

Original Article

MULTIVARIATE ANALYSIS BETWEEN THE PHYTOCHEMICAL FEATURES AND ANTIOXIDANT PROPERTIES OF THE STEMS OF *BAUHINIA GLABRA* JACQ. (FABACEAE)

RANIERI CAMPOS*, VINICIUS BEDNARCZUK DE OLIVEIRA, CRISTIANE DA SILVA PAULA, ROBERTO PONTAROLO, JOSIANE DE FATIMA GASPARI DIAS, MARILIS DALLARMI MIGUEL, SANDRA MARIA WARUMBY ZANIN, OBDULIO GOMES MIGUEL*

Programa de pós-graduação em Ciências Farmacêuticas, Departamento de farmácia, Universidade Federal do Paraná-UFPR, Av. Prof. Lothário Meissner, 632 - Jardim Botânico, CEP 80210-170, Curitiba, PR, Brazil.
Email: raniericampos@uol.com.br

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ABSTRACT

Objective: To provide information about the phytochemical features of the crude extract (CruE) and fractions of the forage *Bauhinia glabra* Jacq., Fabaceae, and present the multivariate correlation between its metabolites and the antioxidant properties.

Methods: Studies were carried out by extraction with ethanol, sequential partition with hexane, chloroform and ethyl acetate, determination of the total yield of extraction, qualitative and quantitative estimation of phytochemicals. Then, the evaluation of antioxidant properties by four methods: 1,1-diphenyl picrylhydrazyl free radical scavenging assay (DPPH), the reduction power, the evaluation of inhibition of lipid peroxidation (TBARS) and the evaluation of recovery of content of methemoglobin in erythrocytes.

Results: Phytochemical analysis on CruE revealed the presence of chlorophylls, carotenoids, coumarins, phytosterols, anthocyanins, tannins and flavonoids. Quantitative estimation of metabolites on CruE showed high concentration of phytosterols (42.21 ± 2.34) mg/gdw and total phenolic (58.50 ± 1.98) mg/gdw. The best results on the antioxidant properties on each assay were the chloroform fraction (ChlF) with 13.76 ± 0.23 and methanolic fraction (MetF) with 13.46 ± 0.45 of half-maximal inhibitory concentration (IC₅₀) values (DPPH). The HexF ($60.99 \pm 1.76\%$) and ChlF ($64.40 \pm 1.73\%$), both based on reduction power of ascorbic acid. The HexF ($73.54 \pm 2.74\%$) of inhibition of lipid peroxidation (TBARS), and HexF with $7.28 \pm 0.36\%$ amount of methemoglobin.

Conclusion: Based on multivariate analysis, HexF that presented the highest concentration of phytosterols, has the highest reduction power and was more effective for inhibition of lipid peroxidation and in recovering methemoglobin content. Therefore, the mechanism of these activities seems to be related to the reduction power presented by phytosterols.

Keywords: *Bauhinia glabra* Jacq., Fabaceae, Antioxidant, Methemoglobin, Phytosterols, Phytochemical features, Multivariate analysis.

INTRODUCTION

The genus *Bauhinia* belongs to family Fabaceae, being pan tropical with distribution in Africa, Asia, and in several countries in Latin America [1]. According to the literature, several medicinal properties have been assigned to the species of *Bauhinia*, which include antidiabetic, anti-inflammatory, analgesic/antinociceptive, hypocholesterolemic and most of these species have been frequently used in folk medicine to treat diabetes [2; 3]. Chemical features previously reported for *Bauhinia* include the isolation of alkaloids, tannins, terpenoids, sterols, triterpenes, and more frequently flavonoids [3; 4; 5; 6; 7].

The species *Bauhinia glabra* Jacq., Fabaceae, also known as "cipó-de-escada", has a leguminous bent stem, which is staggered and simulates a stair. The only report found in the literature suggests that this species is used as feed for ruminants in Brazil, but no studies have been found reporting on its chemical composition, only bromatological analysis was conducted [8].

According to [9] and [10], oxidative stress is one of the most important issues related to the tenderness of meat. During the production of the meat, oxidative stress can intervene in a negative way affecting the quality of production of collagen and therefore the softness of meat. Using a convenient feed that contains antioxidant properties will result in a better response from the animal related to several inflammatory reactions between immunologic parameters that can make the necessity of antibiotics minor. In addition, phenolic compounds can change the ruminant microbiota reducing the incidences of diarrhea and obesity [11]. The objective of this research is to provide information about the phytochemical features of the crude extract (CruE) and the fractions of *B. glabra* and the

multivariate correlation between its metabolites and the antioxidant properties.

