CHARACTERIZATION OF CONDENSED TANNINS FROM NATIVE LEGUMES OF THE BRAZILIAN NORTHEASTERN SEMI-ARID

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ABSTRACT: Despite the possible influence of tannins on the nutritional value of the forages from Caatinga vegetation, there are few studies that evaluated their tannin concentration. This study was conducted to characterize condensed tannins present in the legumes species *Mimosa hostilis* (Jurema Preta), *Mimosa caesalpinifolia* (Sabiá) and *Bauhinia cheilantha* (Mororó), at three stages of their phenological cycle. The concentration of soluble tannin (ST), bound tannin (BT) and total tannin (TT) were determined using the butanol-HCL method; astringency was by the radial diffusion method, and the monomeric composition of purified tannins by a high-performance liquid chromatograph with delphinidin, cyanidin and pelargonidin as standards. Concentration and astringency of purified condensed tannins, as well as their monomeric composition varied between species, and in some cases among phenological cycles. The values observed were always above the limits considered beneficial for ruminal digestion (i.e. 5%). Jurema Preta presented the highest values (30.98% TT and 22% astringency at full growth stage), and Mororó the lowest (10.38% TT and 14% astringency during fructification). Jurema Preta presented a mean relationship prodelfinidin (PD): procyanidin (PC) of 97:3, which did not vary during the phenological cycle, showing the high astringent capacity of these tannins. Sabiá presented a relationship of 90:20 during full growth and flowering stages, decreasing to 40:50 at fructification. In Mororó the PD:PC relationship was more equilibrated, around 40:50 during full growth and flowering stages, decreasing to 35:60 During fructification. Propelargonidin was not detected or was present at low concentration in the three species.
Key words*:* proantocyanidin, antinutritional factors, native species, ruminant

CARACTERIZAÇÃO DOS TANINOS CONDENSADOS EM LEGUMINOSAS NATIVAS DO SEMI-ÁRIDO DO NORDESTE BRASILEIRO

RESUMO: Apesar da possível influência do tanino sobre o valor nutritivo das forrageiras da Caatinga, poucos são os estudos que avaliam a concentração de taninos nestas plantas. O objetivo do presente estudo foi caracterizar os taninos condensados presentes nas espécies *Mimosa hostilis* (Jurema Preta), *Mimosa caesalpinifolia* (Sabiá) e *Bauhinia cheilantha* (Mororó), em três fases do ciclo fenológico. As concentrações de tanino solúvel (TS), tanino ligado ao resíduo (TL) e tanino total (TT) foram determinadas pelo método butanol-HCl; a adstringência foi avaliada pelo método de difusão radial e a composição de monômeros dos taninos purificados através do sistema de cromatografia líquida de alta resolução, utilizando delfinidina, cianidina e pelargonidina como padrões. A concentração e adstringência dos taninos condensados purificados, assim como sua composição monomérica, variou entre as espécies e, em alguns casos, entre os ciclos fenológicos. Os valores foram superiores aos considerados benéficos a digestão ruminal (5%). Jurema Preta apresentou os maiores valores (30,98% TT e 22% de adstringência na vegetação plena) e Mororó os menores valores observados (10,38 TT e 14% de adstringência na frutificação). A Jurema Preta apresentou uma relação prodelfinidina (PD):procianidina (PC) média de 97:3 que se mostrou pouco variável, indicando uma alta capacidade adstringente dos taninos desta espécie em todas as fases do ciclo fenológico. O Sábia apresentou uma relação de 90:20 nas fases de vegetação plena e floração, diminuindo para 40:50 na fase de frutificação. A relação PD:PC do Mororó foi mais equilibrada, oscilando em torno de 40:50 nas fases de vegetação plena e floração e reduzindo para 35:60 durante a frutificação. A propelargonidina esteve ausente ou em pequena concentração nas espécies estudadas.
Palavras-chave: espécies nativas, fatores antinutricionais, proantocianidina, ruminantes

INTRODUCTION

Due to its abundance and resistance to drought, semi-arid native vegetation has been historically used for raising livestock, constituting frequently the only protein and energy source for the local ruminants. Caatinga is the low thorn vegetation that covers the Brazilian northeastern semi-arid region, which comprises an area of about one million square kilometers. Small trees and shrubs that lose their leaves during the dry period dominate the landscape. Around seventy percent of woody species from some ecological sites contribute significantly to bovine, ovine and goat diets (Araújo Filho et al., 1993). Nevertheless, these plants are constantly submitted to several stressing agents such as, high temperature, water shortage, intense solar radiation, and nutrient deficiencies, which modify their morphology and development rates, limiting their production and altering their nutritional quality (Buxton & Fales, 1994).

