Effect of pH on Protein Characterization in Brazilian Cerrado Plant Extracts

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In this paper the effect of extractant on the biochemical composition of Brazilian Cerrado biome plant species (Sapindus *saponaria* L., *Guazuma ulmifolia* Lamb., *Eugenia dysenterica* Mart. and Stryphnodendron adstringens Mart.) was presented. About 50% of S. *saponaria, G. ulmifolia, E. dysenterica* and *S. adstringens* were mainly consisted of lipids. There was a great variety of protein content among the tested species and presence of peroxidase activity (903.54 EU/g). About the antinutritional factors, considerable quantity of lectins (10,752 UH/g) and trypsin inhibitor was also detected (139 IU/g). These plants were considered as source of biomolecules that can largely be explored for biotechnological applications.

Keywords: *plant proteins; Brazilian Savannas; enzymes.*

Neste trabalho, foi apresentado o efeito da solução extratora sobre a composição bioquímica de espécies de plantas do cerrado brasileiro (*Sapindus saponaria* L., *Guazuma ulmifolia* Lamb., *Eugenia dysenterica* Mart. e *Stryphnodendron adstringens* Mart.). Cerca de 50% de *S. saponaria, G. ulmifolia, E. dysenterica* e *S. adstringens* consistem de lipídeos. Observou-se uma grande variação na concentração de proteínas nas espécies testadas e a presença de peroxidases (903,54 UE/g). Fatores antinutricionais, como lectinas (10.752 UH/g) e inibidores de tripsina (139 UI/g) foram também detectados. Estas espécies vegetais foram consideradas como uma fonte de biomoléculas que pode ser largamente explorada para fins biotecnológicos.

Palavras-chave: *proteínas de plantas; savana brasileira; enzimas.*

Introduction

Cerrado is the second largest biome in Brazil, representing 23% of the Brazilian territory. Because of the different phytophysiognomies found in this biome, Cerrado is considered a mosaic of vegetation, including grasslands (native pasture, herbaceous) and forests (named Cerradão)[1]. This large biodiversity reflects the potential of Cerrado species for feeding, medical and biotechnological approaches. On the other hand, about 40 % of Cerrado original area has been converted to anthropic activities in the last 40 years, but 2% of this surface are protected by law [1-3].

There are few studies about the Cerrado flora, especially those applied to biotechnological potential of the species. Torralbo et al.[4] isolated and characterized pectins from *Solanum lycocarpum* St. Hill. and compared to the citrus commercial form. The *S. lycocarpum* presented 100% of emulsifying capacity, high esterification degree and could be used to prepare sauces and jams.

Some recent efforts considering the medicinal use of the Cerrado plants have been published based on the ethnopharmacological studies: cytotoxicity against cell cancer[5], gastroprotective action[6], anti-inflammatory activity[7] and antidiabetic activity[8]. Many fruits are used by the local population to prepare desserts, juices, ice cream and can also be consumed *in natura*[2]. They are sold in some traditional fairs and regional groceries, according the local occurrence of the fruits[2].

The industrial applications of enzymes from Cerrado are not yet explored. Some authors reported enzyme applications from soil microorganisms[9,10]. According to Kirk et al.[11], the attractive enzymatic properties and the development of biotechnology have already introduced these molecules into several industrial processes and products. Enzymes mediate a variety of biotechnological procedures and can be industrially used in food processing[12-15], bio-detergent formulation[14,16-18], production of syrup[19-21] and can be recovered from the bulk using simple techniques.

Carneiro et al.[22,23] prepared a darkeness-inhibitory procedure to *Syagrus* oleracea Becc., a palm usually consumed in Cerrado, by inhibition of the polyphenol oxidase activity detected in the stems. One year later Caramori et al.[24] conducted a preliminary study using eight Cerrado plant seeds that presented proteases, trypsin inhibitor, peroxidases and lectin activities.