MATERIAL AND METHODS

Chemicals

All the chemicals used were of analytical grade and purchased from Aldrich and Merck. The spectrophotometer used was SHIMADZU - UV 1601 PC.

Plant Material

The botanical material (stem) was collected in the month of October, at Rondonópolis, (state of Mato Grosso do Sul, Brazil). The samples were dried in a greenhouse at 30°C for 72 hours, and grinded in a hammer mill. The botanical determination was conducted by biologist Osmar dos Santos Ribas, in the Municipal Botanical Museum in Curitiba (Paraná-Brazil) by comparison with a voucher registered under the number 287876.

Extraction Procedures

The plant material (2kg) was submitted to extraction in a Soxhlet apparatus with 95% ethanol at 78.4°C for 24 hours, followed by filtering. The resulting solution was concentrated under reduced pressure down to 300ml and then left to dry at room temperature, to yield the corresponding crude extract (CruE). This sample was used to determine the extractive yield, by the use of the following equation:

$$\text{Yield (\%)} = \left(\frac{\text{dry weight of extract}}{\text{dry weight of plant powder}} \right) \times 100$$

This CruE was liquid-liquid partitioned yielding a hexane fraction (HexF), chloroform fraction (ChlF), ethyl acetate fraction (EthF), Methanol fraction (MetF) and the remaining fraction (RemF). These samples were fully dried and used for the tests. The yields of partition were deduced using the following equation:

$$\text{Yield (\%)} = \left(\frac{\text{dry weight of fraction}}{\text{dry weight of extract}} \right) \times 100$$

Preliminary Phytochemical Analysis

The qualitative evaluation of the presence of groups of metabolites was accomplished for the CruE, according to the methods described by [12] and [13].

Quantification of Metabolites

The CruE and the five fractions were evaluated quantitatively to estimate the total contents of chlorophylls A and B (TChlCa and TChlCb) and carotenoids (TCarC) [14], phytosterols (TPhyC) [15], total phenolic content (TPheC) [16], anthocyanins (TAnthC) and tannins (TTanC) [17], and flavonoids (TFlaC) [18] following the well-established methods.

DPPH (2,2-diphenyl-1-picrylhydrazyl) Radical Scavenging Activity

This assay was carried out according to [19]. Five methanol solutions of CruE and fractions were prepared, with concentrations ranging from 2.0 to 12.5 µg/ml, of which 2.5 ml was added to 1 ml of 0.03 mmol/ml DPPH methanol solution. For the HexF, five solutions that concentration ranged from 100 to 300 µg/ml were used.

A blank reagent with 2.5 ml of the sample solution and 1 ml of methanol was prepared for each sample. In parallel, a negative control containing 2.5 ml of methanol and 1 ml of DPPH solution was prepared. After 30 minutes, readings were conducted in a spectrophotometer at 518 nm. The positive control was ascorbic acid treated at the same as the samples. The ability of samples on reducing the DPPH radical was calculated as follows:

$$\text{C\%} = 100 - \left[\frac{(\text{A sample} - \text{A blank reagent}) \times 100}{\text{A control}} \right]$$

Phosphomolybdenum Assay (Reduction Power)

This method was prepared based on [20]. A methanolic solution at 200 µg/mL was prepared for crude extract and each fraction. Three hundred microliters of each sample was added to 3 ml of reagent solution of phosphomolybdenum complex. Tubes were shut and kept in a boiler at 95°C for 90 minutes and after cooling, the reading at 695 nm was run. Reactants without the tested samples were used as blank. The reduction power of the samples was compared to ascorbic acid (200 µg/ml) which the reduction power was considered 100%.

TBARS Assay (Lipid Peroxidation Evaluation)

The assay was performed according [21] with modifications. The samples, CruE, fractions and the positive control (BHT) were prepared at 500 ppm in methanol. A sample without treatment was prepared as a negative control. In order to determine the percentage of inhibition of lipoperoxidation, 0.4 ml of H₂O, 0.5 ml of 10 % egg yolk solution in water, 50 µl of 0.07 M ABAP and 1.5 ml of 20% acetic acid (pH 3.5) were added to the samples, followed by 1.5 ml of 0.8% thiobarbituric acid in 1.1% SDS (sodium dodecyl sulfate) solution.

