**Characterization and biological activity of condensed tannins from six tropical legumes**

**Abstract -** The objective of this study was to characterize condensed tannins (CT), their monomers propelargonidin, prodelphinidin and procyanidin, as well as fractions of soluble CT (ECT), protein-bound CT (PBCT) and fiber-bound CT (FBCT), molecular weight, degree of polymerization, polydispersity index and biological activity by protein precipitate phenols (PPP) and butanol-HCl method of legumes leaves namely *Cajanus cajan* (pigeon pea), *Gliricidia sepium* (gliricídia), *Stylo* (*Stylosanthes capitata* x *Stylosanthes macrocephala* ), *Flemingia macrophylla* (flemíngia), *Cratylia argentea* (cratília), *Mimosa Caesalpiniifolia* (sabiá), and the bark of this latter species. The ECT fraction was not confirmed in cratília, but leaves of sabiá reached 79 g kg-1. The PBCT fraction varied 1 g kg-1 through 53 g kg-1. The FBCT fraction was not detected in any of the legumes studied, but the total condensed tannin and PPP had variations 23 through 124 g kg-1 and 3 through 160 g kg-1. The weight-average molecular weight (Mw) ranged 737 through 1168 Da and number-average molecular weight (Mn) ranged 271 through 436 Da (*P*≤0.05). The ratio of prodelphinidin and procyanidin varied 10:80 (stylo) through 65:35 (flemíngia), propelargonidin was only determined in cratília. This difference in the concentration, chemical structure, and biological activity indicates different responses when offering these tanniniferous tropical legumes.

**Index terms**: forage legumes, procyanidins, prodelphinidins, protein precipitable phenolics.

**Caracterização e atividade biológica de taninos condensados de cinco leguminosas tropicais**

**Resumo** - Objetivou-se neste estudo caracterizar os taninos condensados (TC), seus monômeros propelargonidina, prodelfinidina e procianidina, assim como as frações de taninos extraivel (ETC), taninos ligados à proteína (TCPB) e a fibras (TCFB), análise do peso e massa molecular, grau de polimerização, índice de polidispersidade e atividade biológica dos taninos através do método de fenóis precipitados por proteína (PPP) e butanol-HCl em folhas de leguminosas: *Cajanus cajan* (guandú), *Gliricidia sepium* (gliricídia), *Stylosanthes spp* (Stylo), *Flemingia macrophylla* (flemíngia), *Cratylia argentea* (cratília), *Mimosa Caesalpiniifolia* (sabiá), e na casca desta última. A fração de TCE não foi confirmada na cratília, porém as folhas do sabiá apresentaram 79 g kg-1. Já TCPB variou de 1 a 53 g kg-1. A TCFB não foi determinada em nenhuma das leguminosas estudadas, porém o tanino condensado total e PPP apresentaram variações de 23 a 124 g kg-1 e de 3 a 160 g kg-1. O peso molecular variou de 737 a 1168 Da e massa molecular variou de 271 a 436 Da (P≤0,05). A relação prodelfinidina e procianidina variaram de 10:80 no estilosantes a 65:35 na flemíngia. A propelargonidina somente foi determinada na cratília. Esta diferença na concentração, estrutura química e atividade biológica sugere diferente respostas com ofertas destas leguminosas tropicais taniníferas.

**Termos para indexação:** Leguminosas forrageiras, procianidina, prodelfinidina, proteínas precipitadas por fenois.

**Introduction**

The shortage of great quality forage and livestock feed resources is a limitation for ruminant production in some developing countries. The wider use of herbaceous, shrubby and arboreal legumes intercropped with grasses or as protein pool may provide a solution (Muir et al., 2014). These can improve ruminant nutrition because they have great protein amount and digestibility that improve grass digestibility in rumen. Legumes can also serve as a food reserve for dry seasons or drought years in addition to supply Nitrogen (N) in pasture ecosystem, thereby decreasing dependence on expensive fertilizers compared to grass monoculture (Nepomuceno et al., 2013).

Many tropical legumes have low palatability during rainy through dry season (Andersson et al., 2006), wherein grasses begin reducing quantity and quality, which can limit ruminant intake acceptability of tropical forage legumes is often related to secondary metabolic compounds synthesis such as tannins, saponins, terpenes and lignin, designated as "anti-nutritional factors" due to reduction on intake, feed nutritional value (Nepomuceno et al., 2013). As well as affecting digestibility, nutrient uptake or utilization, and, if ingested in high concentrations, can harm ruminant health (Lamy et al., 2011).

