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Influence of environmental factors on the concentration of phenolic compounds in leaves of *Lafoensia pacari*

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Abstract: *Lafoensia pacari* A. St.-Hil., Lythraceae, a plant from the Cerrado known as pacari or dedaleiro, is widely used as an antipyretic, wound healing, anti-inflammatory, antidiarrheal and in the treatment of gastritis and cancer. Notable among the metabolite groups identified in leaves of *L. pacari* are the polyphenols, such as tannins and flavonoids, related to the pharmacological activities of pacari. Studies on the influence of environmental factors over production of major groups of secondary metabolites in pacari are important because they contribute data for its cultivation and harvest, and establish quantitative parameters of secondary metabolites in the plant drug. The objective of this study was to evaluate the influence of environmental factors on concentrations of phenolic metabolites in the leaves of *L. pacari*. Compounds quantified in the leaves were: total phenols, tannins by protein precipitation, hydrolysable tannins, total flavonoids, ellagic acid and mineral nutrients, while soil fertility was also analyzed, all over a period of one year. The data were analyzed using multivariate analysis, and the results suggest that metabolite concentrations in the leaves of this plant are influenced by seasonal factors, in particular the temperature and foliar micronutrients (Cu, Fe, Mn, Zn).

Article

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Introduction

Among the Brazilian ecosystems that of the Cerrado stands out for its marked and conspicuous biodiversity. Data compiled by several authors suggest that, depending on the taxonomic group considered, the percentage of species that occur in the Brazilian Cerrado may represent between 20 and 50 % of the total found in the country (Machado et al., 2004). Many native plants of the Cerrado are popularly used for medicinal purposes, such as *Lafoensia pacari* A. St.-Hil., Lythraceae, known as dedaleiro, didal, mangabeira-brava or pacari in Brazil (Mundo & Duarte, 2007) and as morosyvó in Paraguay (Sólon et al., 2000). Its leaves and stem bark are used by the population in Brazil for their antipyretic, healing, and tonic effects (Mundo & Duarte, 2007), to treat backache, gastritis, ulcers (Nunes et al., 2003), and as antidiarrheal agents (Coelho et al., 2005), while in Paraguay they are used to treat cancer (Sólon et al., 2000).

Studies with extracts from the stem bark and leaves of *L. pacari* have shown antioxidant activity (Sólon et al., 2000), anti-inflammatory, antinociceptive, and antiedematogenic actions (Rogério, 2006, Guimarães, 2008), antidyspeptic activity (Menezes, 2006), antimicrobial (Lima et al., 2006; Porfírio et al., 2009; Silva Jr et al., 2010) and antidepressant-like activity (Galdino et al., 2009), which demonstrates the strong potential of this plant as a source of new phytomedicines.

The pacari is a plant rich in phenolic compounds, particularly tannins and flavonoids (Sólon et al., 2000; Santos et al., 2000; Galdino et al., 2009), while ellagic acid (a product of acid hydrolysis of ellagitannins) is also found in extracts of the leaves and stem bark of *L. pacari.* These phenolic compounds, especially ellagic acid, are believed to be responsible for many of the biological activities shown by such extracts, such as antioxidant (Sólon et al., 2000), anti-inflammatory (Rogério, 2006; Guimarães, 2008), antimicrobial (Silva Jr et al., 2010) and antidyspeptic actions (Menezes, 2006).

Phenolic compounds, as well as other secondary metabolites (responsible for the pharmacological effects of plants), represent a chemical interface between plants and the environment, and their synthesis is often affected by environmental factors (Gobbo-Neto & Lopes, 2007). This, it is important to try to understand how the environment can affect the concentrations of phenolic compounds present in tissues of *L. pacari* used by the population for therapeutic purposes, especially those of

tannins, flavonoids and ellagic acid.

The tannins are a group of secondary metabolites that occur in many species of higher plants (Santos & Mello, 2004), and that appear to be greatly influenced by environmental factors from the standpoint of both quantitative and qualitative factors such as seasonality (season), insect attack, air pollution (Monteiro et al., 2005), water availability and nutrients (macro and micronutrients) (Gobbo-Neto & Lopes, 2007). These appear to directly affect the chemical composition and specifically the tannin content found in plants, which can be explained by the fact that the production of these compounds is related to a type of chemical plant response to the environment.

The flavonoids are another group of phenolic compounds whose levels in plant tissues are influenced by environmental factors. They are mainly accumulated in superficial tissues (*e.g.* epidermis, subepidermal layer, hair cuticle and epicuticular material) and used by the plant as UV filters, because they absorb UV-B without changing the photosynthetically active radiation and an increase in the production of these metabolites, which therefore act as sunscreens for the plant, is controlled by enzymes of the phenylpropanoid biosynthetic pathway (phenylalanine ammonia-lyase and chalcone synthase, among others), which may have their gene expression induced by light (Gobbo-Neto & Lopes, 2007).

Based on the information already in the literature and in view of the lack of data on the influence of environmental factors on the production of phenolic metabolites in leaves of *L. pacari*, this work was carried out in order to obtain new data that would inform the use of these leaves as a plant drug in the development of a phytomedicine, as well as studies on the appropriate cultivation and sampling of this plant.