The samples were heated in a water bath at 95° for 1 hour. After cooling, 5 ml of 1-butanol was added and the samples were centrifuged at 3000 rpm for 10 minutes. The absorbances of supernatant were measured at 532 nm. The inhibition of lipoperoxidation (%IP) was calculated as follows:

$$\%IP = \left[1 - \left(\frac{\text{Absorbance of samples}}{\text{Absorbance of negative control}} \right) \right] \times 100$$

Antioxidant Activity over Erythrocytes

The CruE and fractions were submitted to this assay, which is based on [22] with modifications. A 10% sheep red blood cell suspension (Newprov®) was prepared in pH 7.4 phosphate buffer. The tested samples were diluted in 0.9% NaCl, and tested at concentration of 1000 µg/ml. A volume of 200 µl of the blood cell suspension was added to 500 µl of the samples, which were then mixed slowly and left to rest for 3 hours at 37°C. Subsequently, the samples were totally hemolyzed with 100 µl of 1% saponin. Solvent and phosphate buffer was used as a negative control.

The results were reported as percentage of methemoglobin based on the sum of the absorbances at 540 nm and 630 nm after hemolysis. The results were compared to ascorbic acid (100 µg/ml), and the percentage of methemoglobin was calculated as follows:

$$\% \text{Methe} = \left(\frac{\text{Abs } 630 \times 100}{\text{Abs } 540 + \text{Abs } 630} \right)$$

Statistical Analysis

All the experiments were accomplished in triplicate and expressed as means ± SD. The means were compared by one-way ANOVA and the values were considered to be significantly at P < 0.05. IC₅₀ values were also calculated by linear regression analysis. The multivariate analysis was performed by the use of software "Statistica 10.0".

RESULTS

Phytochemical Features

The chosen method to prepare CruE and the fractions proved to be suitable for the goals of this study. The yield of extraction is shown in table 1.

Table 1: It shows the Yield of CruE and fractions of *B. glabra* Jacq.

Sample	Solid Content (g/kg)
CruE	92.85 ± 1.44
Samples	Yield (%)
HexF	21.39 ± 0.90
ChlF	20.06 ± 1.02
EthF	5.68 ± 0.87
MetF	40.23 ± 1.62
RemF	12.64 ± 1.57

*Data are expressed as means ± SD (n=3)

The preliminary phytochemical analysis of CruE revealed the presence of phytosterols, coumarins (not quantified), anthocyanins, tannins, and flavonoids, according to table 2.

Table 2: It shows the total contents of metabolites of CruE of *B. glabra* Jacq.

Samples	Total content of metabolites (mg/g)
TChlCa	0.01 ± 0.002
TChlCb	0.01 ± 0.003
TCarC	1.35 ± 0.03
TPhyC	42.21 ± 2.34
TAnthC	10.50 ± 1.26
TPheC	58.50 ± 1.98
TTanC	5.24 ± 1.02
TFlaC	2.83 ± 0.06

*Data are expressed as means ± SD (n=3)

In order to establish the relationship between the phytochemical features of the extract of *B. glabra* Jacq., with the antioxidant properties of it, the total content of the groups of metabolites was evaluated in each fraction. The data are shown in table 3.

Table 3: It shows the total content of metabolites per fraction of the CruE of *B. glabra* Jacq.

Metabolites (mg/g)	HexF	ChlF	EthF	MetF	RemF
TChlCa	0.45±0.03	0.64±0.02	n.d.	n.d.	n.d.
TChlCb	0,42±0.04	0.65±0.03	n.d.	n.d.	n.d.
TCarC	0,29±0.02	6.42±0.03	n.d.	n.d.	n.d.
TPheC	n.d.	n.d.	165.50±0.40	79.50±0.40	135.38±0.03
TPhyC	106,34±0.40	68.02±0.50	n.d.	n.d.	n.d.
TAnthC	n.d.	n.d.	n.d.	n.d.	83.08±0.50
TTanC	n.d.	n.d.	6.02±0.70	6.02±0.80	19.65±0.80
TFlaC	n.d.	n.d.	43.00±0.90	n.d.	n.d.

