté significativement. Les extraits $G_{0,5}$, $GN_{1,5}$, $G_{1,0}$, et $G_{1,5}$ ont diminué le nombre de protozoaires de 25,4, 25,4, 30,5 et 37,3%, respectivement, tandis que ceux du thym et de fenouil l'ont augmenté. Cette étude suggère que les extraits végétaux peuvent affecter l'efficacité de la fermentation. L'extrait de graine noire, surtout, pourrait être un agent prometteur de l'atténuation de méthane.*

Mots-clés. *Extrait végétal – Production de gaz – Méthane – Protozoaires – Dégradation.*

I – Introduction

The public concern over the routine use of antibiotics and growth promoters in livestock production

has increased recently because of the risk of the antibiotic residues presence in milk and meat and its effect on human health. These led to its prohibition in the European Union in 2006 in animal feeding. Accordingly, there is greater interest in using plants and plant extracts as alternatives to feed antibiotics to manipulate ruminal fermentation, improve feed efficiency and animal productivity. Many plants produce secondary metabolites such as phenolic compounds, essential oils, and sarsaponins (Calsamiglia *et al.*, 2007) that affect microbial activity. Although many plant extracts have been shown to affect microbial activity (Patra *et al.*, 2006), growth performance of growing lambs (Chaves *et al.*, 2007) and on milk production (Benchaar *et al.*, 2007). The objective of this study was to evaluate the potential of several natural plant extracts as antiprotozoal and antimethanogenic. The fermentation pattern has been also studied by the *in vitro* gas production test.

II – Materials and methods

The investigated herbal plant extracts were thyme [*Thymes capitus*, (T)], fennel [*Foenicum vulgary*, (F)], ginger [*Zingiber officinale*, (G)] and black seed [*Nigila sativa*, (BS)].

1. Procedure of plant extracts preparation

The plant extracts were prepared according to Patra *et al.* (2006) with some modifications. The plant materials were dried at 50°C and ground in mills to pass a 1 mm sieve and 100 g placed in 1000 ml of methanol solvent (95/100 ml). The flasks of all the solvents were stoppered and agitated with a magnetic stirrer for 24 h at room temperature. Then the solutions were centrifuged at 3000 g for 10 min. The residue was re-extracted with 500 ml of methanol for 24 h stirring at room temperature and centrifuged again at 3000 g for 10 min. The plant extracts were combined. Methanol was evaporated from the solution at approximately 42°C by using a rotary-evaporator. The concentrated extract was dissolved in distilled water and stored at 4°C for further use.

2. Treatments and experimental design

The different levels of plant extracts were added to the diet sample. Three levels (0.5, 1.0 and 1.5 ml/75 ml buffered rumen fluid) of each plant extract were investigated as follow: (i) no additive; (ii) thyme ($T_{0.5}$, $T_{1.0}$, $T_{1.5}$); (iii) fennel ($F_{0.5}$, $F_{1.0}$, $F_{1.5}$); (iv) ginger ($G_{0.5}$, $G_{1.0}$, $G_{1.5}$); and (v) black seed ($BS_{0.5}$, $BS_{1.0}$, $BS_{1.5}$). A mixed ration (50% roughage:50% concentrate) was used as substrate. Its chemical composition was 922.4, 131.0, 718.0, 343.0 and 20.0 g/kg for dry matter, crude protein, neutral-detergent fibre, acid-detergent fibre and ether extract, respectively. Untreated and treated samples of substrate were incubated with buffered rumen fluid (2:1, v/v) in 160 ml serum bottles for 24 h. Five adult rumen cannulated sheep grazing tropical grass pasture and a supplement based on maize and soybean meal (0.7 kg/100 kg of live weight, 20% crude protein) plus a mineral mixture were used as inoculum donor. Samples of rumen content containing solid and liquid fractions (50:50) were collected before the morning feeding through the cannula using a stainless steel probe (2.5 mm screen) attached to a large capacity syringe.

The *in vitro* gas production (GP) assay was carried out using a pressure transducer and data logger (LANA/CENA-USP, Piracicaba, SP, Brazil) for measuring the gas produced in 160 ml serum bottles incubated at 39°C (Mauricio *et al.*, 1999). Ground samples (0.5 g) were incubated in 75 ml of diluted rumen fluid (25 ml of mixed rumen fluid + 50 ml of Menke's buffered medium) in 160 ml serum bottles (Longo *et al.*, 2006). Once filled, all the bottles were closed with rubber stoppers shaken and placed in the incubator at 39°C. The bottles were shaken manually after the recording of the gas headspace pressure at 6, 14 and 24 h incubation using a pressure transducer (Theodorou *et al.*, 1994). Methane determination was done in a Shimadzu 2014 gas chromatograph equipped with a thermal conductivity detector. Separation was achieved using a shincarbon ST micro packed column, helium was the carrier gas with a flow rate of 10 ml/min. The detector and column temperature were 250 and 60°C, respectively. The test of linearity and calibration were

accomplished using the standard gas curve in the range of probable concentration of the samples. After incubation the contents of two bottles were used for determination of *in vitro* dry and organic matter degradability (DMD, OMD). The contents of other two bottles were used for determining the partitioning factor (PF, mg truly degraded substrate/ml gas produced) according to Blümmel and Becker (1997) and Blümmel *et al.* (1997). The NH_3-N concentration was measured according to Preston (1995) and, protozoa were microscopically counted following the procedure described by Kamra *et al.* (1991).