Material and Methods

Plant material

The plant material was collected quarterly from five specimens of the plant located in different municipalities in the state of Goiás, Brazil: Pires do Rio (17° 13'03, 0" S, 48° 18'49, 5" W, 832 m) Urutaí (17° 22' 04.4" S, 48° 12' 19.6" W, 848 m), Bela Vista de Goiás (16º 58' 48.3" S, 48° 56' 06.8" W, 840 m), Anápolis (16° 22' 40.4" S, 48° 56' 35.5" W, 912 m) and Nova America (15º 00' 16.7" S, 49° 58' 40.9" W, 701 m), from April 2009 to April 2010, totaling twelve months. The specimens of *Lafoensia pacari* A. St.-Hil., Lythraceae, were native to the Cerrado, and samplings were performed from the same specimen in each locality. The samples were identified by Prof. Dr. Heleno D. Ferreira from the Department of Biology, Federal University of Goiás (UFG). Five voucher specimens were deposited, one for each specimen in the herbarium of UFG, under the numbers: 43182 (Urutaí), 43183 (Pires do Rio), 43184 (Bela Vista de Goias), 43185 (Anápolis) and 43186 (Nova América). Soil sampling was also performed for each specimen used in the study, following a standardized protocol, by the Laboratory of Soil and Foliar Analysis of the School of Agronomy, for analysis of soil nutrients. The plant material collected was dried in an oven with circulating air at a temperature of 40 ºC. After drying, the material was processed in a steel-bladed grinder with a number 100 screen (150 µm), and the powder obtained for each sample was packed in plastic bags properly identified and stored in a cool place and protected from the light.

Assays for quantification of phenolic compounds

The reagents used in the analysis (acetone, acetic acid, hydrochloric acid, sulfuric acid, isopropyl alcohol, ethyl ether, methanol, sodium lauryl sulfate, triethanolamine and pyridine) were provided by Vetec, bovine serum albumin (BSA) provided by Aldrich, and the standard substances used were: tannic acid (Vetec) for quantification of total phenols, tannins by protein precipitation and hydrolysable tannins; rutin (Sigma) for quantification of total flavonoids, and ellagic acid (Alfa Aesar).

Determination of total phenols (TP) and tannins by protein precipitation (TPP)

For the determination of TP and TPP aqueous extracts were prepared from 0.75 g of the sample (plant drug), this material was transferred to a 250 mL Erlenmeyer flask and 150 mL of distilled water were added. The mixture was heated to boiling and kept in a water bath at between 80 and 90 ºC for 30 min. After cooling under running water, the contents of the flask were transferred to a 250 mL volumetric flask and the volume was made up with distilled water. The flask was then left for a sediment to form and the liquid was filtered through qualitative filter paper. The first 50 mL of filtrate was discarded. Each sample was prepared in triplicate. The aqueous extracts obtained were used for the determination of TP and TPP by the method of Hagerman & Butler (Waterman & Mole, 1987a,b).

Determination of hydrolysable tannins (HT)

For the determination of HT, extracts were prepared from 0.5 g of the sample (plant drug). This material was transferred to a 250 mL Erlenmeyer flask, 20 mL of a solution of acetone:water (7:3 v/v) were added and the flask was covered with plastic film before being shaken at 60 rpm for one hour. Next, the residue was decanted and filtered through filter paper. A further

aliquot of 20 mL of the solvent solution was added to the residue in the flask and the procedure was completed in the same way, adding the second filtrate to the first. In all the process was repeated three times. After extraction, the filtrate was transferred to a rotary evaporator and the acetone was evaporated at a temperature of 40 °C. The resulting aqueous extract was extracted with three aliquots of 30 mL of ethyl ether in a separation funnel and the ether layer was discarded. The aqueous extract, free of grease and chlorophyll (after extraction as ethyl ether), was frozen in an appropriate vial and lyophilized. After lyophilization of the extract, the yield was calculated. The complete procedure was performed in triplicate. The lyophilized extracts obtained were then dissolved in distilled water (1.0 mg extract/mL) and used to assay HT by the potassium iodate method described by Willis & Allen (1998).

Determination of ellagic acid (EA)

For the determination of EA it was necessary to subject the material to acid hydrolysis, in which samples (20 mg of plant drug) were placed in identified vials with a 2N solution of H_2SO_4 (10 mg sample/mL acid), the vials were frozen and later became a vacuum inside of them, these were then sealed and transferred to an oven at a temperature of 100 ºC for a period of 20 h. After hydrolysis, the vials containing the samples were cooled to room temperature, opened, and placed in an ice bath for 10 min. The samples were then filtered under vacuum and the residue was washed with a cold wash solution [acetone/water/concentrated HCl (70/30/1 v/v/v)] and then dried outdoors. Once dry, both residue and filter paper were transferred to a beaker and extracted with 10 mL of pyridine. Subsequently, the samples in pyridine were filtered again through glass wool in an analytical funnel. All samples were prepared in triplicate. The filtrate was used for assay of ellagic acid by the method described by Wilson & Hagerman (1990).

Determination of total flavonoids (TF)

For the quantification of total flavonoids in the leaves of *L. pacari*, extracts were prepared from 0.25 g of the sample (plant drug) and then transferred to a 125 mL flask abraded off. A volume of 50 mL of methanol:acetic acid 0.02 M (99:1 v/v) was added and the mixture was incubated in a water bath under reflux at 90-100 °C for 40 min before being filtered. All samples were prepared in triplicate. The resulting filtrate was used for assay of TF by the method described by Rolim et al. (2005).

