Journal of Medicinal Plants Research Vol. 5(12), pp. 2559-2565, 18 June, 2011
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2010 Academic Journals

Full Length Research Paper

Loganin production in *Palicourea rigida* H.B.K. (Rubiaceae) from populations native to Brazilian Cerrado

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Accepted 31 March 2011

***Palicourea rigida* HBK, known as "douradinha", "bate-caixa" and "douradão" is endemic to Brazilian Cerrado and belongs to the Rubiaceae family. The aqueous and hydroalcoholic extracts of *P. rigida* have been traditionally used in the treatment of urinary tract disorders. The aim of this work was to study the chemical diversity of eight populations of *P. rigida* native to Brazilian Cerrado regions in the states of São Paulo, Goiás and Minas Gerais. The loganin quantification was performed by (High-performance liquid chromatography) HPLC and the correlation among iridoid contents and geographic (latitude, longitude and altitude), biotic (diameter and height) and abiotic (soil's macro and micronutrient) factors was estimated using Pearson's correlation coefficient. The concentration of loganin varied (20.09 to 101.63 mg/ g d.w.) among and within populations. The main factors related to this divergence were latitude, longitude and nutrient-poor soils.**

Key words: Medicinal plant, iridoid, Cerrado, Rubiaceae.

INTRODUCTION

Palicourea rigida H.B.K. (Rubiaceae), commonly known as "douradinha", "bate caixa" and "douradão" is endemic to Brazilian Cerrado and belongs to the Rubiaceae family. This medicinal species has been traditionally used in the treatment of kidney pain and other urinary tract disorders (Vieira and Martins, 2000).

Previous phytochemical screenings of *Palicourea* extracts lead to the isolation of phytosterols (stigmasterol, sitosterol and campesterol); alkaloids (strictosidinic acid and vallesiachotamine); coumarin (scopoletin); (3-friedelanone and 30-hydroxyfriedelan-3-one) and iridoids (secologanin, sweroside and loganin) (Bolzani et al., 1992; Vencato et al., 2 006; Rosa, 2009). Among those compounds, loganin is the major constituent biosynthesized in *Palicourea* leaves (Lopes et al., 2004). That monoterpenoid compound apparently seems to be formed in plants by an alternative cyclization of geranyl diphosphate (Sampaio-Santos and Kaplan, 2001).

Several pharmacological activities have been attributed to loganin as immunomodulatory (Mathad et al., 1998), anti-inflammatory (Park et al., 2007); analgesic; hepatoprotective and renoprotective (Xu, 2006; Yamabe et al., 2010). Studies suggest that loganin can increase the contents of osteocalcin and collagen type I, being indicated in the treatment of osteoporosis (Li et al., 2010).

Besides its pharmacological importance, the iridoid loganin is also considered ecologically significant on the control and proliferation of herbivorous insects. That class of compounds is able to limit feedings or reduce the development of generalist herbivore insects. Moreover, iridoids attract specialized herbivorous insects that feed

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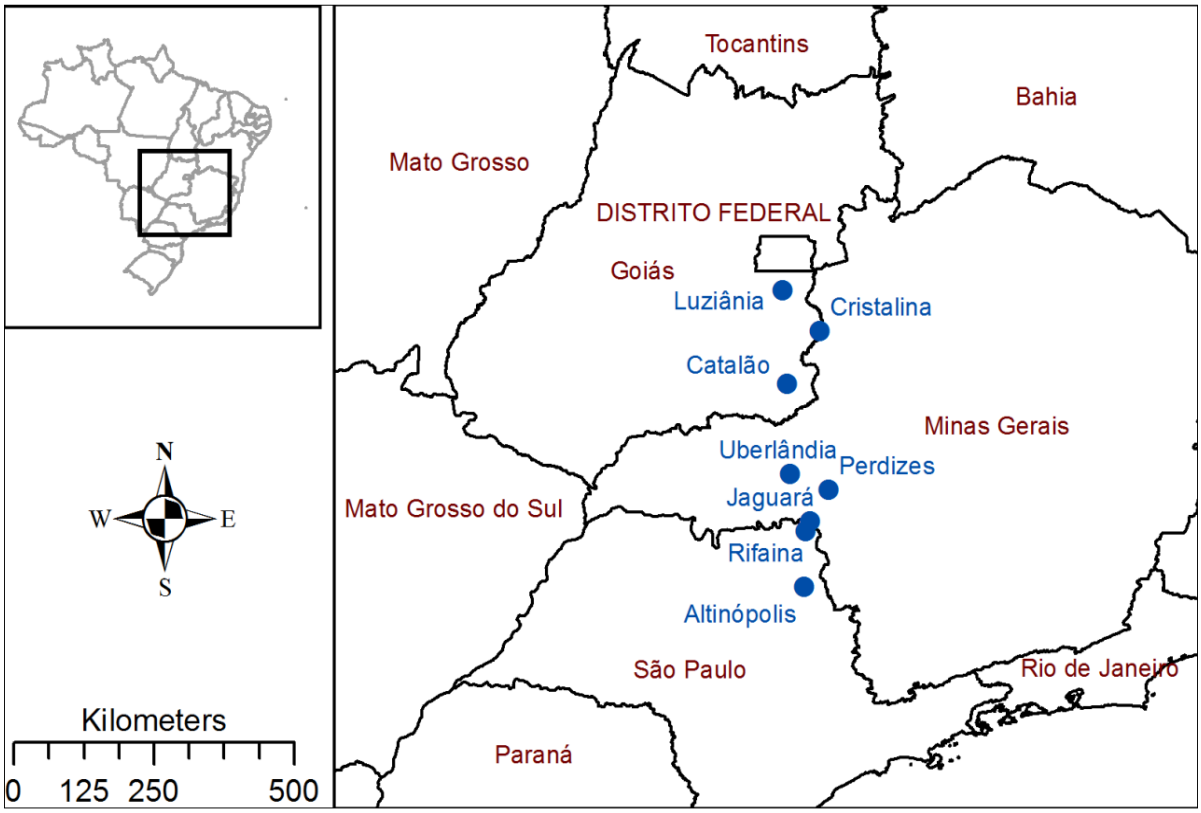