3. Statistical analysis

Data were subjected to analysis of variance (ANOVA), using the General Linear Model procedure, and significant differences between individual means were identified using the Tukey test (SAS, 2000).

III – Results and discussion

The effect of incubating the substrate *in vitro* during 24 h with different levels of plant extracts on gas and methane production (ml/g DM) is shown in Table 1.

Table 1. Effect of different levels of plant extracts on gas (ml/g DM) and methane production (ml/g DM) after *in vitro* incubation for 24 h

Treatments	Levels	GP	% change†	CH_4	CH_4 %††
No additive	–	127.3^{gh}	–	13.0^{d}	10.2
Thyme	$T_{0.5}$	170.3^{cde}	33.7	40.5^{b}	22.7
	$T_{1.0}$	183.4^{bc}	44.0	46.1^{b}	24.5
	$T_{1.5}$	203.4^{a}	59.7	60.3^{a}	27.3
Fennel	$F_{0.5}$	164.2^{cde}	28.9	37.1^{b}	19.9
	$F_{1.0}$	182.5^{bc}	43.3	43.3^{b}	22.4
	$F_{1.5}$	190.9^{ab}	49.9	43.2^{b}	21.6
Ginger	$G_{0.5}$	174.5^{bcd}	37.0	39.2^{b}	21.7
	$G_{1.0}$	154.6^{ef}	21.4	33.7^{bc}	20.1
	$G_{1.5}$	121.5^{h}	-4.6	22.6^{c}	15.6
Black seed	$BS_{0.5}$	158.6^{def}	24.6	32.0^{bc}	18.3
	$BS_{1.0}$	142.3^{gf}	11.7	15.6^{d}	10.8
	$BS_{1.5}$	98.7^{i}	-22.5	3.8e	3.7
SEM†††	–	6.9	–1.5	–	

†Percentage change in GP (%) = (observed GP in control substrate – observed GP in treated substrate)/observed GP in control substrate × 100.
††Methane proportion in total GP.
†††SEM: standard error of difference between means.
^{a,b,c,d,e,f,g,h,i}Means with different superscript, within column, are different (Tukey test; $P < 0.05$).

There were differences ($P < 0.05$) in cumulative GP after subtracting the blank gas volume for the different extracts levels. All the plant extracts and levels increased GP from 127.3 to 203.4 ml/g DM for the control substrate with no additive and $T_{1.5}$, respectively, except $G_{1.5}$ and $BS_{1.5}$ supplementation which decreased GP by 4.6 and 22.5%, respectively.

The plant extracts increased the methane concentration in compare to the control substrate from 53 to 166% except $BS_{1.5}$ which inhibited methane production by 64.1%. In general, rumen microbial activity has been shown to be affected by the use of plant extracts and secondary plant metabolites that may provide an alternative to ruminal modifiers for their ability to improve energy or protein use in the rumen (Kamel, 2001). As in this study, Patra *et al.* (2006) found *in vitro* that GP increased significantly with plant extracts supplementation. The increase in gas production might be partly due

to the addition of soluble sugars in the reaction mixture through inclusion of the extracts. Patra *et al.* (2006) reported that extracts of plants in methanol and water had more soluble sugars than with ethanol. The seed pulp of *Terminalia chebula* extracted by methanol reduced methane emission by 95% with the lower dose (0.25 ml/30 ml incubation medium) and the inhibition was almost complete at the double level of extract (Patra *et al.*, 2006). Phenolic acids such as p-coumaric, ferulic, cinnamic and, phloretic acids and some monomeric phenolic have been found to decrease methane, acetate and propionate production (Asiegbu *et al.*, 1995). The effects of different levels of plant extracts on PF, dry and organic matter degradability, NH_3-N concentration and protozoa count are presented in Table 2. Efficiency of microbial protein production *in vitro*, estimated by the partitioning factor at 24 h incubation, decreased with all the investigated plant extracts when compared to the control except $BS_{1.5}$ that improved it. The plant extracts affected ($P < 0.05$) the dry and organic matter *in vitro* degradation (DMD and OMD). The $G_{1.0}$, $G_{1.5}$ and $BS_{1.5}$ decreased significantly ($P < 0.05$) the DMD and OMD. On the other hand, the $F_{1.5}$ improved significantly ($P < 0.05$) DMD and OMD compared to that of the control. The $G_{0.5}$, $BS_{1.5}$, $G_{1.0}$ and $G_{1.5}$ extracts decreased the protozoa number by 25.4, 25.4, 30.5 and 37.3%, respectively, while the thyme and fennel extracts increased the protozoa number. The black seed, fennel extracts and $G_{0.5}$ decreased ($P < 0.05$) the NH_3-N concentration, while the three levels of thyme and $G_{1.5}$ increased it ($P < 0.05$). A depression in feed degradability by the extracts of thyme, ginger and black seed could be due to phenolic compounds such as tannins, galic acids, elagic acids and tannic acids. Tannins have been implicated for their inhibitory effect on feed digestion, microbial population and enzymes activity in many experiments (Makkar *et al.*, 1995; McSweeney *et al.*, 2001; Patra *et al.*, 2006). The observed reduction in ammonia N in the present trial suggests that plant extracts reduced amino acid deamination, as was indicated by Broderick and Balthrop (1979) with thymol.