Despite the possible influence of tannins on the nutritional value of these forages, few were the studies that attempted at evaluating their tannin concentration. Condensed tannins are polyphenols present in the majority of tropical legumes. They are associated to a reduction in voluntary intake and in the digestibility of dry matter, organic matter, protein and fiber. Climatic conditions and soil fertility influence not only the concentration of tannins, but their monomeric composition and molecular weight as well (Lascano et al., 2001), characteristics that may determine the effect of these phenols on the nutritional quality of the plants.

Therefore, the objective of the present study was to characterize the condensed tannins present in three native legume species of the Brazilian northeastern semi-arid, namely: Jurema Preta, Sabiá and Mororó, at three stages of their phenological cycle (i.e. full growth, flowering and fructification).

MATERIAL AND METHODS

Forage sampling

Jurema Preta (*Mimosa hostilis*), Sabiá (*Mimosa caesalpinifolia*) and Mororó (*Bauhinia cheilantha*) were sampled in a sucessional Caatinga area in Sobral, State of CE (03°40' S, 40°21' W). The region is characterized by a rainy (January to June) and a dry (July to December) season. The annual precipitation of the collection year was of 997 mm, and the minimum and maximum temperatures were 28 and 32°C, respectively. Plant samples were collected three times during the year, which were representative of three phenological stages, i.e. full growth, flowering and fructification, as observed by Pereira et al. (1989) and Araújo Filho et al. (1998). Seven plants of each species were randomly sampled. Two hundred grams of leaves were taken from the apical part of each plant, simulating the natural grazing of the animals. Sampled leaves were immediately frozen, subsequently lyophilized and ground (1 mm) to proceed analyses.

Characterization of condensed tannins

Levels of condensed tannins (soluble and bound in solid residue) of each legume were determined using the method described by Terrill et al. (1992). Soluble condensed tannins were extracted from 10 mg samples, in duplicate, using a mixture containing 2.5 mL of aqueous acetone 70% and ascorbic acid 0.1% and 2.5 mL of diethyl ether. Upon evaporation of the solvents the extracts were brought to 5 mL with distilled water, centrifuged and separated from the residue. Subsequently, 1.8 mL of butanol-HCL (5% v/v) were added to extract aliquots of 0.5 mL, which were placed in a water bath at 95°C for 70 minutes. Condensed tannins bound to the solid residue were determined by the addition of 0.7 mL of distilled water and 4.2 mL of butanol-HCl (5%) in a water bath, as described above. Absorbance was read in a spectrophotometer at the wavelength of 550 nm and the results were converted to percentage of condensed tannins based on the tannin standard regression curve of each species. These regression curves were obtained using Sephadex LH-20 purified tannins as described by Rosales (1999). The total concentration of tannins was obtained by the addition of the soluble fraction and the fraction bound to the residue.

Tannin astringency was measured by the radial diffusion method (Hagerman, 1987), evaluating tannins diffused trough an agarose gel, forming a ring of precipitate. The diameter of the ring was considered proportional to their capacity to precipitate proteins (astringency). Aliquots of 8 mL of plant extract were placed in petri dishes containing 9.5 mL of a mixture of agarose (1% w/v) and BSA (0.1% w/v) in acetic acid buffer solution (0.3% v/v) containing 0.001 g of ascorbic acid (pH 5.0). The quantity of active tannins that reacted with the BSA was determined by the size of the ring formed after incubation for 96 hours at 35°C, and the quantity of protein precipitated was calculated as follows: volume (mL) x protein concentration (mg/mL) / sample weight. The volume is determined by the height of the agar and by the length of the radius before and after incubation.