Figure 1. Geographic localization of the *Palicourea rigida* populations investigated in this work.

from plant tissues rich in iridoids and are protected against carnivorous insects (Puttick and Bowers, 1988; Nieminen et al., 2003; Banden and Dobler, 2009).

The study on the distribution of iridoids in plants is of important significance in chemosystematics since those compounds are of great help in plant classification, phylogeny and evolution, being used as chemical markers for the Corniflorae, Gentianiflorae, Loasiflorae and Lamiiflorae superorders (Sampaio-Santos and Kaplan, 2001).

The aim of this work was to investigate and correlate the influence of geographic, biotic and abiotic factors on the accumulation of loganin in *P. rigida* leaves from native populations and also to determine valuable populations with most productive *P. rigida* assessions rich in iridoid contents.

MATERIALS AND METHODS

Plant samples

Assessions from eight natural populations of *Palicourea rigida* (20 plants per population) were randomly collected in the Brazilian states (cities) of Goiás (Catalão, Cristalina and Luizânia); Minas Gerais (Jaguará, Perdizes and Uberlândia) and São Paulo (Rifaina and Altinópolis). All the assessions were collected during the summer of 2009 and biometric data (stem diameter and plant height) were recorded. Figure 1 represents the geographic localization of the populations investigated.

The voucher speciments (HPMU - 1416) are preserved at the Herbarium of Universidade de Ribeirão Preto (UNAERP, Ribeirão Preto, Brazil).

Soil analysis

Soil samples were collected at a depth of 0.2 m, in all the collection sites. The levels of P, B, S and organic matter were estimated by the colorimetric test using Spectrophotometer CELM (model E215D); Ca and Mg were quantified by titration; Cu, Mn, Fe and Zn were measured by atomic absorption by Spectrophotometer Perkin Elmer (Analyst Model 100); K was measured by photometric flame emission on a Flame Photometer Micronal (model B462) and the pH was determined with $CaCl_2$ solution (0.01M). All the analyses were performed following the methodology of Raij et al. (1987) and Abreu et al. (1994).

Isolation and identification of loganin

P. rigida dried and powdered aerial parts (32 g) were extracted with EtOH and the concentrated extract (5.0 g) was partitioned in Et_2O-H_2O (Lopes et al., 2004). The aqueous phase was concentrated allowing crude extract. This crude extract was fractionated by preparative TLC eluted with $CHCl_3$: MeOH (9:3, V/V) and the fraction containing loganin was recovered from the silica gel with MeOH. The monitoring of the iridoid in the samples was carried out

Table 1. ^{1}H and ^{13}C RMN data for loganin.