Considering the high potential of enzymes in their innumerous applications, it is important to find different fonts for these molecules. In this paper it is presented a set of protocols established to extract different enzyme activities, using variations on the pH. Most of the activities detected here are a target to the large-scale biotechnology.

Materials and Methods

Casein, bovine serum albumin, pyrogallol, hydrogen peroxide, trypsin, and buffers were purchased from Sigma-Aldrich (St. Louis, USA). All chemicals were used from analytical grade.

The plant samples used for the analysis were obtained from Cerrado, collected in the municipalities of Goiânia, Rio Quente and Pirenópolis, Goiás, Brazil (Table 1). It was considered the mature fruits (for the fruit analysis) collected from five different trees located at least 10 m far from each other. The parts used in the analysis were those used for feeding and popular medicinal purposes.

S. saponaria, G. *ulmifolia* and E. *dysenterica* mature fruits were collected and taken to the Laboratory of Protein Chemistry at the Universidade Federal de Goiás. The rind, pulp and seeds were milled in separately and

Table 1. List of plant material, their scientific and popular names, part used and their local origin.

Scientific Name	Popular Name	Part Used	Origin
Sapindus saponaria	Saboneteira	Seed	Rio Quente/Goiânia
Guazuma ulmifolia	Mutamba	Rind/pulp	Goiânia
Eugenia dysenterica	Cagaita	Rind/pulp	Goiânia
Striphnodendrum adstringens	Barbatimão	Caulis rind	Pirenópolis/Goiânia

stored in a refrigerator (-10 ºC) for further analyses. G. *ulmifolia* seeds were not used here because they were already analyzed[24]. S. *adstringens* stalk rind were collected, broken and milled. The resultant flour was stored in a refrigerator for later analysis.

The determination of humidity, ashes (minerals), lipids and total protein concentration were carried out by elemental analysis, according to Instituto Adolfo Lutz [25].

The soluble proteins and phenols concentration of *S. saponaria, G. ulmifolia* and S. adstringens were measured. Samples of the tree species were stirred for 1 h, at 4 ºC, in the ratio of 1.0 g to 10 mL with 0.1 mol L-1 glycin (pH 2.6 and 9.0) and 0.1 mol L-1 sodium phosphate (pH 7.0) buffers. Following, the samples were centrifuged (Himac CR 21E - Hitachi) in 12,000 x g for 15 min. The precipitated was discharged and the supernatant was used as crude extract. E. dysenterica samples were macerated with the extraction buffer in the ratio of 1.0 g to 2.0 mL and submitted to vacuum filtration. The remained solid part in the Watman filter paper (# 1) was rejected and the filtered liquid was used as a crude extract.

The soluble protein concentration was determined according to methodology described by Bradford[26], using bovine serum albumin as standard. This parameter was used to calculate the specific enzyme activities.

The presence of proteolytic activity and trypsin inhibitor was determined according to Kunitz modified by Arnon [27]. Casein 1% (w/v) was used as substrate and trichloroacetic acid (TCA) 5.0 % (w/v) was the stopper. Peroxidase activity was determined according to Halpin and Lee[28], using pyrogallol and hydrogen peroxide as substrates.

The presence of lectins in the extracts was determined by hemagglutinant activity against rabbit red blood cells according to Moreira and Perrone[29] with modifications. Briefly, the extracts (0.2 mL) were diluted in 2-fold dilution series against a 0.15 mol L^{-1} NaCl solution containing 0.1 mol L^{-1} $CaCl_2$ and 0.1 mol L^{-1} $MnCl_2$. Two hundred microliter of 2% (v/v) rabbit erythrocytes was added to an equal volume of the sample and the mixture incubated at 37 ºC for 30 min followed by 30 min of resting at room temperature (25 ºC). The tubes were centrifuged (Excelsa 2 – Fanen Ltda.) at 90.0 x g for 1 min and the last tube that showed visible agglutination was considered the point of equivalence to determination of minimal hemagglutinant activity. The lectin concentration was measured using hemagglutinanting units (HU g^{-1} of flour).