Condensed tannins (CT) are phenolic compounds classified as flavan-3-ol polymers with widely differing molecular weights (Mueller-Harvey, 2006). They are often present in tropical legumes, which as act as chemical defense mechanisms against pathogens, insects and herbivores (Terril et al., 1992). Their ability to bind with proteins and other macromolecules, reversible at acid pH (Dentinho and Bessa, 2016), allows them to play important roles in ruminant nutrition by increase plant amino acid absorption in small intestine (protein bypass). They also can reduce gastro-intestinal parasite; mitigate rumen methanogenesis, which improve energy efficiency; and alter the N elimination route from urine to feces, providing less negative effects on the environment (Naumann et al., 2014).

Due to highly heterogeneous legume phenolic chemical structures (Mueller-Harvey, 2006), CT from different plant species may cause different responses in nutrient availability and utilization by ruminants, even when consumed at the same concentration (Dentinho and Bessa, 2016). However, Beelen et al. (2006) reported that one of the factors that promote this difference in biological activity among plants is the greatest concentration of prodelphinidin monomer, which due to its greater hydroxyl radicals in structure, promotes greater biological activity and astringency.

There are different methods for assaying CT amount and biological activity. Most quick colorimetric methods provide little information about biological activity (Mueller-Harvey, 2006). Gea et al. (2011) suggested to use high-performance liquid chromatography (HPLC) to determine degree of polymerization (DP), prodelphinidin:procyanidin ratio (PD:PC ratio) and molecular weight (Mw). Although these procedures are laborious and costly, they are most reliable demonstration of CT activity of forage legumes for ruminant feed. Range effects of different plants on ruminant nutrition and health are caused by different types and quantities of CT (Naumann et al., 2014)

Our objective was to characterize and compare CT biological activity of six tropical forage legumes

**Material and Methods**

Approximately 2 kg dry matter (DM) of leaves, each from pigeon pea, gliricídia, stylo, flemíngia, cratília, and sabiá plants, as well as bark from sabiá branches with up to 12 cm diameter were collected at different maturity stages, in the experimental area of EMBRAPA Agrobiology, in Seropédica, RJ, Brazil, in October and November 2013. The material was dried in a ventilated hangar with average 28 °C temperature for 1 wk. A representative 100 g subsample was ground through a hammer mill fitted with a 1-mm sieve, and shipped to Texas A&M AgriLife Research in Stephenville, Texas, USA for CT analysis.

Purification of CT from each legume species was performed according to Wolfe et al. (2008), extracted from plant tissues (25 g). Purified CT from each species were used to develop specific patterns, as well as to determine molecular weight (Mw) and monomers of CT. Precipitable protein phenols (PPP) were analyzed, from plants crude extracts (50 mg) in duplicate, as described by Hagerman and Butler (1978), well-correlated with biologically active CT on animals. Crude extract was used due to limitations of purified CT amount.

Fractions of CT were determined as described by Terrill et al. (1992), wherein extractable condensed tannin (ECT) was determined with 10 ml of acetone:water solution (70:30) followed by 10 ml of diethyl ether. Fraction of protein-bound CT (PBCT) were extracted from ECT residue with 10 ml of sodium dodecyl sulfate-mercaptoethanol containing 0.01 M Tris-HCl. The fiber-bound CT (FBCT) was determined using the residue after ECT and PBCT analysis. Those fractions were determined based on absorbance at 550 nm in sequence with butanol-HCl reaction (5% v/v HCl). The dried phenolic-protein residue was analyzed for N through Vario Macro Elementary C:N (Elementary Americas, Inc., Mt. Laurel, NJ, USA). Nitrogen percentage was multiplied by 6.25 to estimate crude protein.

To determine CT molecular mass, a gel permeation chromatography attached in HPLC modular system was used as described by Huang et al. (2010). Molecular weights were calculated based on the calibration curve established with PolystyreneLowEasiVials standards (Agilent Technologies, Santa Clara, CA, USA), with Mw ranging from 162 to 38,640 Da. The weight-average molecular weight (Mw) and number-average molecular weight (Mn) were integrated and quantified by Breeze Software (Waters Corporation, Milford, MA). Degree of polymerization is the estimated number of monomers that make up the purified CT polymer according to Williams et al. (1983), in which a single unit of proanthocyanidin has approximately 500 Da of Mw, while the polydispersy index (PDI) represents the molecular weight distance distributed from the polymer and it was assessed using the equation Mw/Mn.

We determined the PD:PC ratio of CT from six plants using anthocyanidin monomers as described by Naumann et al. (2015), which use purified CT added to butanol acid. Delphinidin and propelargonidin (PP) (Sigma-AldrichCo., St. Louis, MO) were used as Standards in HPLC controlled by Breeze Software (Waters Corporation, Milford, MA).

