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POLYPHENOLIC COMPOUNDS OF NUTRACEUTICAL TREES AND THE VARIABILITY OF THEIR BIOLOGICAL ACTIVITY MEASURED BY TWO METHODS

[CONTENIDO DE COMPUESTOS POLIFENOLICOS EN ÁRBOLES FORRAJEROS Y VARIABILIDAD DE SU ACTIVIDAD BIOLÓGICA MEDIDA CON DOS MÉTODOS]

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SUMMARY

The aim was to determine the differences and variability in the quantity of polyphenolic compounds (PCom) and their biological activity (BA) amongst and within three tannin-rich tree species namely, *Acacia pennatula*, *Lysiloma latisiliquum* and *Piscidia piscipula*. *Acacia pennatula* had the highest levels of total phenols (TP) ($P<0.001$) and BA measured with two techniques ($P<0.01$). Total phenols showed strong association with BA measurements. There was significant variability in PCom and BA amongst trees of the same species as well as between species. Future studies are required to understand the origin of these variations better before implementing the use of these browse trees as nutraceuticals under farm conditions.

Keywords: Tree fodders; polyphenolic compounds; tannins; biological activity; *in vitro* gas production; variability

RESUMEN

El objetivo del presente trabajo fue estudiar las diferencias y la variabilidad en el contenido de compuestos polifenólicos (PCom) y su actividad biológica (BA) dentro y entre tres especies de árboles forrajeros ricos en taninos, *Acacia pennatula*, *Lysiloma latisiliquum* y *Piscidia piscipula*. *Acacia pennatula* tuvo el mayor contenido de fenoles totales (TP) ($P<0.001$) y la mayor BA.Los fenoles totales mostraron una fuerte asociación con el valor de BA. Se encontró una variabilidad significativa en PCom y BA entre árboles de la misma especie y entre especies. Se requieren estudios que ayuden a entender el origen de las variaciones antes de poder implementar el uso de estos follajes como nutraceúticos en condiciones de campo.

Palabras clave: Arboles forrajeros; compuestos polifenolicos; taninos; actividad biológica; producción de gas in vitro; variabilidad.

INTRODUCTION

Acacia pennatula, *Lysiloma latisiliquum* and *Piscidia piscipula* have been suggested as plants of nutritional and healthy properties for small ruminants. These tannin-rich plants (TRP) are an important source of nutrients, especially protein (Sotelo et al., 1995, Sandoval-Castro et al., 2005). The intake of the leaves of these three species by goats and sheep seems to be regulated by their digestible fibre fraction, rather than by their polyphenolic (PCom) compounds content (Alonso-Díaz et al., 2008a, 2009). Additionally, acetone/water extracts of *A. pennatula*, *L. latisiliquum* and *P. piscipula* have showed an *in vitro* anthelmintic (AH) effect against important abomasal (*Haemonchus contortus*) (Alonso-Díaz et al., 2008b) and intestinal (*Trichostrongylus colubriformis*) (Alonso-Díaz et al., 2008c) parasitic nematodes related with their bioactive compounds (tannins).

Accordingly, these plants have been suggested as nutraceuticals (non-conventional anthelmintics) for small ruminants within sustainable grazing systems. However, in agreement with Hoste *et al*. (2008), Athanasiadou *et al*. (2007) and Makkar *et al*. (2007), the study of the variability of bioactive compounds contents in plants is an indispensable step towards a sustainable tool for the management of gastrointestinal nematodes in ruminants. The concentrations of plant secondary metabolites and their biological activity (BA) may vary with factors

inherent to the plant and/or environmental conditions in which the plants are growing (Makkar et al., 2007).

The objectives of this study were: i) to determine the differences of polyphenolic compounds (PCom) and their BA of *Acacia pennatula*, *Lysiloma latisiliquum* and *Piscidia piscipula*, ii) to determine the variability of the BA within each tree species, and iii) to evaluate the relationship amongst PCom and BA.