Chemical analysis of leaves and soil

Chemical analysis of soil and leaf was performed at the Laboratory of Soil and Foliar Analysis, School of Agronomy, Federal University of Goiás, following standard procedures (Silva, 2009). For the analysis of foliar nutrients, 15 g of each sample were packed in plastic bags, carefully labeled and sent for foliar analysis at the Laboratory of Soil and Foliar Analysis. The nitrogen (N) was extracted by digestion with H_2SO_4 and catalysts. The minerals phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sulfur (S), copper (Cu), iron (Fe), manganese (Mn) and zinc (Zn) were extracted by digestion with $HClO_4$ and HNO_3.

For the analysis of nutrients in the soil samples were collected at a depth of 0-20 cm in four locations around each specimen of *L. pacari,* subsequently homogenized and then air dried. A mass of 500 g was packed in plastic bags, carefully labeled and sent for analysis of soil nutrients at the Laboratory of Soil and Foliar Analysis. The pH was determined in a volume of water-soil at 1:1. Ca, Mg and Al were extracted with KCl 1M, and P, K, Zn, Cu, Fe and Mn were extracted with Mehlich's solution. Organic matter (OM), cation exchange capacity (CEC), potential acidity (H+Al), base saturation (V) and aluminum saturation (m) were determined by standard methods (Silva, 2009).

The quantitative determination of minerals in leaves and soil was performed according to the methodology described by Silva (2009). Nitrogen was determined by distillation (semi-micro Kjeldahl method), phosphorus by colorimetry, potassium by flame photometry and sulfur by turbidimetry. Calcium, magnesium, copper, iron, manganese and zinc were determined by atomic absorption.

Climate data

The monthly climate data (average temperature and average daily precipitation) for the study period were collected from the official site of the National Institute for Space Research (*Instituto Nacional de Pesquisas Espaciais*).

Statistical analysis

The experimental data underwent pre-treatment prior to statistical analysis. Organic-chemical variables (phenolic compounds), organic matter (OM), aluminum saturation (m) and base saturation (V) (expressed in %) were transformed by arcosine $(x/100)^{1/2}$, and the other environmental variables (soil/foliar analysis/climate), except pH, were transformed by log (x +1). The statistical software used was Canoco Version 4.5 for Windows in association with CanoDraw for Windows 4.1, SYSTAT 10 and STATISTICA 7.

The Detrended Correspondence Analysis (DCA) was used to measure the environmental gradient.

Canonical Redundancy Analysis (RDA) was used to assess the variable inflation, the correlation between metabolites and environment, and subsequently used to select the appropriate environmental variables. Hierarchical Cluster Analysis (HCA) was performed to study the similarities of the samples on the basis of the distribution of the constituents. The hierarchical clusters were formed according to Ward's Minimum Variance Method (Ward, 1963).

Canonical Discriminant Analysis (CDA) was used to propose the classification of groups, and the percentage of correct classification was determined by the Jackknife test. The significance for the Canonical Redundancy Analysis was determined by a Monte Carlo test (with 999 permutations under a reduced model). *p*-Values below 0.05 were considered significant.

Results

The data obtained after completion of the sampling and assay tests were separated into two groups: environmental variables [climate data (Table 1), foliar nutrients (Table 2) and soil (Tables 3 and 4)] and organic-chemical variables [phenolic compounds (Table 5)]. They were then analyzed statistically to determine the relationship between the environment and the phenolic compounds present in leaves of *L. pacari.*

Table 1. Climate data for the collection sites over the period from April 2009 to April 2010 - Mean precipitation (mm) and mean temperature (ºC).

Samples*	Precipitation (mm)	Temperature (ºC)
PR-Apr09	5.11	22.02
UR-Apr09	5.11	22.02
BV-Apr09	4.75	24.26
AN-Apr09	3.18	21.46
NA-Apr09	10.08	25.79
PR-Aug09	2.82	21.25
UR-Aug09	2.82	21.25
BV-Aug09	1.29	24.13
AN-Aug09	0.54	21.12
NA-Aug09	2.83	24.17
PR-Dec09	5.42	24.31
UR-Dec09	5.42	24.31
BV-Dec09	6.80	26.08
AN-Dec09	11.98	21.47
NA-Dec09	7.92	27.53
PR-Apr10	1.35	23.39
UR-Apr10	1.35	23.39
BV-Apr10	4.75	23.75
AN-Apr10	3.58	22.12
NA-Apr10	1.71	26.51

*PR: Pires do Rio; UR: Urutaí; BV: Bela Vista; AN: Anápolis; NA: Nova América.

Table 2. Levels of foliar macronutrients (Nf, Pf, Kf, Caf, Mgf, Sf in g/kg) and micronutrients (Cuf, Fef, Mnf, Znf in mg/kg) in the leaves of *Lafoensia pacari* from each collection site over the period from April 2009 to April 2010.

