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Gas production technique to evaluate the nutritive value of tannin-containing shrub species from a mountain area in northern Tunisia

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SUMMARY – The effect of tannins on the gas production kinetics of a mixture of leaves and fine green stems of *Arbutus unedo, Calicotome villosa*, *Erica arborea, Myrtus communis, Phillyrea angustifolia, Pistacia lentiscus and Quercus suber,* harvested in spring from a grazing mountain area of the northwest of Tunisia (Nefza), was investigated. Chemical composition and kinetics of *in vitro* gas production measured in presence and absence of polyethylene glycol (PEG 6000) were assessed. *C. villosa* had the highest crude protein (CP) and the lowest cell wall and phenolic contents; while *P. lentiscus* showed the lowest CP and the highest cell wall and phenolic contents. PEG supply resulted in the largest increase of gas produced at 24 h (G24) with *P. lentiscus* (71%). Foliage of tanniniferous species such as *A. unedo* and *P. lentiscus* could have a nutritive potential value as when as its tannin content is inactivated by using a tannin-binding agent.

Keywords: Browses, tannins, PEG, *in vitro* gas production.

RESUME – *"Technique de production de gaz pour évaluer la valeur nutritive des espèces d'arbustes contenant des tannins, d'un secteur de montagne au nord de la Tunisie". L´effet des tannins sur les cinétiques de production de gaz du mélange de feuilles et de brindilles de* Arbutus unedo, Calicotome villosa, Erica arborea*,* Myrtus communis*,* Phillyrea angustifolia, Pistacia lentiscus *et* Quercus suber *collectées au printemps dans la montagne du nord-ouest de la Tunisie (Nefza), a été étudié. La composition chimique et la cinétique de production de gaz, mesurées en présence et absence de polythylène glycol (PEG 6000), ont été déterminées.* C*.* villosa *a prouvé sa richesse protéique et sa faible concentration en composés phénoliques et paroi cellulaire. En revanche,* P. lentiscus *a montré des caractéristiques nutritives opposées. Suite à l´addition du PEG au milieu d´incubation, la production de gaz à 24 h (G24) a augmenté largement chez* P. lentiscus*. Les arbustes riches en tannins, tels que* P. lentiscus*, pourraient avoir un potentiel nutritif important si les tannins présents étaient inactivés moyennant l'utilisation d'agents inhibiteurs.*

Mots-clés : *Arbustes, tannins, PEG, production de gaz* in vitro.

Introduction

Herbivores, which fed on feedstuffs of widely variable quality, must match their nutritive needs and intake rates to the varying conditions of pasture. Along with grass in ruminant diets, brush plants are promising sources of protein if used as supplement to ruminants receiving low-quality forages (Devendra, 1990). However, many of the shrub species are problematic as feed supplements because they often contain antinutritional compounds such as tannins, saponins and non-protein amino acids, which are either toxic to rumen microbes or to the animal, or their metabolic products are toxic (Lowry *et al*., 1996). Thus, the quantification of tannins is important for predicting their effects on the nutritive value of tannin-containing browses and browsing animals. Nevertheless, there are problems with colorimetric analysis, which are caused by the variable structures of tannin polymers and the absence of satisfactory standards (Ammar, 2002). Recently, biological assays based on the use of tannin-binding agents such as polyethylene glycol (PEG), which strongly binds to tannins and thus inhibits their biological effects, have been developed (Jones and Palmer, 2000). Comparison can then be made between the biological properties of tannin-containing browses with and without PEG, the difference being a measure of the effect of tannins found in these browses.

The principal objective of the present study was to carry out a preliminary evaluation of shrubs, which have not been previously investigated, throughout the quantification of their tannin content and the study of their biological activity measured by the use of *in vitro* gas production technique with and without PEG (molecular weight 6000).

Material and methods

Leaves and current twigs of the season from the shrub plants *Arbutus unedo, Calicotome villosa*, *Erica arborea, Myrtus communis, Phillyrea angustifolia, Pistacia lentiscus* and *Quercus suber* were collected from the uplands of Taref in the delegation of Nefza (Norwest of Tunisia) and taken in spring of 1998. The climate is Mediterranean (mean annual rainfall and temperature is 900 mm and 21ºC, respectively). The browse plants were clipped with scissors harvesting a mixture of leaves and fine green stems (Ø <5 mm). In the laboratory, both leaves and stems were handily separated from the original samples, then they were immediately oven-dried at 40ºC and milled in a hammer mill using a 1-mm sieve for their later analysis.

Chemical analysis

Nitrogen content was determined using the Kjeldahl method (AOAC, 1995). Neutral detergent fibre (NDF) was determined according to the technique proposed by Van Soest *et al*. (1991) following the modifications proposed by ANKOM (1998). Total phenols (TP) were determined according to the method of Julkunen-Tiitto (1985) using the tannic acid as a standard. Total tannins (TT) were estimated indirectly after being adsorbed to insoluble polyvinylpyrrolidone (PVP) and measuring the remaining total phenols (non-precipitable phenols NPP) as described by Makkar *et al*. (1993). Free and bound condensed tannins (FCT, BCT, respectively) were measured using the butanol-HCl assay (ECT-but) reported by Porter *et al*. (1986) with the modifications of Makkar (2000). The purified quebracho tannin was used as a standard. Concentrations of all phenolics were expressed in g/kg DM, standard equivalent. The concentration of total condensed tannins (TCT) was therefore calculated as follows: TCT = FCT + BCT.