Determination of the presence and concentration of procyanidin (PC), prodelphinidin (PD) and propelargonidin (PP) in the purified tannin extracts was performed as described by Rosales (1999). Aliquots of 300 mL of purified tannins were hydrolyzed by the

addition of 1.8 mL of butanol-HCL and subsequent boiling in a water bath at 95°C for 70 minutes. One milliliter of each sample was completely evaporated. The dry antocynidins were then dissolved in 1 mL of a methanol solution containing 1% HCL (v/v). Percentages of cyanidin, delfinidin and pelargonidin were determined by injecting 20 μL of the solution containing antocyanidins into a HPLC system adjusted with a Nova Pack C18 column of 8 × 10 cm. Two solvents were used: A. Aqueous ascorbic acid (5%) and B. Pure methanol. Samples were eluted following a linear gradient of 30-100% of B at 525 nm during twenty minutes and pulses identified in comparison to the time of retention and maximum wavelength absorbance of the antocyanidin standards (Cyanidin chloride, Delfinidin chloride and Pelargonidin chloride - Apin Chemical, UK). The percentages were calculated considering only the pulses corresponding to known substances.

Proximate analyses

Samples were analyzed for dry matter (DM), crude protein (CP), ash, neutral detergent fiber (NDF), acid detergent fiber (ADF), lignin (LIG), and insobuble nitrogen in acid detergent (N-ADF). CP was analyzed according to the Dumas method with the aid of a protein analyzer. NDF, ADF and LIG according to the Van Soest method using a fiber analyzer, and N-ADF using the ADF residue without adding sodium sulfite. The NDF-ADF-LIG determination was performed by the sequential method, adding 0.5 g of sodium sulfite to the samples to removing tannin-protein complexes (Terrill et al., 1994).

Statistical analysis

The effect of species and phenological stage on the characteristics of the tannins and on the concentration of CP, ash, NDF, ADF, LIG and N-ADF present in the legumes was analyzed using a completely randomized model in subdivided parcels, where the legume species were distributed in three parcels and the phenological stages also in three sub-parcels. ANOVA analysis and comparison of means by the Tukey test were conducted using the PROC GLM of the Statistical Analysis System (SAS, 1990).

RESULTS AND DISCUSSION

Tannins might be associated with adverse effects as anti-nutritional factors, causing lower dry matter intake and reduced digestion of protein and fiber. The effects depend on tannin concentration in the plant and also other factors, such as type of tannin, animal species, physiological status and diet composition (Schofield et al., 2001; Makkar, 2003). Nevertheless, the presence of moderate levels of condensed tannins in the rumen is related to the protection of dietary protein against degradation by ruminal microorganisms, increasing the flux of dietary protein to be absorbed in the intestines (Min et al., 2003).

Purification of condensed tannins from the species Jurema Preta, Sabiá and Mororó resulted in 17%, 13% and 7% of tannins, respectively. The standard curves obtained with the purified tannins are shown in Figure 1.

The utilization of external standards is one of the major problems encountered for the accurate determination of condensed tannins (Mupangwa et al., 2000). In the present study this problem was solved by the utilization of standards composed of tannins purified from the studied species. The standard curves built with the purified tannins demonstrated that the type of standard used could influence seriously the results. For example, if the purified tannin from Jurema Preta had been chosen as the standard for all species, the output obtained for the Mororó extract from 0.5 to 550 nm would be of 20.3% of condensed tannin, against a true value of 9% of condensed tannin obtained with the tannin standard obtained from the Mororó itself. This would have caused a super estimation of 225.5% in the concentration of soluble tannin of Mororó.

The regression equations of the standard curves generated by the butanol-HCL assay were: $Y = 1.1851x + 0.0238$; $R^2 = 0.994$ for Jurema Preta; $Y = 1.522x + 0.0173$; $R^2 = 0.999$ for Sabiá; and $Y = 2.6155x - 0.0297$; $R^2 = 0.996$ for Mororó.

On the other hand, regression curves built with purified tannins of the same species, but at different phenological stages did not interfere on the obtained tannin concentration (results not shown). Therefore, regression curves built with purified tannins extracted at the full growth stage were used to calculate the tannin concentration at all stages of the phenological cycle for each forage species.