Samples*	Nf	Pf	Kf	Caf	Mgf	Sf	Cuf	Fef	Mnf	Znf
PR-Apr09	14.3	0.93	9.2	9.0	2.0	0.1	4.0	325.0	167.0	20.9
UR-Apr09	13.7	1.33	9.0	4.0	2.0	0.1	7.0	486.0	89.0	21.5
BV-Apr09	14.0	1.23	9.0	9.0	2.0	0.4	5.0	467.0	161.0	23.8
AN-Apr09	12.6	1.33	9.6	8.0	2.0	0.1	6.0	479.0	190.0	21.1
NA-Apr09	17.9	1.13	8.6	8.0	1.0	0.1	5.0	304.0	121.0	19.4
PR-Aug09	4.5	1.33	8.6	13.0	1.0	0.4	52.0	423.0	207.0	14.7
UR-Aug09	12.6	1.33	8.2	5.0	2.0	0.1	41.0	407.0	184.0	10.6
BV-Aug09	10.6	1.44	8.4	5.0	3.0	0.1	51.0	358.0	247.0	15.5
AN-Aug09	16.8	2.67	10.0	6.0	2.0	0.4	31.0	313.0	55.0	12.4
NA-Aug09	9.0	1.23	8.2	7.0	1.0	0.1	49.0	293.0	155.0	12.2
PR-Dec09	12.6	1.76	9.0	5.0	2.0	0.4	22.0	336.0	51.0	16.7
UR-Dec09	13.2	1.65	8.2	3.0	2.0	0.1	20.0	314.0	65.0	14.8
BV-Dec09	10.1	1.98	8.4	4.0	2.0	0.3	20.0	335.0	85.0	21.9
AN-Dec09	11.2	1.76	9.8	8.0	2.0	0.4	14.0	403.0	49.0	21.3
NA-Dec09	14.0	1.44	8.4	5.0	2.0	0.3	18.0	304.0	41.0	16.7
PR-Apr10	12.0	1.00	10.0	15.0	6.5	1.1	8.0	94.0	86.0	19.0
UR-Apr10	14.0	0.50	8.4	4.4	2.8	1.8	9.0	114.0	101.0	20.0
BV-Apr10	9.4	0.60	8.6	4.4	2.2	0.8	6.0	163.0	140.0	24.0
AN-Apr10	9.6	1.00	17.0	7.5	3.2	1.0	9.0	111.0	170.0	35.0
NA-Apr10	10.8	0.80	6.8	7.5	2.6	1.0	8.0	68.0	107.0	30.0

*PR: Pires do Rio; UR: Urutaí; BV: Bela Vista; AN: Anápolis; NA: Nova América.

Table 3. Levels of mineral nutrients and fertility parameters of soil from each collection site (Part A).

Samples*	Cu mg/dm³	Fe mg/dm³	Mn mg/dm³	Zn mg/dm³	P mg/dm³	K mg/dm³	O. M. %	pH ($CaCl_2$)	Ca cmolc/dm³	Mg cmolc/dm³
PR-Apr09	1.70	47.00	19.70	0.90	0.80	50.00	1.5	4.5	0.90	0.20
UR-Apr09	0.70	46.60	20.00	1.00	0.80	42.00	1.3	5.8	0.70	0.20
BV-Apr09	1.50	43.30	14.20	1.50	0.30	52.00	2.1	4.5	0.60	0.20
AN-Apr09	1.30	52.20	16.20	0.60	0.80	50.00	2.7	4.5	0.60	0.20
NA-Apr09	0.30	65.20	14.40	0.90	1.10	60.00	1.6	4.7	1.20	0.40
PR-Aug09	1.70	47.00	19.70	0.90	0.80	50.00	1.5	4.5	0.90	0.20
UR-Aug09	0.70	46.60	20.00	1.00	0.80	42.00	1.3	5.8	0.70	0.20
BV-Aug09	1.50	43.30	14.20	1.50	0.30	52.00	2.1	4.5	0.60	0.20
AN-Aug09	1.30	52.20	16.20	0.60	0.80	50.00	2.7	4.5	0.60	0.20
NA-Aug09	0.30	65.20	14.40	0.90	1.10	60.00	1.6	4.7	1.20	0.40
PR-Dec09	1.70	47.00	19.70	0.90	0.80	50.00	1.5	4.5	0.90	0.20
UR-Dec09	0.70	46.60	20.00	1.00	0.80	42.00	1.3	5.8	0.70	0.20
BV-Dec09	1.50	43.30	14.20	1.50	0.30	52.00	2.1	4.5	0.60	0.20
AN-Dec09	1.30	52.20	16.20	0.60	0.80	50.00	2.7	4.5	0.60	0.20
NA-Dec09	0.30	65.20	14.40	0.90	1.10	60.00	1.6	4.7	1.20	0.40
PR-Apr10	1.70	47.00	19.70	0.90	0.80	50.00	1.5	4.5	0.90	0.20
UR-Apr10	0.70	46.60	20.00	1.00	0.80	42.00	1.3	5.8	0.70	0.20
BV-Apr10	1.50	43.30	14.20	1.50	0.30	52.00	2.1	4.5	0.60	0.20
AN-Apr10	1.30	52.20	16.20	0.60	0.80	50.00	2.7	4.5	0.60	0.20
NA-Apr10	0.30	65.20	14.40	0.90	1.10	60.00	1.6	4.7	1.20	0.40

*PR: Pires do Rio; UR: Urutaí; BV: Bela Vista; AN: Anápolis; NA: Nova América.

Table 4. Levels of mineral nutrients and fertility parameters of soil from each collection site (Part B).