The phenolic compounds were determined according to Price and Butler[30], with modifications. The crude extract (0.1 mL) was placed in contact with 0.1 mol L-1 ferric chloride (0.3 mL) and 0.008 mol L^{-1} potassium ferricyanide (0.3 mL) until the appearance of a green coloration. After 15 min of reaction the samples were spectrophotometricaly quantified in the wavelength of 720 nm. Catechol (10 μg mL^{-1}) was used as standard (r^2 = 0.9997).

All experiments were conducted in triplicates and mean values were reported. Statistica software (Statistica 6.0, StatSoft Inc., Okla., U.S.A.) was used to perform analysis of variance (ANOVA) followed by the Tukey test to determine the significant differences among the means. The level of significance used was 95% ($P<0.05$).

Results and Discussion

The results obtained from the elemental analysis of the samples are presented in Table 2. *E. dysenterica*, a juicy fruit, presented humidity of 90% above. Lipids and protein where similar to those found by Cardoso et al.[31], in a nutritional study of *E. dysenterica* fruits.

Table 2. Elemental analysis – humidity, ashes, lipids, proteins and carbohydrates of the plants.

Plant	Humidity (%)	Ashes (%)	Lipids (%)	Proteins (%)	Carbohydrates* (%)
S. saponaria	1.15 ± 0.05	5.07 ± 0.03	46.99 ± 0.25	38.4 ± 1.27	8.39 ± 1.3
G. ulmifolia	10.44 ± 0.07	3.11 ± 0.05	55.27 ± 0.37	15.58 ± 1.81	15.6 ± 1.88
E. dysenterica	90.62 ± 0.12	1.00 ± 0.07	0.54 ± 0.03	0.87 ± 0.01	6.97 ± 0.19
S. adstringens	10.36 ± 0.07	2.03 ± 0.03	54.08 ± 0.49	21.52 ± 0.18	12.01 ± 0.63

Table 2 also shows the lipid content of *S. saponaria, G. ulmifolia* and *S. adstringens*, close to 50%. This finding indicates these materials as possible sources to be explored. Previous delipidation, on the other hand, can be considered, once the protein content of these plants is the focus of the scientific investigation. *S. saponaria* and S. *adstringens* showed the major protein content, presenting 38% and 21%, respectively. The protein content found in these samples was similar than all Leguminosae seeds analysed by Caramori et al.[24]. The authors found mean values around 22%.

Figure 1 shows the results of the soluble protein concentration. This analysis indicates variations in the results among different species and in different pH extractants. *E. dysenterica* pulp presented low protein content for all values of pH used, following by *G. ulmifolia*, without expressive variations. *S. saponaria* and *S. adstringens* presented higher soluble protein concentration, as well as demonstrated for the results in Table 2. On the other hand, it is interesting to notify the pH selectivity for S. adstringens. Both 2.6 and 9.0 showed similar protein content, but pH 7.0 was not appropriated as extractant for this purpose. Different extractants have been used to isolate same protein family, just varying the plant material font[32-34]. Depending on the cell compartment, the protein extracted in different strategies may be employed, as presented here.

The proteolytic activity showed great variation among the species, also showing the influence of the way the extraction was carried out, except for *G. ulmifolia*, where the activities were not statistically different at 5% (Figure 2). *S. saponaria* and *S. adstringens* presented the best activities when the crude extract was prepared at pH 7.0. In addition, the specific activity in Figure 3 (calculated by the ratio between enzymatic activity and the soluble protein concentration), for *E. dysenterica* and *G. ulmifolia,* presented the highest values because of the low extracted protein from these plants. This finding is an excellent factor to be considered for purification purposes.