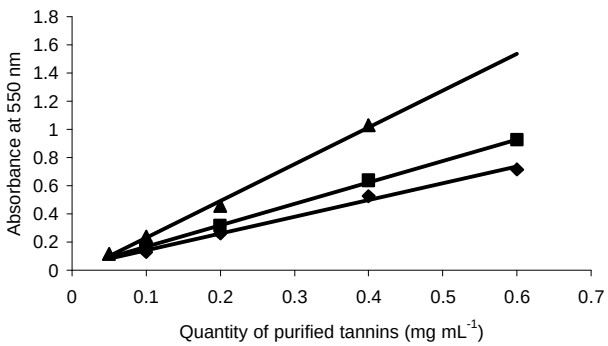

Figure 1 - Standard curve of purified tannins from the legume species ◆ Jurema Preta, ■ Sabiá and ▲ Mororó.

The concentrations of condensed tannins present in the three native legumes at all phenological stages are shown in Table 1. Significant differences were found between species in relation to the concentration and astringency of tannins. Jurema Preta presented the highest values (30.98% TT and 22% of astringency at full growth), and Mororó the lowest (10.38% TT and 13.49% of astringency at fructification). Tannin concentration and its fractions varied in function of the phenological cycle for Jurema Preta ($P < 0.05$), but stayed constant for Sabiá ($P > 0.05$). The three native legumes presented high tannin concentrations at all stages of the phenological cycle. Tannin levels of Jurema Preta were higher in the full growth stage. Sabiá presented intermediate levels and Mororó the lowest ones.

As opposed to Jurema Preta, where a decrease in soluble tannin concentration and astringency was observed with the maturation of the leaves, Sabiá and Mororó showed the highest concentrations during flowering. Although this was not statistically significant, it may be an indication that these two species have increased the production of phenolic compounds during the reproduction period, aiming at protecting themselves from being consumed by herbivores at a stage of fundamental importance for the species. Even with a decrease in tannin concentration, with maturation Jurema Preta still presented concentrations as high as Sabiá (17.68% of dry matter), which is considered very high in the literature.

Studies have shown that the consumption of plants presenting a concentration of 3-4% of condensed tannins is associated to positive effects on digestion, i.e. protection of proteins against excessive ruminal degradation without affecting voluntary intake or fiber digestion (Barry & McNabb, 1999) and that protein digestibility is not affected at tannin concentrations lower than 2% DM (Poncet & Rémond, 2002). Nevertheless, Vitti et al. (2005) stated that it can not be generalized that tannin concentration between 2% and 4% of DM are benefic for digestion and that values above 5% are deleterious to the metabolism, stressing the importance of taking into account other aspects related to plant composition.

More than tannin concentration, its molecular weight, the monomer composition (Lascano et al., 2001) and the spatial distribution of these monomers in the molecules of tannins (Rosales, 1999) can be characteristics that may determine the effect of these phenols on the nutritional quality of the plants.

However, total condensed tannins of the three species were higher than 10% of dry matter in all phenological stages, demonstrating thus their role as important anti-nutritional factors.

The monomeric composition, percentage of prodefinidin (PD), procyanidin (PC) and propelargonidin (PP) of purified condensed tannins are shown in Figure 2. The monomeric composition of tannins (relationship PD:PC) varied considerably between species, and in the case of Sabiá and Mororó also between the stages of the phenological cycle. Jurema Preta presented a PD: PC mean relationship of 97:3 without great variation. Sabiá presented a PD: PC relationship of 90:20 during full growth and flowering stages, but decreasing to 40:50 during fructification.