Samples*	H+Al cmolc/dm³	Al cmolc/dm³	CEC cmolc/dm³	M %	V %	Ca/Mg-	Mg/K-	Ca/K-	Ca/CTC %	Mg/CTC %	K/CTC %
PR-Apr09	3.10	0.000	4.30	0.0	28.4	4.5	1.6	7.0	20.8	4.6	3.0
UR-Apr09	2.50	0.100	3.50	9.0	28.7	3.5	1.9	6.5	20.0	5.7	3.1
BV-Apr09	3.10	0.100	4.00	9.7	23.1	3.0	1.5	4.5	14.9	5.0	3.3
AN-Apr09	3.50	0.000	4.40	0.0	21.0	3.0	1.6	4.7	13.6	4.5	2.9
NA-Apr09	2.80	0.100	4.60	5.4	38.5	3.0	2.6	7.8	26.4	8.8	3.4
PR-Aug09	3.10	0.000	4.30	0.0	28.4	4.5	1.6	7.0	20.8	4.6	3.0
UR-Aug09	2.50	0.100	3.50	9.0	28.7	3.5	1.9	6.5	20.0	5.7	3.1
BV-Aug09	3.10	0.100	4.00	9.7	23.1	3.0	1.5	4.5	14.9	5.0	3.3
AN-Aug09	3.50	0.000	4.40	0.0	21.0	3.0	1.6	4.7	13.6	4.5	2.9
NA-Aug09	2.80	0.100	4.60	5.4	38.5	3.0	2.6	7.8	26.4	8.8	3.4
PR-Dec09	3.10	0.000	4.30	0.0	28.4	4.5	1.6	7.0	20.8	4.6	3.0
UR-Dec09	2.50	0.100	3.50	9.0	28.7	3.5	1.9	6.5	20.0	5.7	3.1
BV-Dec09	3.10	0.100	4.00	9.7	23.1	3.0	1.5	4.5	14.9	5.0	3.3
AN-Dec09	3.50	0.000	4.40	0.0	21.0	3.0	1.6	4.7	13.6	4.5	2.9
NA-Dec09	2.80	0.100	4.60	5.4	38.5	3.0	2.6	7.8	26.4	8.8	3.4
PR-Apr10	3.10	0.000	4.30	0.0	28.4	4.5	1.6	7.0	20.8	4.6	3.0
UR-Apr10	2.50	0.100	3.50	9.0	28.7	3.5	1.9	6.5	20.0	5.7	3.1
BV-Apr10	3.10	0.100	4.00	9.7	23.1	3.0	1.5	4.5	14.9	5.0	3.3
AN-Apr10	3.50	0.000	4.40	0.0	21.0	3.0	1.6	4.7	13.6	4.5	2.9
NA-Apr10	2.80	0.100	4.60	5.4	38.5	3.0	2.6	7.8	26.4	8.8	3.4

*PR: Pires do Rio; UR: Urutaí; BV: Bela Vista; AN: Anápolis; NA: Nova América.

Table 5. Phenolic compounds contents (total phenols, tannins by protein precipitation, hydrolysable tannins, total flavonoids and ellagic acid) in leaves of *Lafoensia pacari* (g/100 g expressed as mean±standard deviation).

Samples[1]	TP*	TPP*	HT*	TF**	EA***
PR-Apr09	38.85±0.31	16.91±0.28	35.68±0.47	10.05±0.06	3.84±0.02
UR-Apr09	33.18±0.49	18.75±0.54	33.00±1.49	8.13±0.16	4.58±0.14
BV-Apr09	29.70±1.09	14.06±0.42	31.61±0.67	8.34±0.21	3.50±0.06
AN-Apr09	28.58±0.47	13.57±0.41	40.09±0.30	7.80±0.40	3.58±0.01
NA-Apr09	27.00±0.21	11.87±0.09	32.45±0.29	8.54±0.29	3.29±0.14
PR-Aug09	30.84±0.93	15.87±0.72	29.23±0.23	7.35±0.25	5.29±0.19
UR-Aug09	36.86±0.04	17.31±0.16	52.28±1.00	8.27±0.09	7.85±0.29
BV-Aug09	29.87±0.62	14.15±0.68	44.16±0.39	6.42±0.22	7.29±0.35
AN-Aug09	39.15±0.89	19.54±0.27	30.20±0.77	9.23±0.07	10.81±0.47
NA-Aug09	26.36±0.54	11.01±0.31	35.16±1.02	7.51±0.11	2.49±0.08
PR-Dec09	37.82±0.85	18.82±0.49	48.54±1.82	9.26±0.13	6.18±0.14
UR-Dec09	40.77±0.98	18.18±0.51	41.33±1.33	9.50±0.45	9.05±0.38
BV-Dec09	29.90±1.03	11.91±0.30	50.33±1.75	7.45±0.14	6.73±0.20
AN-Dec09	33.15±0.97	18.01±0.22	48.05±2.15	9.26±0.07	7.54±0.33
NA-Dec09	32.63±0.37	15.23±0.49	42.09±1.07	9.00±0.22	7.17±0.29
PR-Apr10	38.52±1.05	14.49±0.34	54.58±1.01	8.18±0.38	4.96±0.17
UR-Apr10	30.69±0.82	14.87±0.18	42.51±1.44	6.28±0.30	6.44±0.09
BV-Apr10	28.26±0.21	14.39±0.42	13.02±0.36	7.28±0.27	6.67±0.21
AN-Apr10	30.08±0.69	11.52±0.15	41.15±1.17	8.09±0.29	7.17±0.32
NA-Apr10	23.84±0.49	11.29±0.50	30.60±0.66	7.50±0.16	6.23±0.03

*Results in tannic acid equivalent percentage (g/100g) of dry leaf. **Results in rutin equivalent percentage (g/100 g) of dry leaf. ***Results in ellagic acid equivalent percentage (g/100 g) of dry leaf. [1]PR: Pires do Rio; UR: Urutaí; BV: Bela Vista; AN: Anápolis; NA: Nova América.