*Data are expressed as means±SD (n=3); n.d. = not detected

Table 4: It shows the results of the evaluation of antioxidant properties of CruE and fractions of *b. glabra* Jacq.

Samples	DPPH	Phosphomolibdenium (%)	TBARS (%)	Methemoglobin (%)
Pos. control	5.24 ^a ±0.03	100.00 ^a	52.31 ^d ±2.45	5.75 ^a ±0.32
Neg. control	-	-	-	10.62 ^d ±0.43
HexF	162.29 ^a ±0.72	60.99 ^b ±1.76	73.54 ^a ±2.74	7.48 ^b ±0.33
ChlF	13.76 ^b ±0.23	64.40 ^b ±1.73	56.02 ^{cd} ±2.92	10.77 ^d ±0.45
EthF	37.54 ^c ±0.85	19.13 ^d ±1.66	15.86 ^e ±2.05	10.92 ^d ±0.42
MetF	13.46 ^b ±0.45	42.79 ^c ±1.52	9.81 ^{ef} ±2.58	10.81 ^d ±0.38
RemF	58.17 ^d ±0.61	9.20 ^e ±1.63	7.20 ^f ±2.09	9.25 ^e ±0.30
CruE	52.32 ^c ±0.45	37.54 ^c ±0.47	59.3 ^{bc} ±2.69	8.97 ^c ±0.32

*Data are expressed as means±SD (n=3 per group); Means were compared by Tukey Test (P< 0.05); a-f: Samples with no statistical difference; Positive controls: DPPH (Ascorbic acid), Phosphomolibdenium (Ascorbic acid); TBARS (BHT), Methemoglobin recovery (Acid Ascorbic); Negative controls: Data with no treatment.

Antioxidant Properties

All the results of the research on the antioxidant properties of CruE and fractions of *B. glabra* Jacq. are summarized in table 4.

DPPH (2,2- diphenyl -1- picrylhydrazyl) Radical Scavenging Activity: This assay was accomplished for CruE and fractions (Table 4), is an indicator of their respective antioxidant capacity. The means were grouped in five categories, in which ascorbic acid has shown the best results, followed by ChlF and MetF. The third category includes CruE, followed by RemF, and then HexF.

Phosphomolybdenum Assay (Reduction Power)

This essay presents the reduction power of CruE and fractions (Table 4). The means were grouped in five categories, in which ascorbic acid represents 100% of reduction power. Both fractions, HexF and ChlF, showed the higher results, followed by MetF and CruE, EthF, and then RemF.

TBARS Assay (Lipid Peroxidation Evaluation)

This method presents the percentage of inhibition of lipid peroxidation present by CruE and fractions (Table 4). The means were grouped in six categories, in which Butyl hydroxyl toluene (BHT) inhibited 52.31% of peroxidation, being equal to ChlF. There is no significant difference between ChlF and CruE activities, but CruE activity is clearly higher than that presented by BHT. The best result was 73.54% of inhibition of lipid peroxidation presented by HexF. The fractions EthF and MetF are grouped showing low activity, followed by RemF. There are no significant differences between MetF and RemF activities also.

Antioxidant Activity over Erythrocytes

The evaluation of antioxidant activity over erythrocytes presents the capacity of the samples on recovering of Methemoglobin (Fe³⁺) into oxihemoglobin (Fe²⁺). The means were grouped in four categories, in which ascorbic acid presents the higher activity, followed by HexF. Both fractions, CruE and RemF, showed a little activity. The ChlF, EthF and MetF were not different from the negative control (Table 4).

DISCUSSION

The chemical composition and antioxidant properties of CruE and fractions were correlated by multivariate analysis (Fig. 1), and divided into four distinct groups based on similarity. The group 1

includes HexF and ChlF. Group 2 that exhibits similarity related to phenolic content. Group 3, formed by only EthF, and group 4, by RemF. The group 1 (both negative components) and the groups 2 and 3 (both positive components) are in opposition, which suggests distinct features and properties among them. The group 4 has one positive and one negative component suggesting similarity with all the other groups in long distance.