The experimental design was completely randomized with seven treatments (leaves from six species plus sabiá bark) replicated four times. Dependent variables included percentage of ECT, PBCT, FBCT, and TCT; structural characteristics including PP, PD and PC, DP, PDI, Mw, Mn, PPP and PB. These were submitted to analysis of variance and Tukey test mean using RStudio. Differences were considered significant at P≤0.05.

**Results and Discusson**

Concentrations of ECT, PBCT, TCT, and PPP differed among species ( P < 0.05). No differences were found for FBCT in none of the studied legumes (Table 1). The ECT fraction is the largest TCT component in tropical legumes (Terril et al., 1992). ECT fraction ranged from non-detected in cratília and gliricídia up to 79 g kg-1 in sabiá leaves, which was significant greater than 41, 2.5, and 15 g kg-1 found in flemíngia, pigeon pea, and sabiá bark, respectively.

Concentrations of PBCT, in decreasing order, were 53, 44, 35, 35, 14, 8 e 1 g kg-1 (P<0.05) for flemíngia, sabiá leaves, sabiá bark, gliricídia, stylo, pigeon pea, and cratilia, respectively. No differences were observed between gliricídia and sabiá bark (P > 0.05).

The TCT concentration ranged from non-detected (cratília) to 124 g kg-1 in sabiá leaves, which was greater than the TCT concentration found in the other legumes and sabiá bark (P<0.05), demonstrating differences in this compound among different species and plant fractions within the same species (Viteli et al., 2007). Flemíngia had 93 g kg-1 and sabiá bark, 50 g kg-1, glirícidia and pigeon pea had an average concentration of 35 g kg-1, and stylo, 23 g kg-1.

The legumes in our study had widely differing PPP concentrations (P<0.05) with sabiá leaves containing the greatest levels. The PPP amount in pigeon pea and flemíngia were similar, but greater than stylo leaf and sabiá bark. Cratília and gliricídia had no PPP.

Sturm et al. (2007) observed CT values in cratília and flemíngia similar to those reported in our study. Their results for flemíngia were 5% of ECT, but TCT was lower (5%) and no FBCT and PBCT. Cano et al. (1994) studied the biological activity of tropical legumes CT and reported 79 % of ETC, 14% of PBCT and 7% of FBCT. The values of ECT, PBCT and FBCT were greater than those found in our study; however, these authors did not report the percentage of FBCT.

Balogun et al. (1998) reported only traces of ECT in gliricídia (<1%), but 2% PBCT and 4% TCT, values similar to those found in our study. However, Lara et al. (2000) reported for gliricídia 4 g kg-1 of ECT, 30 g kg-1 of PBCT, 5 g kg-1 of FBCT and 40 g kg-1 of TCT. Nozella (2001) evaluated tannin concentrations in plants with forage potential for ruminants and reported that gliricídia had low levels of phenols (13.72 g kg-1 DM), tannins (6.86 g kg-1 DM) and CT (0.3 g kg-1 DM).

Optimum ruminant performance is often observed with diets containing moderate levels ranging from 20 to 50 g CT kg-1 (Animut et al. 2008; Muir, 2011). Benefits normally derive from plant protein protection from microbial degradation in rumen (by-pass) with a corresponding increase in absorption of plant amino acids in the gut and improved animal performance results (Patra and Saxena, 2011) e efeito anti-helmintico (IQBAL et al., 2002).

Even though, forage legumes without CT are a good source of protein, since they serve as an input of N to ruminants, representing a possible alternative to more expensive dietary inputs, especially when feed quality is limiting (Muir et al., 2014). Leaves of stylo, pigeon pea, and gliricídia, all of which contained between 20 and 50 g kg-1 TCT, if fed as sole feeds in ruminant diets, should be beneficial CT sources. According to Gonçalves et al. (2010), sabiá bark has potential as a source of CT. Although the bark is not considered forage, it was included in this study because it might be used as a potential tannin additive in ruminant diets to enhance livestock performance (Sartor Neto et al., 2011).

Diets containing more than 50 g CT/kg DM may negatively affect feed intake and digestibility, so high-CT legumes, such as sabiá and flemíngia, should be offered with other forages containing no CT (Littlefield et al., 2011). For example, lambs fed quebracho (*Schinopsis* spp.) extract in diets containing 89 g CT/kg DM had reduced DM intake of fresh vetch (*Vicia sativa*) compared to lambs fed the same forage without CT (768 vs. 956 g/day) (Patra and Saxena, 2011). The tannin content found in our study for the sabiá was 12%, lower than 17% found by Beelen et al. (2006), who report that that CT contents greater than 10% on DM basis may have detrimental effect to ruminants. Lamy et al. (2011) mentioned that certain herbivores, such as goats, can secrete proline and other salivary proteins as mechanism of temporary CT inactivation. However, *in vitro* analyses followed by *in vivo* trials should confirm such assertions because biological activity of specific legume CT can vary.