The result of the Detrended Correspondence Analysis (DCA) is presented in Table 6. Small values were observed for the lengths of the gradients of all axes, which indicates low dispersion of the data.

Table 6. Data from the Detrended Correspondence Analysis (DCA) to measure the environmental gradient.

Axes	1	2	3	4	Total Inertia
Eigenvalues	0.005	0.002	0.000	0.000	0.009
Lengths of gradient	0.299	0.142	0.099	0.137	

Table 7 presents the results obtained from the Canonical Redundancy Analysis (RDA) for measurement of inflation of the variable and the chemical-environment correlation. The summary of the RDA is presented in Table 8. Table 9 provides a summary of the Monte Carlo test used in determining significance for the Canonical Redundancy Analysis (RDA).

After the RDA to assess the inflation of the variable and the chemical-environment correlation, some variables, such as Fe (198.1419), were seen to have very high inflation factors, indicating an effect of excess variables describing the same phenomenon. Table 8 shows that the first two RDA axes together explain 80.1% of the variance found in the system through the chemical-environment correlation; however, the p-value obtained from the Monte Carlo test was higher than 0.05, which indicates that the results obtained through this preliminary RDA are not statistically significant, so variables were selected according to their respective inflation factors (inflation factor<20) to perform a new RDA with variable selection and significance of the first eigenvalue (RDA-1). The results of this RDA are presented in Table 10.

The summary of the RDA with variable selection is presented in Table 11, while Table 12 presents a summary of the Monte Carlo test used to determine the significance of the first eigenvalue (RDA-1).

The selection of variables, excluding those with the highest inflation factors, resulted in a set of eleven variables all with values for the inflation factor below 20, with the first two RDA axes together explaining 86.3% of variance total system through the chemical-environment correlation and a *p*-value equal to 0.014, indicating that the results are statistically significant.

From the environmental variables selected by the RDA and the organic-chemical variables, proposed to groups for the samples analyzed using Canonical Discriminant Analysis (CDA). Four groups were obtained

for analyzed samples of *L. pacari* based on sampling periods, as follows: G1f (April 2009), G2f (August 2009), G3f (December 2009) and G4f (April 2010), and the variables were used as discriminants for the groups: total phenols (TP), foliar copper (Cuf), foliar iron (Fef) and temperature (Temp). The percentage of correct classification for all proposed groups was equal to 100%, according the discriminant analysis of groups for leaves of *L. pacari*.

The Hierarchical Cluster Analysis (HCA) employing Ward's method was used to confirm the groups by constructing a dendogram (Figure 1) to represent the hierarchical clustering.

The trend of distribution of climate data, foliar and soil nutrients, contents of phenolic compounds and samples (organized by location and sampling time) compared to the first two RDA axes (first axis: RDA-1; and the second axis: RDA-2) is shown in Figure 2. From Figure 2 it can be seen that there was a high concentration of groups of phenolic metabolites during the months of lowest temperature, as indicated by the fact that the vectors representing the phenolic compounds were driving in the opposite direction to the vector representing the temperature.

Table 7. Canonical Redundancy Analysis (RDA) for assessment of the inflation of variable and chemical x environment correlation for the leaves.

N	Name*	Mean (weighted)	Standard Deviation	Inflation Factor
1	Nf	1.1061	0.1108	8.8090
2	Pf	0.3567	0.0885	42.7442
3	Kf	1.0013	0.0669	3.7872
4	Caf	0.8697	0.1506	21.9899
5	Mgf	0.4945	0.1224	14.3794
6	Sf	0.1456	0.1166	42.3314
7	Cuf	1.1742	0.3361	31.7707
8	Fef	2.4289	0.2478	14.2403
9	Mnf	2.0416	0.2245	31.3404
10	Znf	1.2968	0.1192	17.3392
11	Cu	0.3070	0.1182	30.2939
12	Fe	1.7104	0.0609	198.1419
13	Mn	1.2488	0.0603	19.2616
14	Zn	0.2922	0.0623	61.1194
15	P	0.2402	0.0682	0.0000
16	K	1.7116	0.0485	0.0000
17	MO	0.1348	0.0182	0.0000
18	pH	4.8000	0.5060	0.0000
19	Ca	0.2518	0.0528	0.0000
20	Mg	0.0924	0.0268	0.0000
21	H+Al	0.6006	0.0366	0.0000
22	Al	0.0246	0.0201	0.0000
23	CTC	0.7112	0.0331	0.0000
24	M	0.1714	0.1427	0.0000
25	V	0.5546	0.0668	0.0000
26	Prec	0.6730	0.2382	10.7829
27	Temp	1.3881	0.0328	20.0404

*Foliar nutrients identified by the letter f (eg, Nf).

Discussion

The leaves of *Lafoensia pacari* A. St.-Hil., Lythraceae, were considered in four groups based on the month in which the plant material was collected, as follows: Group I (G1-f) - April 2009, Group II (G2-f) – August 2009, Group III (G3-f) - December 2009 and Group IV (G4-f) - April 2010. First, through the RDA, it can be seen that all groups of phenolic compounds showed a strong trend towards higher concentrations in the months of 2009, with more modest values for the month of April 2010, contrasting with the temperature, which

Table 8. Summary of Canonical Redundancy Analysis (RDA) for assessment of the inflation of variable and of the chemical x environment correlation for the leaves.