MATERIAL AND METHODS

Study area

This study was conducted in the Faculty of Veterinary Medicine and Animal Science, University of Yucatan, Mexico (N 22° 30', W 89° 30'). Climate of the area is AW_0 (tropical warm sub-humid with summer rainfall). The soil type of the area is a mixture of litosol and rendzina (Flores and Espejel, 1994). The browsing area of collection was a 10 ha sub-humid tropical forest with 15 to 20 years of growth. The vegetation of this area was previously described by Casanova-Jimenez (2000). The average annual temperature ranges from 26 to 27.8 °C, and annual precipitation ranges from 940 to 1100 mm (Garcia, 1988).

Plant material collection

All the plant material was collected between June and July 2006 from a 10ha browsing area of 20 years under continuous use by goats and sheep. A sample of at least 500 g of fresh leaves were taken from each of thirteen trees of each of the species viz. *A. pennatula*, *L. latisiliquum* and *P. piscipula*. Trees were randomly selected from the browsing area only taking care to collect as evenly as possible from all the 10 ha and do not concentrate collection in a small subplot. Botanists in the FMVZ-UADY herbarium did the taxonomic identification of the trees. After harvesting, plant materials were placed in a cool box and were delivered to the laboratory in less than one hour. Each sample was oven dried at 50 ºC for 72 h and then ground to pass through to 1.0 mm sieve.

Extraction from plant materials

Extracts from each individual plant were obtained to measure their PCom content and their BA. The extraction was made using acetone:water (70:30 v/v) adding ascorbic acid (1 g L^{-1}) to avoid oxidation of the extract. The mixture was then sonicated for 20 min in a water bath (Branson 5510®), then filtered using a filter paper to obtain the extract. Pigments were removed from the extracts with methylene chloride. The final extracts were refrigerated at 4 °C in airtight containers until use for biochemical and biological assays.

Chemical analysis

Total phenols (TP) and Total Tanins (TT) present in the plant extracts were determined using the Folin-Ciocalteu method (Makkar 2003). Firstly, the TP were determined. Then polyvinyl polypyrrolidone (PVPP) was added to the extract to calculate TT, by difference of measurements from the same material. Tanins were quantified with a diode array spectrophotometer (Agilent 8453®). Standard solutions were formulated with the Folin-Ciocalteu reagent and the calibration curve was made with tannic acid. The quantification of TP and TT was made at 725 nm. The tannins were expressed as tannic acid equivalent.

The condensed tannin (CT) content of the extracts was quantified using the Butanol assay. (Makkar, 2003). This method is based on the oxidative cleavage of the interflavan bonds in the presence of mineral acids in alcoholic solutions at 95-100 °C. The quantification of CT was made by spectophotometry at 550 nm (Agilent 8453®). The CT were expressed as leucocyanidin equivalent.

Biological activity

Two complementary techniques were used to assess the biological activity of the extract. The radial diffusion assay is a measure of astringency of the compounds and their ability to bind to protein molecules. The *in vitro* gas production technique detects possible effects upon rumen microbial communities and hence modification or impairment of fermentation.

Radial Diffusion Assay

Biological activity was determined with a radial diffusion assay (BARD) (Hagerman, 1987) as modified by Reyes (1993). The technique determined the protein precipitation capacity of tannins by the formation of insoluble protein–tannin complexes in a protein-containing agar plate. Agar was prepared with 1% agarose (Baker A247-05®) in acetate buffer and bovine haemoglobin (Sigma H-2625®) (100 mg per liter of agar). The pH was adjusted to 5.0 with NaOH. Ten ml of agar were placed in Petri dishes (10 cm diameter). On each Petri dish, five wells (4 mm diameter each) were made in the agar (one in the middle and four in the outer regions). The outer wells were used to place 15 µl of a solution of each extract (0.1 g of each extract was re-suspended in 10 ml acetone solution 70%). Then, 15 µl of a resorcinol solution (5 g of resorcinol

in 5 ml of a methanol solution 70%) was placed in the centre well as a standard. Samples were incubated for 24 h at 25 °C. The area of agar within which the tannins precipitated the haemoglobin was indicative of the BA of the respective extract. The diameter of radial diffusion was measured with a digital caliper and activity was expressed relative to the standard.