According to Lin et al. (2007), changes in CT structure probably decrease the protein binding capacity. Low PPP in mature and senescing plants may result from increasing degree of polymerization and reduced active sites on CT molecules resulting in less likelihood of complexation with proteins. Beleen et al. (2006) confirmed that CT variation can occur in the different stages of phenological cycle of plants. This may or may not have been a factor in our study because the age of the species was not evaluated.

Terrill et al. (1994) showed that increases in sample drying temperature decreased ECT from *Calliandra calothyrsus* and *Lespedeza cuneata* and increased PBCT and FBCT fractions. According to Muetzel and Becker (2006), drying in a greenhouse did not negatively affect the extraction and biological activity of CT but decreased the solubility of cell wall and protein, reducing its degradability in the rumen. Lin et al. (2007), studying different plant fractions and phenological stages, found that CT fractions, TCT and PPP decreased as plant maturity advance. PPP determines the amount of bioactive condensed tannins of interest in ruminant nutrition (Cooper et al., 2014), however, the extent of the bioactivity depends upon other factors such as tannin structure and concentration, physical status of the animal, and diet-related factors such as protein concentration (Naumann et al., 2014).

DR, PDI, Mw, and Mn varied among species (P < 0.05) (Table 2). No differences were observed for PD in stylo, flemíngia, and sabiá bark, however, the values were greater (P < 0.05) than the ones observed for cratília, gliricídia, pigeon pea, and sabiá leaves, with no differences found within this last group of legumes. PDI in decreasing order was presented by sabiá (both bark and leaves), pigeon pea, cratília, stylo, flemíngia, and gliricídia.

Stylo presented the greatest Mw (1168 Da) and gliricidia the lowest (737 Da), whereas flemíngia, sabiá bark, sabiá leaves, pigeon pea, and cratília presented, in a decreasing order, the intermediate values, respectively. No differences were found between flemíngia and sabiá bark, and between sabiá leaves and pigeon pea (P > 0.05). Stylo, flemíngia, sabiá, cratilia, pigeon pea, and gliricidia presented decreasing values for Mn, respectively. No differences were observed for Mn between sabiá fractions and between cratilia and gliricídia (P > 0.05).

The Mw of CT ranges from 500 to > 20,000 Da (Mane et al., 2007), the concentration as well as the chemical structure being determinants of their biological activity (Beelen et al., 2006). Naumann et al. (2014) found weak correlations between legume CT Mw and PPP. According to Cano et al. (1994), CT ability to bind and precipitate proteins increases as DP and Mw increases. Huang et al. (2010) working with *Leucaena hybrids*, found that CT Mw ranged from 2737 to 2871 Da but were unable to attribute CT binding affinity to proteins directly to Mw because, according to the authors, the chemical structure can also play a vital role in this feature. In the case of the legumes we studied, stylo CT had low PPP (Table 1) but high Mw (Table 2), which was not the case with flemíngia or pigeon pea.

Naumann et al. (2014) evaluating perennial legumes from warm climates in southern North America, found ECT Mw ranging from 552 to 1483 Da, values close to those found in our study (Table 2). They found that Mw did not, however, explain biological activity as measured by *in vitro* rumen methanogenesis, suggesting the need for further research on Mw in PBCT and FBCT. Other factors, including chemical structure, may play an important role to explain results as well.

Proportions of the monomers PP, PD, and PC, as well as, PD:PC ratio from legumes were presented using a descriptive analysis (Table 3).