Axes	1	2	3	4	Total variance
Eigenvalues:	0.539	0.179	0.106	0.046	1.000
Chemical-environment correlations:	0.974	0.921	0.914	0.842	
Cumulative percentage variance					
of chemical data:	53.9	71.8	82.4	87.0	
of chemical-environment relationship:	60.1	80.1	91.8	96.9	
Sum of all eigenvalues					1.000
Sum of all canonical eigenvalues					0.898

Table 9. Summary of a Monte Carlo test (999 permutations in the reduced model) for the Canonical Redundancy Analysis (RDA).

Test of significance of first canonical axis:	Eigenvalue	0.539
	F-ratio	3.509
	p-value	0.2110
Test of significance of all canonical axes:	Trace	0.898
	F-ratio	1.642
	p-value	0.2140

Table 10. Canonical Redundancy Analysis (RDA) with variable selection for leaves.

N	Name	Mean (weighted)	Standard Deviation	Inflation Factor
2	Pf	0.3567	0.0885	8.0464
5	Mgf	0.4945	0.1224	3.1691
6	Sf	0.1456	0.1166	7.2105
7	Cuf	1.1742	0.3361	4.1179
8	Fef	2.4289	0.2478	8.0542
9	Mnf	2.0416	0.2245	3.6730
10	Znf	1.2968	0.1192	4.5890
13	Mn	1.2488	0.0603	9.1879
22	Al	0.0246	0.0201	19.3758
23	CTC	0.7112	0.0331	14.4842
27	Temp	1.3881	0.0328	5.5505

Table 11. Summary of Canonical Redundancy Analysis (RDA) with variable selection for leaves.

Axes	1	2	3	4	Total variance
Eigenvalues:	0.578	0.122	0.051	0.044	1.000
Chemical-environment correlations:	0.940	0.920	0.782	0.728	
Cumulative percentage variance					
of chemical data:	57.8	70.0	75.1	79.5	
of chemical-environment relationship:	71.3	86.3	92.6	98.1	
Sum of all eigenvalues					1.000
Sum of all canonical eigenvalues					0.811

Table 12. Summary of Monte Carlo test (999 permutations in the reduced model) to determine the significance of the first eigenvalue (RDA-1).

Test of significance of first canonical axis:	Eigenvalue	0.578
	F-ratio	10.960
	p-value	0.0140
Test of significance of all canonical axes:	Trace	0.811
	F-ratio	3.116
	p-value	0.0060

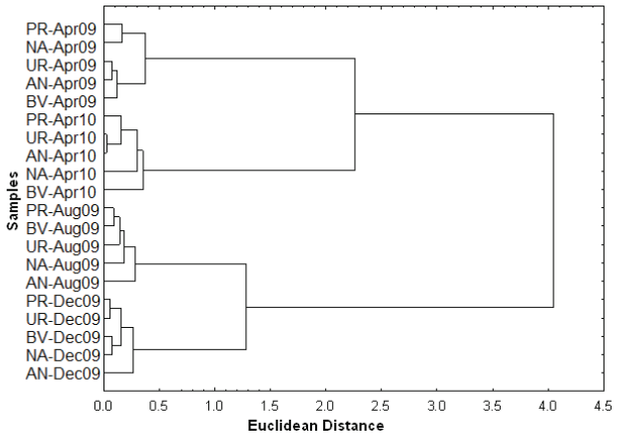

Figure 1. Dendrogram representing hierarchical clustering of samples of leaves of *Lafoensia pacari*, linking the climate data, soil and foliar nutrients, and phenolic compounds formed with the Euclidean distance of samples according to Ward's Method of Minimum Variance.

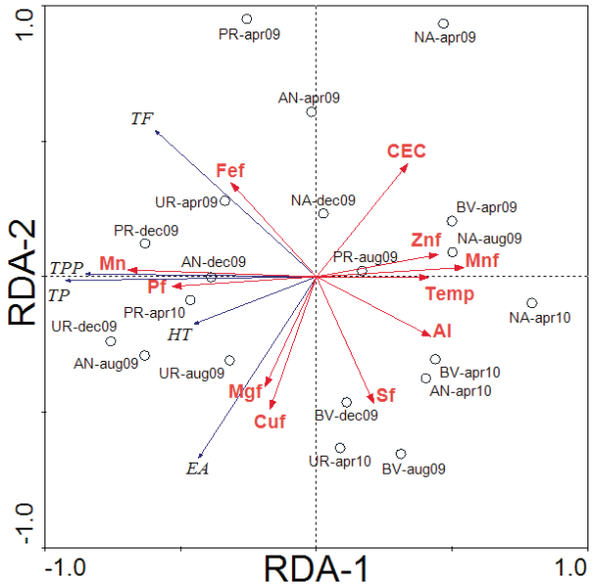

Figure 2. Distribution trend of climate data, soil and foliar nutrients, phenolic compounds and samples (organized by location and collection time) compared to the first two axes of RDA (RDA-1 and RDA-2). The dots represent the scores of samples and the discriminating variables are represented by vectors.

showed a trend towards higher values in 2010. This may be related to the fact that exposure to high temperatures inhibits photosynthesis in the leaves before respiration, as a result of which photosynthesis cannot replace the carbon consumed in respiration and there is a decrease in the availability of carbohydrates in the plant (Taiz & Zeiger, 2004). As the synthesis of secondary metabolites is dependent on the products of photosynthesis (glucose metabolism) (Santos, 2004), a decrease in photosynthetic levels and a consequent reduction in the supply of carbohydrates may have caused a decline in secondary metabolism in the leaves during the period in which the highest temperatures occurred, with this in turn explaining the lower levels of phenolic compounds in the warmer months.