In vitro gas production. Rumen liquor was obtained from two crossbred cows (*Bos indicus* x *B. taurus*) receiving 700 g kg^{-1} freshly cut forage (*Pennisetum purpureum*) and 300 g kg^{-1} concentrate with 180 g kg^{-1} CP. Rumen liquor was collected before the morning feeding and immediately transported to the laboratory in an airtight container (100 m distance). Preparation of the N rich media and rumen liquor were made as described by Menke and Steingass (1988). *In vitro* gas production (IVGP) was measured as described by Theodorou et al. (1994). Foliage samples (0.5 g DM) were incubated in 100 ml capacity serum bottles with and without 0.5 g Polyethyleneglycol (PEG) (4000 MW, Sigma®) in a single run. Each sample was incubated by triplicate with 54 ml of media and 6 ml of rumen liquor. Readings were made every three hours up to 24-h of incubation. In this study, the difference between the quantity of gas production with and without PEG was used as the BA of tannins measured as gas production (BAIVGP) (Makkar 2003).

Statistical analysis

Analyses of variance (ANOVA) were used to compare the PCom and BA among tree species. Condensed tannins (CT), IVGP (with and without PEG) and BARD data were log-transformed (log10) before statistical analyses to normalize their distribution and/or to equalize variances. Total phenols and TT were analyzed by Kruskal Wallis tests. ANOVA was also used to compare the BA variability within tree species. Radial diffusion data were analyzed using a Kruskal Wallis test. Pearson correlations were used to determine the association between PCom content and the BARD and the IVGP with and without PEG. All data was analyzed with the SAS software (SAS, 1991).

RESULTS

Comparison of polyphenolic compounds and biological activity between the various nutraceutical trees

The PCom content, the BARD and the IVGP with and without PEG varied amongst tree species (Table 1). *Acacia pennatula* had the highest levels of TP and BARD ($P<0.05$). The mean TT and CT of *A. pennatula* were higher than that of *P. piscipula* ($P<0.05$) but similar to *L. latisiliquum* ($P>0.05$). The highest levels of IVGP without PEG were found in *P. piscipula* followed by *L. latisiliquum* and *A. pennatula* ($P<0.05$). With the addition of PEG, *A. pennatula* leaves had the highest increase in BAIVGP ($P<0.05$).

Variability of biological activity within nutraceuticaltree species

The BA of tannins, measured as BARD, varied within the tree species ($P<0.05$) (Table 2). When the BA was measured as BAIVGP, a significant variability was only found for *A. pennatula* ($P<0.05$).

Relationship between PCom and BA in nutraceutical trees

When the three nutraceutical species were included in the same database (n=39), the TP contents were strongly associated to IVGP without PEG ($P<0.01$; $r = -0.90$) and the BA measured as BARD ($P<0.001$; $r = 0.85$) and BAIVGP ($P<0.05$; $r= 0.74$) (Table 3). Within each species, TP content was associated only to IVGP without PEG in *A. pennatula* ($P<0.05$; $r = -0.59$) and in *L. latisiliquum* ($P<0.001$; $r = -0.87$) (Table 4). The PCom contents of *P. piscipula* were not related with IVGP ($P<0.05$) (Table 4). However, the TP and CT of *P. piscipula* were associated with the BARD and BAIVGP (Table 4).

Table 1. Differences between polyphenolic compounds and biological activity in 3 nutraceutical tree species