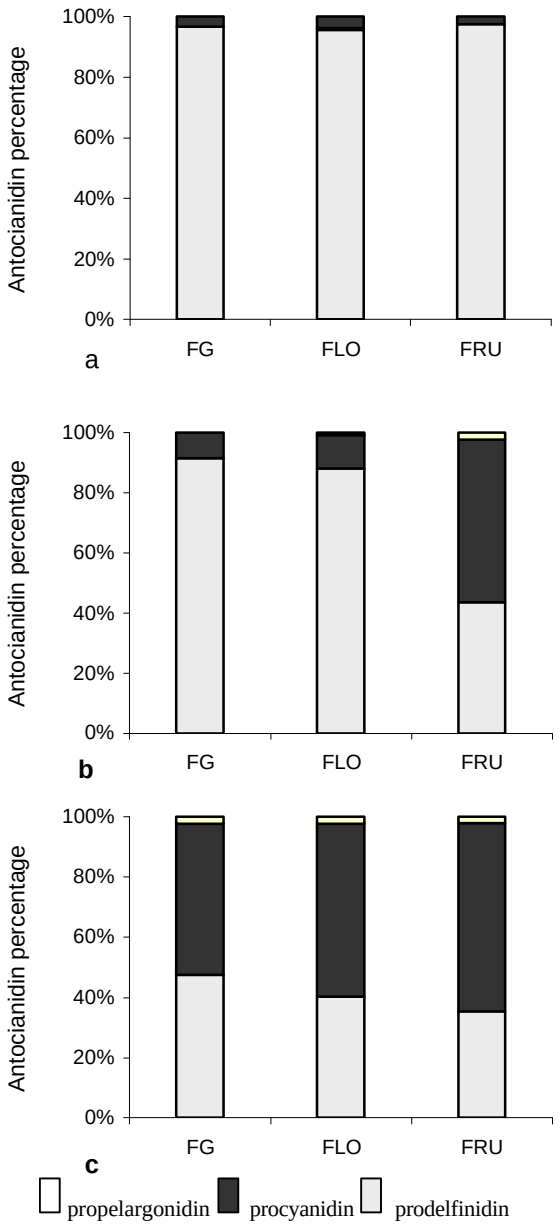

Figure 2 - Percentage of prodelfinidin-procyanidin-propelargonidin of purified tannins from Jurema Preta (A), Sabiá (B) and Mororó (C), at different stages of their phenological cycle. FG: full growth, FLO: flowering, FRU: fructification.

Table 1 - Composition and astringency of condensed tannins and levels of crude protein, ash, fiber fraction, in the leaves of the species Jurema Preta (*Mimosa hostilis)*, Sabiá (*Mimosa caesalpinifolia)* and Mororó (*Bauhinia cheilantha*) at three stages of their phenological cycle.

Species	Stage of phenological cycle			SEM
	Full growth	Flowering	Fructification	
		Soluble tannin (%)		1.76
Jurema Preta	26. 7 cC	17.7 bB	14.1 aB	
Sabiá	15.4 B	17.7 B	16.9 B	
Mororó	8.5 A	9.4 A	7.4 A	
		Tannin bound to residue (%)		0.37
Jurema Preta	4.3 bB	3.0 aAB	3.2 aB	
Sabiá	2.5 A	2.4 A	2.3 A	
Mororó	2.7 bA	3.4 aB	3.0 aB	
		Total condensed tannin (%)		1.72
Jurema Preta	31.0 cC	20.7 bB	17.3 aB	
Sabiá	17.9 B	20.1 B	19.1 B	
Mororó	11.18 A	12.73 A	10.38 A	
		Astringency (% bound protein/ mL tannin)		1.52
Jurema Preta	22.0 C	20.9 B	20.1 B	
Sabiá	18.2 B	20.7 B	20.2 B	
Mororó	14.4 A	16.6 A	13.5 A	
		Ash (%)		0.88
Jurema Preta	4.4 A	4.0 A	4.1 A	
Sabiá	4.8 A	5.5 B	5.9 A	
Mororó	7.9 bB	6.1 aB	8.7 bB	
		Crude protein (%)		1.50
Jurema Preta	16.9 b	13.5 a A	13.2 a A	
Sabiá	17.8	17.7 B	16.3 B	
Mororó	19.0 b	13.4 aA	14.4 aA B	
		Neutral detergent fiber (%)		4.60
Jurema Preta	35.0	36.3	36.8	
Sabiá	46.3	40.0	39.3	
Mororó	42.1	40.3	38.7	
		Acid detergent fiber (%)		1.92
Jurema Preta	15.1 A	16.9 B	17.4 A	
Sabiá	25.9 bB	22.0 aA	22.3 aB	
Mororó	23.8 bB	20.8 abA	19.3 aAB	
		N-ADF (% of total N)		3.13
Jurema Preta	11.8 aA	18.1 b	19.1 bB	
Sabiá	19.4 B	17.0	19.4 B	
Mororó	8.4 aA	15.9 b	11.6 bA	
		Lignin (%)		1.64
Jurema Preta	7.4 A	8.9 A	8.9 A	
Sabiá	13.9 bB	11.1 aB	11.4 aB	
Mororó	7.3 A	6.5 A	6.2 A	

Means with different letters in the same row are significantly different ($P < 0.05$); Means with different capital letters in the same column are significantly different ($P < 0.05$); SEM: Standard error of means.