In *S. saponaria* seeds, the proteolytic activity was low in comparison to the other species for all the tested values of pH. The presence of trypsin inhibitor (63.84 IU g^{-1} of sample) at the acid pH can explain the low proteolytic activity found here. Despite enzymes and their respective

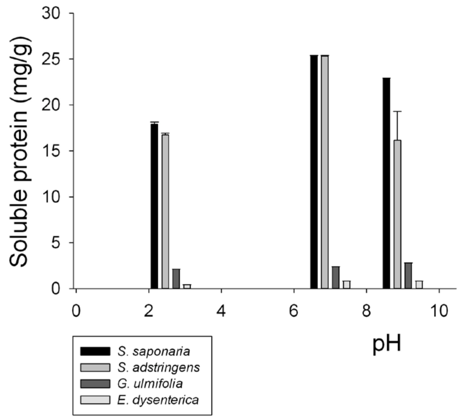

Figure 1. Soluble protein content (mg g-1 of sample) in the tested plants.

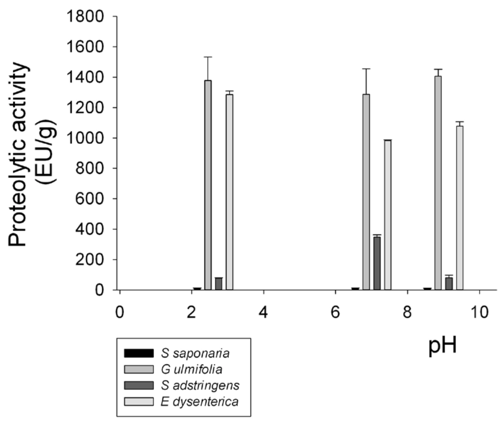

Figure 2. Proteolytic activity using casein (EU g-1 of sample).

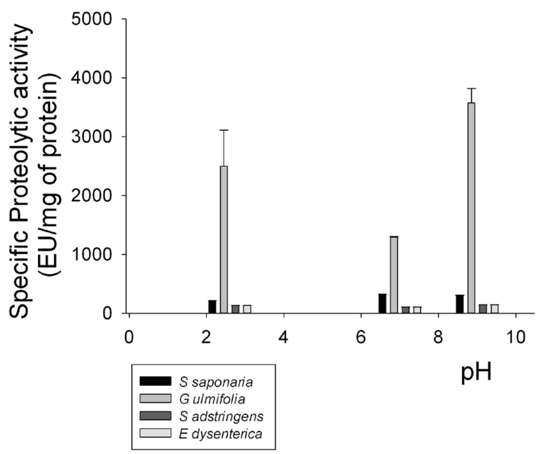

Figure 3. Proteolytic activity using casein (EU g-1 of sample).

inhibitors being generally located in different plant cell compartments, they can find each other when the crude extract is prepared. Similar results were observed for *G. ulmifolia*, that presented low proteolytic activity at pH 7.0, but showed 139.10 IU g^{-1} of trypsin inhibitor in the same condition. On the other hand, the activity found at pH 9.0 is an important finding in order to establish future purification strategies.

Peroxidase activity was only detected in *S. saponaria* and *E. dysenterica,* with major activity found in S. saponaria samples. This sample presented a type of peroxidase that can be better obtained in the neutral pH (Figure 4).

Only *S. adstringens* showed hemagglutinanting activity (10,752.00 HU g-1 of sample). Although phenolic compounds can cause erythrocytes clotting, this activity persists after boiling, due to thermal resistance of phenolics. In this case, heating the samples up to 60 ºC caused hemagglutination disappearance, confirming the hemagglutination occurrency by the presence of lectins.

The presence of lectins in *S. adstringens* was better detected in the samples extracted at the pH 7.0. The dependence of the pH extractant is an important factor in the lectin studies, because it informs about the protein solubility, consequently showing preliminary aspects of the protein structure. The activity found in this sample was approximately three hundred-fold higher than the value found by Charungchitrak et al.[35] in seeds of *Archidendron giringa* Nielsen, and higher (6.25 fold) than *Parkia platycephala* Benth.[36,] both Mimosoideae lectins.