	Acacia pennatula (n =13)				*Lysiloma latisiliquum* (n =13)				*Piscidia piscipula* (n =13)			
	Min	Max	Mean	S.E.	Min	Max	Mean	S.E.	Min	Max	Mean	S.E.
TP[*]	73.6	124.6	97.2[a]	4.6	16.9	74.2	39.3[b]	4.4	12.9	40.3	20.6[b]	2.1
TT[*]	11.2	98.7	31.8[a]	6.3	8.9	28.2	17.3[ab]	1.7	9.1	17.5	12.6[b]	0.8
CT[†]	7.3	30.6	16.3[a]	2.3	5.2	32.4	12.0[ab]	2.1	3.8	15.9	9.2[b]	1.1
BARD[**]	2.3	5.20	3.6[a]	0.2	0.6	1.2	0.9[b]	0.1	0.4	1.5	0.7[c]	0.1
IVGP(-)[††]	27.1	42.2	32.7[a]	1.1	35.7	50.5	43.6[b]	1.3	44.5	61.7	52.3[c]	1.3
IVGP (+)[††]	37.7	59.8	49.2[a]	1.2	40.0	56.7	50.2[a]	1.3	50.9	65.7	56.4[b]	1.1
BAIVGP[††]	5.7	24.5	16.5[a]	0.5	1.1	19.3	6.6[b]	1.3	0.2	12.5	4.1[c]	0.9

TP = Total phenols; TT= Total tannins; CT= Condensed Tannins; BARD= biological activity measured as radial diffusion; IVGP (-)= *In vitro* gas production without PEG; IVGP (+)= *In vitro* gas production with PEG; BAIVGP= biological activity measured as the difference between the quantity of gas production with and without PEG; S.E.= standard error

[*] Expressed as g tannic acid eq. (g kg $^{-1}$ DM)

[†] Expressed as g leucocyanidin eq. (g kg $^{-1}$ DM)

[**] Measured as units of precipitation per g of plant relative to resorcinol standard

[††] L gas kg $^{-1}$ DM

Different letter between columns indicate differences statistically significant (P<0.05)

Table 2. Variability of the biological activity, measured by the radial diffusion method (BARD) and *in vitro* gas production (BAIVGP) for each of the nutraceutical trees

Tree	*Acacia pennatula*		*Lysiloma latisiliquum*		*Piscidia piscipula*	
	BARD[**]	BAIVGP[††]	BARD[**]	BAIVGP[††]	BARD[**]	BAIVGP[††]
1	4.5±0.1	5.6±1.2	1.1±0.05	7.6±2.6	0.7±0.07	4.2±1.5
2	4.6±0.1	7.3±2.5	0.6±0.04	8.0±2.9	0.9±0.16	12.5±0.6
3	4.1±0.2	24.0±2.6	0.6±0.01	-1.9±1.9	0.7±0.09	8.8±1.3
4	2.9±0.1	16.0±3.2	1.1±0.05	1.1±1.5	0.6±0.02	5.2±1.5
5	3.3±0.1	12.0±0.5	0.5±0.01	2.7±2.7	0.7±0.01	4.7±1.6
6	4.4±0.1	11.6±1.4	0.9±0.1	-1.6±1.8	0.7±0.02	0.4±1.4
7	3.5±0.1	24.5±3.3	0.75±0.01	14.5±2.9	1.5±0.1	9.8±0.4
8	3.5±0.2	12.7±2.1	0.91±0.05	4.3±4.1	0.3±0.01	-1.5±0.2
9	3.9±0.4	17.6±2.4	1.1±0.05	6.9±3.1	0.8±0.03	1.6±2.1
10	5.2±0.7	23.8±1.2	0.8±0.03	7.6±3.9	0.7±0.03	1.0±0.9
11	2.6±0.07	15.1±4.3	1.1±0.05	5.9±4.1	0.7±0.04	3.2±0.7
12	2.3±0.1	24.3±2.1	1.2±0.04	9.8±4.4	0.6±0.03	0.1±2.2
13	2.7±0.1	19.8±0.9	1.2±0.04	19.2±2.1	0.5±0.02	3.6±3.4
	P<0.05	P<0.05	P<0.05	P>0.05	P<0.05	P>0.05

BARD= biological activity measured as radial diffusion (mean ± standard error of 4 replicates); BAIVGP= biological activity measured as the difference in gas production with and without PEG (mean ± standard error of 3 replicates)

[**] Measured as units of precipitation per g of plant extract relative to resorcinol standard