	^{13}C	^{1}H
1	97.74	5.27 d (5 Hz)
3	152.16	7.38 s
4	114.07	
5	32.19	3.1 dd (10 e, 5 Hz)
6	42.74	
6a		1.62 ddd (10, 10 e, 5 Hz)
6b		2.22 dd (15 e, 10 Hz)
7	75.10	1.03 m
8	42.20	1.87 m
9	46.52	2.02 ddd (10, 10 e, 5 Hz)
C=O	169.59	
-OCH3	51.67	
-OH		4.59 sl
OMe		3.67 s
1'	100.08	4.64 d (5 Hz)
2'	74.76	3.19 dd (10 e, 5 Hz)
3'	78.06	3.36 t (10 Hz)
4'	71.62	3.27-3.30 (m)
5'	78.40	3.27-3.30 (m)
6'	62.79	
6a'		3.89 dd (10 Hz)
6b'		3.67 dd (5 Hz)

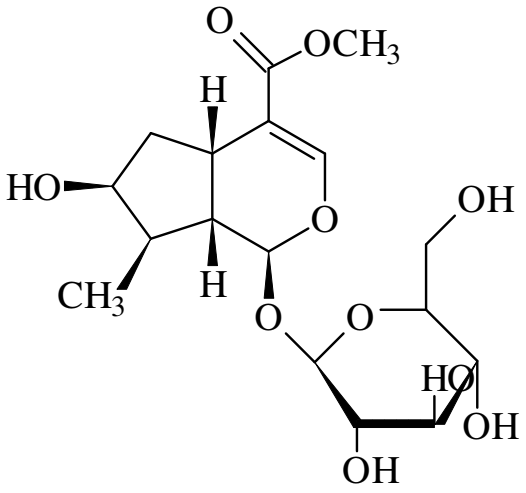

Figure 2. Loganin.

with analytic TLC using loganin (Fluka Analytical™) as reference standard. The iridoid fraction (250 mg) was column chromatographed on silica gel using $CHCl_3$: MeOH gradient system, followed by preparative HPLC purification in Shimadzu Liquid Chromatograph coupled with Photo-diode Array detector SPD-M10A, using a reverse phase column LC-18 (Supelcosil™ 250 mm x 10 mm, 4.6 µm). The gradient program with H_2O (A) and MeOH (B) was a linear gradient starting with 5 to 40%B in 60 min. Detection was at 254 nm and flow rate was 1.0 mL/min. The purified loganin (78 mg) was identified by RMN spectra (^{1}H e ^{13}C) (Table 1 and Figure 2).

Chemicals and reagents

Milli-Q water from Millipore™, Metanol (HPLC degree) from

Table 2. Standard concentration range and calibration curve for the iridoid analyzed.

Standard	Concentration range (µg/mL)	Regression curve	R^2 (n = 6)	LOD µg/mL	LOQ µg/mL
Loganin (Fluka analytical™)	1.0-0.03125	Y = 9124415x+973466	0.9981	94.26	31.42

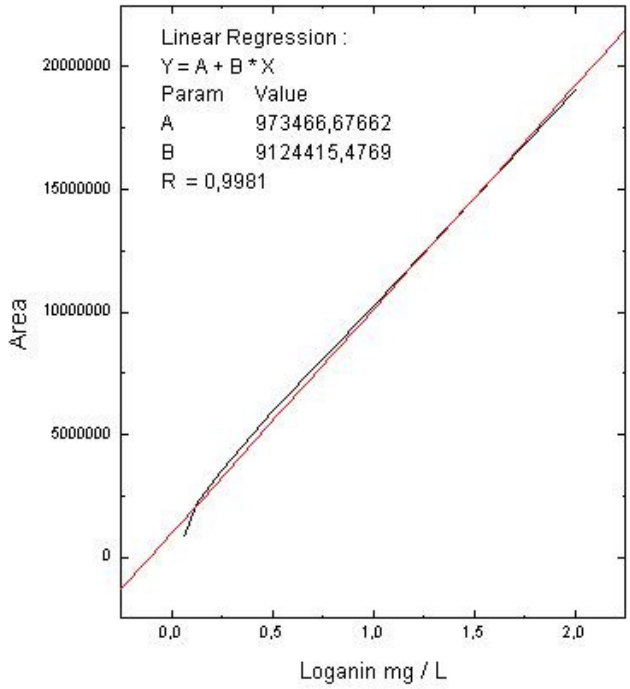

Figure 3. Calibration curve.

J.T.Baker™ and Acetic acid from ShynthLab™.

Reference standard

Loganin (Fluka Analytical™, Germany).

Plant extract

Dried powered *P. rigida* leaves (100 mg) were placed in a conic tube and extracted with MeOH: H_2O (50:50, v/v) by sonication in a sonic bath (Thronton Inpec™) at room temperature for 45 min. Samples were filtered through 0.45 µm Altech™ syringe filter prior to injection in HPLC.

HPLC conditions

Instrumentation consisted of a Shimatzu liquid chromatography system equipped with a Shimatzu Diiode Array Detector SPD-M10A. A reverse phase column LC-18 (Supelcosil™ 250 mm x 4.6 mm, 4.6 µm) was used. Detection wavelength was at 254 nm and flow rate was 1.0 mL/min. The gradient system program with Acetic acid 0.1% (A) and MeOH (B) was: 0 to 50 min a linear gradient 10 to 50% B; from 50 to 52 min a linear gradient 50 to 10% B; 52 to 55 min isocratic condition at B 10%. The volume injected was 20 µL.