In vitro gas production

The method used for the gas production measurements was as described by Theodorou *et al*. (1994). Rumen fluid was obtained from the rumen of four Merino sheep housed in individual cages, fitted with rumen fistula and fed 1 kg alfalfa hay daily and with free access to water and mineral/vitamin licks. A sample of the rumen content was collected before the morning meal in thermos flasks and taken immediately to the laboratory where it was strained through four layers of cheesecloth and kept at 39ºC under a CO_2 atmosphere. Buffer solutions and rumen liquor/buffer (1:4) were prepared as described by Goering and Van Soest (1970). Incubation was carried out in serum bottles with or without addition of 2 ml of PEG solution (50 g PEG/200 ml water). Ground samples (300 mg) were weighed out into serum bottles kept at approximately 39ºC and flushed with CO_2 before use. Six bottles were used for each substrate, three for each treatment (with or without PEG). 50 ml of rumen/buffer mixture were anaerobically dispensed in each bottle at 39ºC. All the bottles were crimped and placed in the incubator at 39ºC, shaking them at regular times. The volume of gas produced in each bottle was recorded at different inoculation times (3, 6, 9, 12, 16, 21, 26, 31, 36, 48, 60, 72, 96, 120 and 144 h) using a pressure transducer (Theodorou *et al*., 1994). In order to compensate for gas production in the absence of substrate three serum bottles containing rumen fluid inoculum with or without PEG were incubated as controls. Incubations were performed in one triplicated run (3 bottles/sample/treatment). In order to estimate the kinetics of gas production, data of the cumulative gas volume produced were fitted to the exponential model proposed by France *et al*. (2000): $G = A\ (1\text{-}e^{-c(t-L)})$, where G (ml) denotes the cumulative gas production at time t; A (ml) is the asymptotic gas production; c (h^{-1}) is the fractional rate of gas production and L (h) is the lag time. A, c and L are constant parameters.

Statistical analysis

Analysis of variance (Steel and Torrie, 1980) was performed on *in vitro* digestibility and gas production kinetics data. The statistical significance of the differences between means was tested using the Duncan test.

Results and discussion

Although many Mediterranean shrubs gained increasing significance as the nutritional value of grass drops, they never reached a prominent place in the diet, since their crude protein content is often less than 10% (Cabiddu *et al*., 2000). In the present study, CP content was particularly low (<100 g/kg DM) in *A. unedo*, *E. arborea*, *P. lentiscus* and *Q. suber* (Table 1). These low contents were probably due to high proportions of mature leaves and twigs included in the analysis. Nonetheless, the remaining species and particularly *C. villosa,* maintained crude protein levels (230 g/kg DM) up to 8.4% that goats need for maintenance and production (NRC, 1981). This may be misleading, however, because not all the protein is digestible and anti-quality factors may further reduce available protein below what crude protein concentration is needed. Therefore, CP should not be the sole criteria of judging the relative importance of a particular species. The high CP found in *C. villosa* may be due to at least to the fact that none of the studied species is leguminous. Therefore, its foliage would be a good protein supplement to low quality Mediterranean fodders. The richness of leguminous species in CP was confirmed earlier (Ammar, 2002). All of the shrubs contained significant NDF. These high levels seem to be overestimated since tannins and tannin-protein complexes may appear as lignin or neutral detergent insoluble nitrogen and therefore will apparently increase the content of neutral detergent fibre in the plant.

Table 1. Chemical composition (g/kg DM) and tannin contents (g/kg DM, standard equivalent) of shrub species

Species	CP	NDF	TP	FCT	BCT	TCT
A. unedo	65	410	208	177	55	232
C. villosa	221	572	92	1	9	10
E. arborea	80	524	249	221	58	279
M. communis	130	383	227	49	55	104
P. angustifolia	110	445	230	2	10	12
P. lentiscus	60	433	261	363	42	405
Q. suber	70	551	161	103	48	151

CP: crude protein; NDF: neutral detergent fiber; TP: total phenol; BCT: bound condensed tannins; TCT: total condensed tannins.