The relationship PD: PC of Mororó was the most equilibrated, being 40:50 during full growth and flowering, and decreasing to 35:65 during fructification. The properlargonidin was absent or in very low concentration in all species, and therefore was not taken into account in the relationships. Jones et al. (1976) attributed the content of prodelfinidin to a higher astringency of the tannins. Ayres et al. (1997) associated the higher percentage of prodelfinidin to an anti-herbivore action of tannins. The authors observed that prodelfinidin had one more hydroxyl than procyanidin and speculated that this was the reason of its higher astringent activity. Even if such a relationship existed, it was not very consistent in the present study. However, this association may be more complex since the astringency of tannins seems to be related not only to their monomeric composition, but also to the spatial distribution of these monomers in their molecules (Rosales, 1999).

The content of proanthocianidin varied considerably between the three species. Results show that the condensed tannins present in the species Jurema Preta and Sabiá are mainly constituted of PD, while the tannin present in Mororó has a relationship PD:PC of approximately 50%.

Proximate values are presented in Table 1. All the studied variables differed between species ($P < 0.05$), but in most cases these differences were dependent on plant phenological stage. Nevertheless, excepting the crude protein of Jurema Preta and Mororó, which decreased more than 20% between the full growth and fructification stages, the variables stayed relatively constant. An interaction between species and phenological stages was found for the variables ash, CP, FDA, lignin and N-ADF. For the species Sabiá, the NDF varied from 46.3 to 39.3% between the full growth and fructification stages, respectively, but the variation was not statistically significant ($P > 0.05$).

The values of NDF (Table 1) were inferior to the ones present in the literature for similar phenological stages: 52.2% for Sabiá at full growth (Vasconcelos, 1997); 49.54% for Mororó and 44.06% for Sabiá at the fructification stage (Vieira et al., 1998); 44.48% for the Jurema Preta at the beginning of flowering (Pereira Filho et al., 2001). These differences may have been caused by the formation of tannin-protein complexes, which were not solubilized during the NDF analysis, and may have led to a super estimation of the cellular wall content (Terrill et al., 1994). In the present study sodium sulfite was added to the NDF solution to remove tannins and protein residues in order to avoid this problem.

Despite a considerable number of studies describing the chemical composition of the main legume species of the Brazilian semi-arid, few of them have assessed their tannin content. Beside the three legume species here studied, several other native legumes probably rich in tannins are largely used for livestock raising in the Brazilian northeastern semi-arid. Therefore, further work is important to: 1. better characterize the tannins present in the native browse legumes, and 2. assess whether or not tannin levels may be influencing the NDF and ADF values in relation to those found in the literature, since they are not taken into account when these routine chemical analyses are performed.

Since the physiological activity of tannins is attributed to their capacity to bind proteins, studies on their characterization should include concentration analyses, but should also contemplate measurements of astringency and digestibility.

Tannin astringency is traditionally measured by the radial diffusion method (Hagerman, 1987). The tannins diffuse through an agarose gel containing bovine serum protein (BSA) originating thus a ring-shaped precipitate. The ring diameter is considered proportional to the protein precipitation capacity (astringency).

More recently, *in vitro* gas measuring techniques are being used in studies with plants containing high levels of tannins. The methodology described by Makkar (2001) assesses the tannin effect by means of an *in vitro* bioassay, which measures the gas production in samples with or without polyethylene glycol (PEG). PEG molecule has a tannin high affinity, which inhibits their complexing activity with other macromolecules (Titus et al., 2000; Villalba et al., 2002). Therefore, the higher the sample gas production in the presence of PEG, the higher is the tannin reactivity. It is shown in the present study that tannins may have distinct chemical and structural characteristics in function of the forage species, and sometimes between their phenological cycles, which may influence their biological action. Therefore, the use of the biological methodologies, in association to the determination of tannin concentration, might give a better evaluation of the anti-nutritional effects of tannins present in the studied species.

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