The PP was detected in cratilia and stylo, however, PP concentration was 4.2 times greater in cratília. Proportion of PD varied from 7.1 in the stylo to 64.1 in the flemíngia, cratília presented 30.7%, sabiá leaves 52.8%, and gliricídia, pigeon pea, and sabiá bark all presented 40%. The PD:PC ratio presented by the studied legumes was 10:80 for stylo, 40:60 for gliricídia, pigeon pea, and sabiá bark, 52:48 for sabiá leaves, 30:25 for cratilia, and 65:35 for flemíngia. The two most common CT found in forages are PC and PD, which have two and three hydroxyls in the B ring of the flavan-3-ol unit, respectively (Klongsiriwet et al., 2015). Greater hydroxyl number in PD results in greater biological activity for this compound (Ayres et al., 1997), reflecting both in the benefits for the animal (IQBAL et al., 2002; Patra and Saxena, 2011) as well as in the adverse effects related to the reduction in DM intake, and reduction in fiber and protein digestion (Beelen et al., 2006). This might be explained by the concentration of this monomer in the plant and by its relationship with procyanidin, considering that in some plants these compounds form mixtures difficult to separate (Klongsiriwet et al., 2015). According to Molan et al. (2003), the PD:PC ratio affects the biological activity. These authors compared CT extracts of *Lotus pendunculatus* and *Onobrychis viciifolia* with a PD:PC ratio of 70:30 and 77:23, respectively, with the extract of *Lotus corniculatus* with a PD:PC ratio of 27:73. They correlated greater activity in the extract with greater proportion of prodelphinidin using these extracts on larvae of *Trichostrongylus colubriformis.* As demonstrated in this research, condensed tannin bioactivity varies with plant species and plant fraction, chemical structure, and concentration, corroborating other results from the literature.

**Conclusions**

Sabiá leaf should not be fed as a sole dietary component due to its great PPP and TCT concentration far in excess of 5%. Flemíngia, on the other hand, despite TCT in excess of 5%, may not pose a danger to ruminants because PPP is lower. At first, this allows its use in a similar way to cratília, pigeon pea, gliricídia, and stylo.

The use of sabiá bark as a source of condensed tannin has to be reviewed, since the low biological activity of its tannins.

All of these potential conclusions, however, should be tested *in vivo* prior to drawing definitive conclusions. Prediction of biological activities, such as methane suppression and larval inhibition, should be studied as predictors of additional benefits for ruminants and environment.

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**Table 1.** Concentration of extractable (ECT), protein-bound (PBCT), fiber-bound (FBCT), and total (TCT) condensed tannins as well as protein precipitable phenols (PPP) in leaf dry matter of the tropical legumes cratília, flemíngia, stylo, gliricídia, pigeon pea, sabiá, and sabiá bark.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Legumes** | **ECT** | **PBCT** | **FBCT** | **TCT** | **PPP** |
|  | g kg-1 | | | | |
| Cratília | ND | 1e | ND | ND | ND |
| Stylo | 9e | 14d | ND | 23e | 03d |
| Flemíngia | 41b | 53a | ND | 93b | 42b |
| Gliricídia | ND | 35c | ND | 36d | ND |
| pigeon pea | 25c | 08d | ND | 33d | 37b |
| Sabiá (leaf) | 79a | 44b | ND | 124a | 160a |
| Sabiá (bark) | 15d | 35c | ND | 50c | 11c |
| CV | 5.7 | 7.6 | - | 8.1 | 6.5 |

Means in columns followed by different letters differed (*P*≤0.05) according to a Tukey test.

ND not detected.

Results presented on a dry matter basis and standard curves based on purified condensed tannins from each species.

**Table 2**. Polymerization degree (DP), polydispersity index (PDI); Relative weight-average molecular weight (Mw), and number-average molecular weight (Mn) for six tropical forage legumes.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Legumes** | **DP** | **PDI** | **Mw** | **Mn** |
| Cratília | 1.6b | 2.9c | 791d | 274d |
| Stylo | 2.3a | 2.8c | 1168ª | 436a |
| Flemíngia | 2.0a | 2.9c | 999b | 351b |
| Gliricídia | 1.5b | 2.8c | 737e | 260e |
| pigeon pea | 1.7b | 3.1b | 838c | 271d |
| Sabiá (leaf) | 1.8b | 3.2a | 917c | 284c |
| Sabiá (bark) | 2.0a | 3.5a | 993b | 284c |
| CV | 2.1 | 16.1 | 2.1 | 18.9 |

Means in columns followed by different letters differed (*P*≤005) according to a Tukey test.

Results presented on a dry matter basis and standard curves based on purified condensed tannins from each species.

**Table 3**. Propelargonidin (PP), prodelphinidin (PD), and procyanidin (CP) of condensed tannin in six tropical forage legumes.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Legumes** | **PP** | **PD** | **PC** | **PD:PC** |
| **%** | | |
| Cratília | 44.5 | 30.7 | 24.7 | 30:25 |
| Stylo | 10.7 | 7.1 | 82.1 | 10:80 |
| Flemíngia | ND | 64.1 | 35.3 | 65:35 |
| Gliricídia | ND | 39.8 | 60.2 | 40:60 |
| pigeon pea | ND | 40.3 | 59.7 | 40:60 |
| Sabiá (leaf) | ND | 52.8 | 47.2 | 52:48 |
| Sabiá (bark) | ND | 40.0 | 60.0 | 40:60 |

ND = not detected