According to Padda & Picha (2008) and Albert et al. (2009) there appears to be a relationship between temperature and levels of phenolic compounds in plant tissues, in which lower temperatures are associated with higher levels of phenolic compounds. The increased levels of phenolic compounds in the leaves may be related to increased activity of phenylalanine ammonia-lyase (PAL) at lower temperatures, given the fact that PAL is an important enzyme in the biogenesis of various phenolic compounds (Padda & Picha, 2008).

In general, macronutrient levels do not appear to have a strong connection with the production of phenolic compounds in the leaves of *L. pacari* since, after the RDA, only foliar phosphorus (Pf), foliar magnesium (Mgf) and foliar sulfur (Sf) were correlated with variation of phenolic metabolites in leaves; however, the fact that Pf and Mgf presented a trend in the direction of phenolic compounds may be related to the fact that the trend of foliar nutrients go into the direction of the months of lower temperature, in which levels of photosynthesis are higher, and also because Pf and Mgf are nutrients highly involved in photosynthesis (Mg is present in the chlorophyll molecule) (Taiz & Zeiger, 2004) and in metabolic processes directly linked to photosynthesis, cellular respiration and synthesis of phospholipids (Marschner, 1997).

The foliar micronutrients [foliar copper (Cuf), foliar iron (Fef), foliar manganese (Mnf) and foliar zinc (Znf)] appear to be highly correlated with the synthesis of phenolic compounds in leaves of *L. pacari*, since all the nutrients in this class, after the RDA, presented a correlation with the chemical variables, and the Fef and Cuf followed the same trend as phenolic compounds, especially in relation to total flavonoids (for the Fef) and ellagic acid (for Cuf), while the micronutrients Mnf and ZnF showed an opposite trend to that of total phenols, heading for the months of higher temperature. The relationship of foliar micronutrients with the production of phenolic compounds in *L. pacari* can be explained by several factors, since these nutrients are actively associated with the process of photosynthesis (mainly Fe) (Taiz & Zeiger, 2004), with the synthesis of phenolic compounds such as simple phenolic acids (shikimic acid pathway and phenylpropanoid pathway) (Kirkby & Hömheld, 2004), as well as the transformation of simple phenols into lignin and other more complex compounds (Marschner, 1997).

Cu is a micronutrient closely linked to production of phenolic compounds in plants, mainly acting as an

activator of the PAL pathway (Kováčik & Klejdus, 2008). Studies by Kováčik & Klejdus (2008) and Kováčik et al. (2009) demonstrated high levels of phenolic acids in tissues rich in copper, suggesting that this mineral is an important enzyme activator for the synthesis of these compounds (by PAL). Another interpretation of the increased levels of phenolic compounds in these tissues, suggested by the same authors, is that this is associated with a mechanism of tolerance to Cu, since Cu is a catalyst for redox reactions that can produce free radicals harmful to the plant; consequently, increased levels of phenolic compounds have two goals: to decrease the concentration of free Cu in plant tissue by the reaction of this with phenols, and to minimize the deleterious effects of free radicals formed, through the antioxidant action of the phenolic compounds.

Phenolic compounds appear to accumulate in tissues rich in Fe and this fact may be related to a mechanism for mobilization of Fe in the plant, since the phenols may complex with Fe (Fe^{3+}) and be transported to other tissues, facilitating the mobilization of this mineral between different tissue types, and also participating in the reduction of Fe^{3+} to Fe^{2+}, aiding reductase-type enzymes (Jin et al., 2007).

Micronutrients Mnf and Znf showed an opposite trend in the groups of phenolic compounds, going toward the warmer months, which is inconsistent with the literature, since both Mn and Zn are involved in processes of synthesis and conversion of phenolics as enzymatic cofactors (metalloproteins) (Marschner, 1997; Yamada, 2004; Kirkby & Römheld, 2004); however, this may indicate that the high temperatures are responsible for limiting photosynthetic rates (Taiz & Zeiger, 2004), reducing the availability of substrates from glucose metabolism (Santos, 2004), and thus affecting the synthesis of phenolic compounds in the leaves, even with high levels of Mnf and Znf.

The abiotic factors related to soil (soil nutrients and fertility) were found to exert little influence on the metabolism of phenols in leaves of *L. pacari*, since only Mn, Al and CEC were correlated with the concentrations of phenolic compounds in plant leaf tissue; in other words, most of the nutrients present in the collected soils and their fertility parameters do not appear to influence significantly the secondary metabolism in *L. pacari* leaves with respect to phenolics.

The correlation between the CEC and the concentrations of phenolic compounds showed, after the RDA, an opposite trend to phenolic compounds, especially in relation to RDA-1, which may be related to the fact that CEC is one of the main parameters of soil fertility, in other words, it is related to biomass production by the plant (primary metabolism) (Lopes & Guilherme, 1994). Thus, in soils with higher values of CEC, the specimens of *L. pacari* would have more fertile conditions for their growth, leading to greater competition between the primary and secondary metabolism of substrates, and resulting in a reduction in the synthesis of secondary metabolites such as phenolic compounds (Taiz & Zeiger, 2004).

This study suggests that there is a great influence of seasonal factors on the production of phenolic compounds in the leaves of *L. pacari*, with the temperature and foliar micronutrients (Cuf, Fef, Mnf, Znf) being the main factors of environmental influence on this plant tissue.

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