Table2. Effect of different levels of plant extracts on partition factor (PF, mg truly digested organic matter/ml gas at 24 h), dry and organic matter degradability (DMD, OMD), NH_3-N concentration (mg/l) and protozoa counts ($\times 10^5$/ml)

Treatments	Levels	DMD	OMD	PF	Protozoa	NH_3-N
No additive	–	427.3^{ab}	400.6^{abc}	3.79^{ab}	2.2^{abc}	160.6^{b}
Thyme	$T_{0.5}$	346.1^{abc}	337.4^{abc}	2.99^{bc}	2.5^{a}	173.6^{a}
	$T_{1.0}$	363.8^{abc}	342.2^{abc}	2.38^{c}	2.4^{ab}	194.6^{a}
	$T_{1.5}$	370.8^{abc}	322.1^{abc}	2.4^{c}	2.7^{a}	147.0^{bc}
Fennel	$F_{0.5}$	418.2^{ab}	382.0^{abc}	2.81^{ab}	2.3^{ab}	135.5^{c}
	$F_{1.0}$	432.2^{ab}	405.4^{ab}	2.56^{c}	2.4^{ab}	126.0^{c}
	$F_{1.5}$	496.1^{a}	464.7^{a}	2.42^{c}	2.1^{abcd}	130.2^{c}
Ginger	$G_{0.5}$	402.1^{abc}	377.3^{abc}	2.7^{c}	1.7^{bcd}	105.0^{c}
	$G_{1.0}$	306.3^{cd}	296.3^{abc}	3.16^{abc}	1.5^{cd}	195.3^{a}
	$G_{1.5}$	247.3^{d}	255.5^{bc}	3.30^{abc}	1.4^{d}	183.4^{a}
Black seed	$BS_{0.5}$	424.2^{ab}	419.7^{ab}	2.88^{ab}	2.3^{ab}	129.2^{bc}
	$BS_{1.0}$	403.2^{ab}	362.8^{abc}	3.14^{abc}	2.7^{a}	100.8^{c}
	$BS_{1.5}$	292.8^{cd}	285.0^{bc}	4.00^{a}	1.7^{bcd}	104.0^{c}
SEM†	–	42.7	40.6	0.35	0.35	8.8

†SEM: standard error of difference between means.
[a,b,c,d]Means with different superscripts, within column, are different (Tukey test: $P < 0.05$).

The inhibition of amino acid deamination has practical implications because it may increase ruminal escape of dietary protein and improve the efficiency of N use in the rumen (van Nevel and Demeyer, 1988). A consistent finding when saponins are supplied to ruminants is a reduction in ruminal ammonia N concentration (Hristov *et al.*, 1999). These effects have been generally attributed to the pronounced antiprotozoal activity of saponins (Francis *et al.*, 2002), protozoa being the primary rumen ammonia producers. The antiprotozoal effect of saponins is due to their capacity

to form irreversible complexes with the cholesterol in the protozoal cell membrane, causing breakdown of the membrane, cell lysis, and death (Francis *et al.*, 2002). However, ruminal ammonia N concentration may increase (Hristov *et al.*, 1999) or decrease (Devant *et al.*, 2000) depending on the amount of degradable protein and on the amount and type of dietary carbohydrates available for microbial use (Russell *et al.*, 1983). This is probably the first report that extract of BS could inhibit rumen protozoa and this may be due to triterpenoids and steroid saponins in the BS extract. The inhibitory effect of these extracts on protozoa could be due to their saponin content. Decreased protozoal counts with supplementation of saponins-rich extracts (Kamra *et al.*, 2000) or saponin-rich forages (Newbold *et al.*, 1997) or fruits (Hess *et al.*, 2003) have been reported saponins possibly binding with sterol of cell membrane of protozoa and change its permeability.

IV – Conclusion

This study suggested that the plant extracts have the potential to affect ruminal fermentation efficiency, and black seed extract could be a promising methane mitigating agent.

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