[††] g kg $^{-1}$ DM

DISCUSSION

The first objective of this study was to determine the difference of PCom content, IVGP and BA amongst the tree species. Of the three species studied *Acacia pennatula* had the highest levels of TP, TT, CT and BA (measured either as BARD or as BAIVGP). The results of PCom content and BA obtained were consistent with previous reports (Alonso-Díaz et al., 2008a, 2009, Monforte-Briceño et al., 2005, Sandoval-Castro et al., 2005). Tropical tannin rich plant (TRP) have been pointed out as trees that contain high levels of protein and tannins (Getachew et al., 2002, Makkar et al., 2007, Muller-Harvey, 2006, Osuga et al., 2007). These characteristics have generated a discussion about their possible use as a protein supplement, in spite of their tannin or PCom content. Tannins may affect the intake of ruminants

due to factors such as astringency, reduced digestibility and possible toxicity (Makkar et al., 2007). The antinutritional effects of the TRP seem to occur when the levels of CT are above 50 g kg $^{-1}$ DM (Mueller-Harvey, 2006). Previous preference studies performed both in goats and sheep, using the same plants as in the present work, showed that the intake was not affected by their PCom content (Alonso-Diaz et al., 2008, 2009). All the plants used in those trials had CT levels below the 50 g kg $^{-1}$ DM threshold (rank = 9.2 – 16.3 g kg $^{-1}$ DM). Therefore, no negative effect on consumption was expected. However, due to the complexity of the tannin structures, other tannins, which have not been measured in those trials (i.e. the hydrolysable tannins) may have also an important impact in animals.

In the present trial, the IVGP without PEG varied amongst tree plants (*A. pennatula*<*L. latisiliquum*<*P. piscipula*). The IVGP method has been used in tropical plants with two main objectives: 1) to evaluate the potential nutritive value of plant species (Getachew et al., 2002, Osuga et al., 2007) and 2) to evaluate the tannin´s BA of browse forages (Monforte-Briceño et al., 2005, Makkar et al., 1995, McSweeney et al., 2001). In the IVGP method, the use of plant substrate for rumen fermentation is reflected in the gas production (Getachew et al., 2002). Differences in the magnitude of the fermentation from various tree species has been related to the differences in their chemical composition including PCom such as tannins (Osuga et al., 2007).

Table 3. Correlations between polyphenolic compounds and biological activity including the three nutraceutical tree species

	TP	TT	CT	BA RD	IVGP/-PEG	IVGP/ +PEG	BA IVGP
TP	1						
TT	0.65	1					
CT	0.59	0.40	1				
BARD	0.85**	0.36	0.42	1			
IVGP/-PEG	-0.90**	-0.51	-0.50	-0.77	1		
IVGP/+PEG	-0.44	-0.21	-0.35	-0.37	0.55	1	
BAIVGP	0.74*	0.44	0.32	0.64	-0.77	0.09	1

TP = Total phenols; TT= Total tannins; CT= Condensed Tannins; BARD= biological activity measured as radial diffusion; IVGP/-PEG= *In vitro* gas production without PEG; IVGP/+PEG= *In vitro* gas production with PEG; BAIVGP= biological activity measured as the difference between the quantity of gas production with and without PEG. *=P<0.05; **=P<0.01

Table 4. Correlations among polyphenolics compounds and biological activity in each nutraceutical plant

	Acacia pennatula			*Lysiloma latisiliquum*			*Piscidia piscipula*		
	-PEG	BA IVGP	BA RD	- PEG	BA IVGP	BA RD	- PEG	BA IVGP	BA RD
TP	-0.59*	-0.05	-0.19	-0.87***	0.67*	0.65*	-0.29	0.64*	0.84***
TT	-0.36	0.14	0.43	0.21	0.13	0.09	0.04	0.28	-0.11
CT	-0.23	-0.37	0.11	-0.38	0.41	0.42	-0.47	0.58*	0.63*
BARD	0.18	-0.27	1	-0.58*	0.30	1	-0.33	0.66*	1

TP= Total phenols; TT= Total tannins; CT= Condensed Tannins; BARD= Radial diffusion; IVGP/-PEG= *In vitro* gas production without PEG; BAIVGP= biological activity measured as the difference between the quantity of gas production with and without PEG. *=P<0.05; ***P<0.001