The highest concentration of phenols was found in *S. adstringens*, with more expressive results in the alkaline pH (Figure 5). These values were comparable to some recognized fonts of phenols, such as *Anacardium occidentale* pseudofruits (28 mg g^{-1}), analyzed by Rufino et al.[37] and were higher than *Myrcaria dubia* rind (6.02 mg g^{-1}), reported by Villanueva-Tiburcio et al.[38]. Phenolic compounds have many benefits, they are known as natural antioxidants[39] that prevent several chronic diseases[40], but a great number of them are considered dangerous substances[41]. This result must be taken into account when the popular medicinal knowledge recommends the ingestion of alcoholic and aqueous extracts of the *S. adstringens* rind[42].

Conclusions

This study discloses the potentiality of selected Cerrado plants and opens perspectives for more studies in

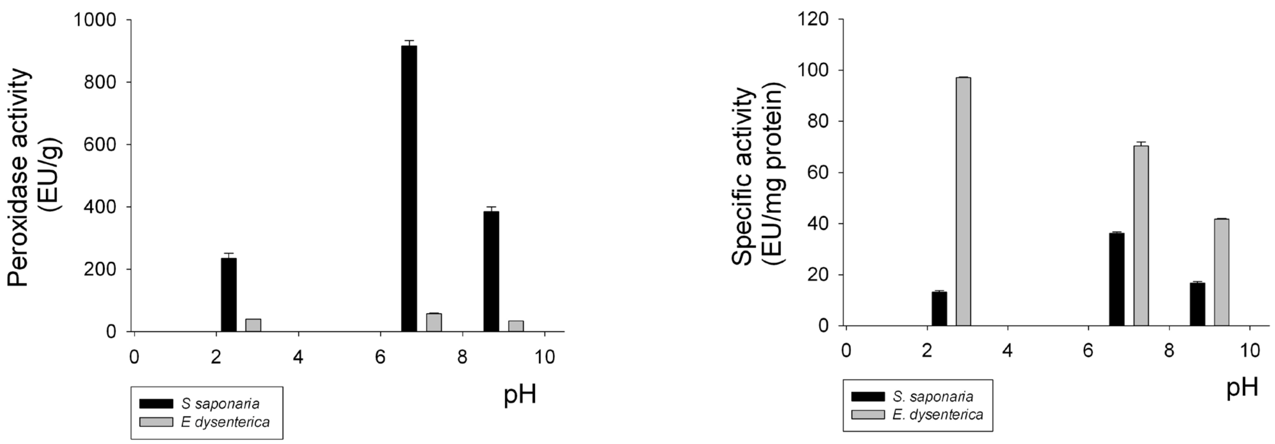

Figure 4. Peroxidase activity (EU g-1 of sample) and specific peroxidase activity (EU mg-1 of sample) in the tested plants.

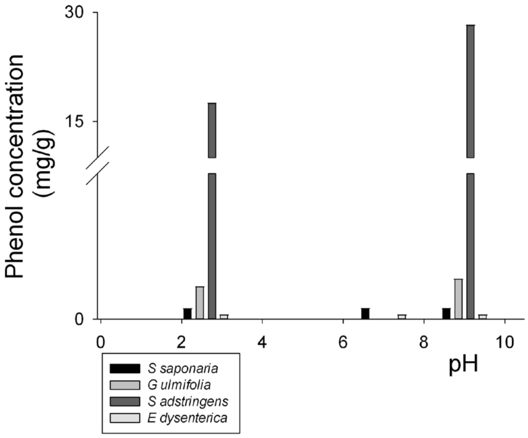

Figure 5. Determination of total phenol content (mg g-1 of sample) in tested plants.

this area. The results indicate plants that can be considered as different rich sources of biomolecules. This study also serves as an alert to actual damages related to antropic action and alerts for the biotechnological potentialities that can be irreversibly destroyed. Finally, it warns about the necessity of deeper knowledge about the biochemical composition of plants used in popular medicine.

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