Tannin contents in examined shrubs revealed a considerable variation between species (Table 1). *P. lentiscus* and *E. arborea* showed relatively high contents in FCT (>20% DM), according to the findings of Tolera *et al*. (1997) who confirmed the richness of *Erica* sp. in CT. These levels are clearly superior to those considered to be potentially detrimental for herbivores. While *C. villosa* and *P. angustifolia* had low contents of FCT which would generally be considered unlikely to significantly affect digestion of nutrients in ruminants. With high protein content and low CT, *C. villosa* would be regarded as having potentially high nutritive value. On the other hand, the presence of evergreen leaves in *E. arborea* would make it more susceptible to consumption throughout the year, which is consistent with their high tannin content throughout the year. It is pertinent to mention that a high proportion of TCT content was recovered as FCT. This result is consistent with the findings reported by Barry and McNabb (1999) and confirmed by a high positive correlation between both fractions (Table 3) ($r = 0.99$, $P<0.001$). It is believed, therefore, that any detrimental effect of CT on microbial fermentation of nutrients in the rumen should be explained by the concept of 'free tannins'.

Furthermore, results depicted in Table 1 displayed a considerable variation between the different phenolics related to the method of analysis. This variation is expected since the chemical properties that are involved in the reactivity of polyphenols determined by the different methods are widely different. The deficiencies in the analytical method used for the quantification of tannin contents can explain partly the unrealistic concentrations of TCT in some browsing samples. For example, it seems very unlikely that TCT concentration will be higher than TP concentration, as there are many other phenolic compounds different from condensed tannins. Therefore, these methods should be used with caution as a quantitative assay.

It is well established that the *in vitro* gas method combined with the use of PEG 6000 is expected to be better than chemical methods for quantification of anti-nutritional factors. The percentage increase in gas production represents the effect of tannins; the higher the biological activity of tannins on rumen microbes, the higher the increase in gas production in presence of PEG. Clearly there were large differences between shrub species in response to PEG in term of kinetics of gas production (Table 2). The largest improvement was observed with *P. lentiscus* followed by *A. unedo*. Addition of PEG to tannin-free plants (i.e. *C. villosa* and *Ph. angustifolia*) did not increase the *in vitro* gas production and may result in negative effects by decreasing the efficiency of microbial synthesis (Getachew *et al*., 2000).

Table 2. Kinetics of *in vitro* gas production in presence and absence of PEG 6000

Species	G24 (mL/g)			A (mL/g)			c (h^{-1})		
	– PEG	+ PEG	s.e.d.	– PEG	+ PEG	s.e.d.	– PEG	+ PEG	s.e.d.
A. unedo	156	224	3.34	263	299	5.05	0.037	0.058	0.00088
C. villosa	211	208	1.52	343	352	2.33	0.040	0.037	0.00015
E. arborea	126	177	1.96	217	248	1.90	0.036	0.052	0.00107
M. communis	151	217	9.76	292	315	12.98	0.030	0.049	0.00046
Ph. angustifolia	197	206	2.82	291	299	4.06	0.047	0.049	0.00028
P. lentiscus	127	217	1.46	190	288	2.31	0.046	0.058	0.00098
Q. suber	122	138	2.61	187	214	4.29	0.044	0.043	0.00155

These results strongly indicate that addition of PEG is advantageous if the tannin content of the feed is high to the extent that it depresses microbial activity and digestibility of feeds drastically. It is pertinent to mention that effect of PEG on kinetics of gas production from *M. communis* was larger than that from *Q. suber* (Table 2), which contained much more tannins (Table 1). Thus, it appears that the extent of positive or negative effect of tannin in the shrubs vary depending not only on the level of tannins in plants but also on their type and their biological activity. If the activity of CT in all shrubs was the same then a positive linear improvement in gas production due to PEG would be expected as the level of CT increased.

As depicted in Table 3, tannin contents and their biological activity show a positive interrelationship, according to what has been reported in our previous study (Ammar, 2002).

Table 3. Correlation coefficients (r) between tannins and their biological activity

	FCT	BCT	TCT	TP	G24PEG	APEG	cPEG
FCT	1.00	0.77	0.99	0.67	0.85	0.87	0.41
BCT		1.00	0.83	0.60	0.84	0.57	0.73
TCT			1.00	0.68	0.88	0.85	0.47
TP				1.00	0.79	0.61	0.57
G24 PEG					1.00	0.78	0.73
APEG						1.00	0.14
cPEG							1.00

For n = 7, $P<0.05$ if $r>0.75$; $P<0.01$ if $r>0.87$; $P<0.001$ if $r>0.95$.
APEG, G24PEG and cPEG denote the effect of PEG on the parameters A (asymptotic of gas production), G24 (gas production 24 h) and c (fractional rate of gas production).

The highest correlation ($P<0.01$) observed between TCT and *in vitro* gas production improvement due to PEG (G24PEG) supports the concept that PEG may replace tannins in pre-existing complexes.

Furthermore, this high correlation confirms the widely believed opinion that the *in vitro* gas production technique coupled with the use of PEG is regarded as a useful tool for screening the potential effects of tannins in shrub species due to its simplicity and accuracy.

Conclusions

It is concluded that the moderate to high crude protein content and low tannin contents found in *C. villosa*, *P. angustifolia* and *M. communis* give theses plants high nutritional value. However, foliage of tanniniferous species such as *A. unedo* and *P. lentiscus* could have a nutritive potential value when their tannins are inactivated by using a tannin-binding agent such as PEG 6000.

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