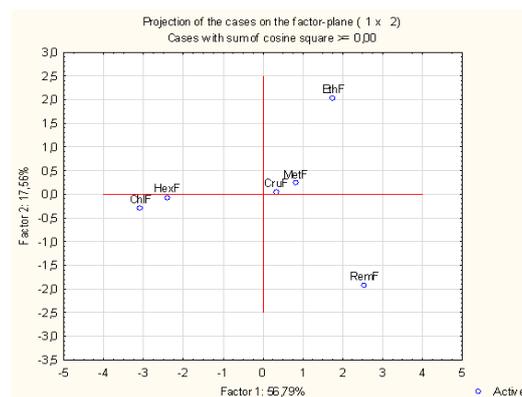


Fig. 1: It shows the principal component analysis (PCA) of similarity between the chemical features and the antioxidant properties of the samples. Samples are considered to be similar if they are located in the same Cartesian quadrant and in a maximum distance of one square, in all sides. Samples are considered to have a linkage if at least one component is at the same signal (positive or negative).

Accordingly, with this classification above, the contribution of each group of metabolites over the antioxidant properties was evaluated by clustering analysis (fig.2).

The samples belonging to group 1, that exhibits the highest content of phytosterols, have a strict relation with the reduction power, results in inhibition of lipid peroxidation and the recovery of the content of methemoglobin. In addition, the content of carotenoids presented in ChlF seems to influence directly the result of this

sample in DPPH assay. It can be explained by the nature of these metabolites, once they are electron transporters.

On the other hand, phenolic compounds like tannins and flavonoids are more related with the results obtained on DPPH assay, in long distance. All metabolites seem to promote a response on DPPH assay, but the most significant were those attributed to phenolic compounds. According to the literature, phenolic compounds are well known for their remarkable ability to acting as antioxidants, and in most cases, they play an important role against oxidative stress. Even further, flavonoids show, anti-inflammatory and immunomodulation activities [23; 24; 25].

The anthocyanins found only on RemF seem to play a role in the recovery of the content of methemoglobin by the reduction power, but the concentration is low to produce a better response. This relation needs to be further evaluated.

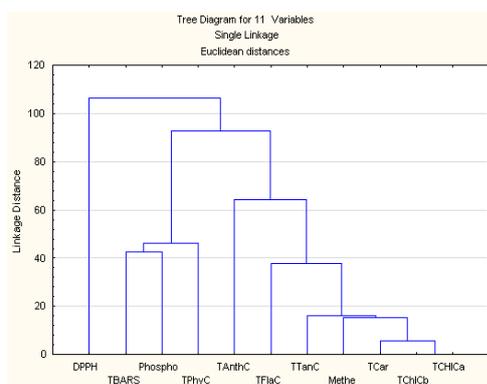


Fig. 2: It shows the Euclidian linkage distances among the chemical features and antioxidant properties of samples. The graphic was analyzed at 60 of linkage distance. The phytochemical features and the antioxidant properties were separated into 4 groups, according to the proximity of correlation

The inhibition of lipid peroxidation is one of the major concerns on the matter of oxidative stress, and the best results on this assay were HexF and ChlF, which are in concordance with literature, once phytosterols are much similar with lipids of the cell membrane, and chlorophylls act like electron transporters. It was observed a striking relationship between this class of metabolites and the processes of lipoperoxidation. Phytosterols chemically act as antioxidants, in solution and physically in the cell membranes by packing and stabilizing them [26].

The activity on recovering the content of methemoglobin was evaluated and only HexF exerted activity. The composition of this fraction seems to be responsible for the activity that CruE showed once any other fraction presented a non-significant response. When analyzing figure 2, the methemoglobin assay exhibits a great dependence on the phosphomolibdenium and TBARS assays. Fractions with good reduction power and the ability of inhibiting lipoperoxidation have increased their activity on recovering methemoglobin content. The best correlation was among TPhyC, the methemoglobin and the TBARS assays, suggesting that this group of metabolites not only exert protective effects, but they also play a part in the activity, by the reduction power of these substances.

CONCLUSION

The present study aimed to provide information about the phytochemical features and the correlation between these, and the antioxidant properties of the CruE and fractions of *B. glabra*. The groups of metabolites found in the samples were chlorophylls, carotenoids, phytosterols, coumarins, and phenolics such as anthocyanins, flavonoids and tannins. The phytosterols seem to be the major components responsible for the results on TBARS and Methemoglobin assays, as well as phenolic compounds produce a better response over the DPPH assay. The anthocyanins seem to be

active in all the accomplished methods and this relationship should be further evaluated. Additionally, the mechanism of these activities seem to be related to the reduction power of the samples.

CONFLICT OF INTERESTS

Declared None

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