Tannin specific inhibitors (*i.e*. PEG) have been used to evaluate tannin BA in browse forages (Makkar et al., 1995). In the current study, *A. pennatula*, *L. latiliquum*, and *P. piscipula* increased the IVGP after the addition of PEG in different proportions. Studies have demonstrated that the intake of TRP is associated with a reduction in the population of the primary fibre-degrading rumen bacteria (Guimarães-Beelen et al., 2005, Makkar et al, 1995). This reduction in the cellulolytic population could be originated via a direct inhibition of the microorganisms (tannin interactions with the cell wall or enzymes) and/or reduced substrate availability (tannins binding to nutrients). The

difference in the increase on IVGP could be related to their quantity of PCom or their chemical structure in each plant. The latter was not elucidated in the present experiment. The BA measured with BARD test showed the same tendency found with BAIVGP (Table 1). The BARD technique measured the affinity of tannins to a single type of protein (in this case, pure hemoglobin). It is worth to notice that this similarity occurred even when using two techniques with different principles.

The second objective was to determine the variability of the BA within each tree species. Different responses were found depending on the tree species and the technique used. The BA measured with the BARD assay showed variability within the three species of trees (Table 2), whereas with BAIVGP, variability was only found within *A. pennatula* (Table 2). The variation in chemical compounds among species is determined by genotypic factors (Makkar et al., 2007). However, within species the influence of environmental conditions could have also modified the biological properties of the various PCom. For example, flavonoids compounds contained in plants are classified as "preformed" and "induced" compounds (Treutter 2006). The "preformed group" are innate compounds that are formed during the normal development of the plants' tissues. And the "induced" compounds are synthesized by plants due to physical injury, infections or stress. For example, the accumulation of flavonoids in some plants can be modified by pathogens, herbivory, light, UV-radiation, temperature, humidity and phytoregulators (Arcas et al., 2000, Makkar et al., 2007, Treutter, 2006). The response mechanisms associated to the interactions of these factors are largely unknown, especially under the conditions of forest vegetation being utilized for browsing. Thus, it is necessary to develop a multidisciplinary approach in order to study the factors that are involved in the BA variability within species.

The third objective was to evaluate the relationship between the PCom and BA. Biological activity showed a closer relationship with TP than with CT in agreement with previous statements (Getachew et al., 2002, Makkar, 2003). These authors pointed out that CT values measured with the Butanol-HCL assay did not seem to reflect their BA while TT and TP were more related. Among the colorimetric chemical assays, the TP method would seem to have some advantages over alternative methods because it can be designed to reflect the ortho-di or tri-phenolic content of the tannins and there is some evidence that this ortho-phenolic content is correlated with BA (Schofield et al., 2001).

A negative relationship between TP and IVGP without PEG was found in *A. pennatula* and *L. latisiliquum* but not for *P. piscipula*. Meanwhile the association between TP and BA, measured with both techniques (BARD and BAIVPG), was found in *L. latisiliquum* and *P. piscipula* but not for *A. pennatula*. The different response could be associated to the structure of the PCom found on each species as discussed above. These effects have also been reported for other types of BA tests such as *in vitro* anthelmintic techniques. When using these same plants, the larval migration of *Haemonchus contortus* and *Trichostrongylus colubriformis* was inhibited (larval migration inhibition test, LMI) by *A. pennatula* and *L. latisiliquum* but not by *P. piscipula*. However, the larval exsheathment was blocked by the three plant extracts (Exsheathment test) (Alonso-Diaz et al., 2008b, 2008c). This suggests that BA variability can be associated to the stereochemical affinities of PCom and the substrate (i.e. protein) involved in every test model. Thus, it seems that not a single BA test can provide a definitive answer of PCom activity. This warrants further investigation.

CONCLUSIONS

This trial showed that there is significant variability in PCom content and BA among trees of the same species. In addition, this trial confirmed that no single biochemical or biological test provide a definitive answer for a potential nutraceutical effect. These facts need to be considered when designing strategies for the use of nutraceutical plants responding to various objectives.

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