Regression curve, limit of detection and calibration

A regression curve for loganin was obtained with 6 different concentrations of the standard, each concentration being injected in triplicate. Calibration curve was set plotting peak area (X) vs. concentration of standard solutions (Y, µg/mL). A good correlation was achieved (R^2> 0.9981). The limit of detection (LOD) was determined analyzing decreasing concentrations of the standard solution in order to establish the lowest concentration that could be detected with a signal noise (S/N) ratio of 3. The limit of quantification (LOQ) was defined as the lowest concentration (S/N = 10) that the method could quantify (data presented in Table 2 and Figure 3).

Statistical analysis

Data were submitted to analysis of variance (ANOVA) and means compared by Scott-Knott's multiple-range tests at $P \leq 0.05$ by using the software SISVAR (Variance Analysis System - Federal University of Viçosa, Viçosa – MG, Brazil). Pearson's coefficient was used to determine the correlation between loganin

Table 3. Correlation between geographic variation and biometric characters on the production of loganin in *P. rigida* plants from 8 native populations.

Sites of collection	Latitude	Longitude	Altitude (m)	Loganine content (mg/ g^{1})	Height (m)	Diameter (cm)
Catalão	-17° 49' 16,7"	-47° 46' 08,2"	940	27.34 c	1.626	6.51
Cristalina	-16° 59' 24,7"	-47° 14' 38,3"	922	41.94 b	1.397	4.51
Luziânia	-16° 21' 06,6"	-47° 50' 01,8"	947	101.,63 a	0.820	2.61
Jaguará	-20° 01' 02,0"	-47° 24' 11,1"	647	21.09 d	1.610	5.36
Perdizes	-19° 31' 01,8"	-47° 05' 41,5"	928	24.80 c	0.721	2.61
Uberlândia	-19° 15' 51,0"	-47° 43' 32,5"	985	40.85 b	1.184	3.56
Altinópolis	-21°03'25,0'"	-47°29'22,7"	626	56.44 b	1.544	4.27
Rifaina	-20° 10' 03,7"	-47° 28' 08,2"	954	43.55 b	0.924	3.76

Means followed by the same letters within each column did not significantly differ by Scott–Knott test at P < 0.05. CV: coefficient of variation.

Table 4. Variation in iridoid production within populations of *P. rigida* collected in the Brazilian Cerrado (States of Goiás, Minas Gerais and São Paulo).

Individual	Catalão	Cristalina	Luiziânia	Jaguara	Perdizes	Uberlância	Altinópolis	Rifaina
1	21.71 c	37.56 e	31.85 d	14.33 d	20.50 f	42.72 d	54.18 c	34.08
2	16.87 d	20.03 g	42.08 d	27.56 b	13.63 g	48.47 c	61.75 b	42.86
3	31.54 b	45.27 c	99.12 c	19.73 c	16.10 g	46.30 c	60.32 b	36.67
4	17.58 d	54.61 b	121.54 c	23.96 b	19.27 f	38.83 e	51.98 c	26.87
5	23.14 c	59.48 b	81.12 c	21.10 c	18.42 f	34.22 e	40.53 d	38.70
6	11.86 e	31.60 e	78.69 c	26.16 b	19.73 f	36.75 e	57.62 b	32.49
7	17.79 d	33.49 e	95.49 c	27.19 b	21.73 e	53.62 b	52.66 c	48.75
8	21.07 c	28.78 f	166.28 b	25.55 b	40.45 a	45.13 c	55.54 c	41.49
9	24.28 c	41.03 d	145.07 b	15.91 d	27.77 d	32.86 f	51.35 d	62.79
10	35.81 a	46.51 c	205.14 a	17.24 d	21.47 e	40.91d	58.76 b	62.35
11	13.06 e	45.13 c	98.32 c	16.18 d	24.74 e	17.64 g	55.44 c	48.79
12	38.58 a	25.56 f	160.01 b	22.94 b	24.03 e	48.50 c	52.53 c	55.77
13	36.07 a	39.95 d	106.41 c	20.44 c	16.91 g	42.22 d	76.86 a	39.45
14	32.71 b	41.83 d	103.21 c	25.11 b	29.77 c	30.92 f	54.47 c	51.61
15	28.99 b	67.49 a	173.74 b	18.96 c	24.41 e	58.31 a	61.61 b	32.29
16	30.38 b	45.39 c	78.62 c	16.47 d	39.88 a	61.92 a	58.84 b	52.42
17	35.50 a	40.52 d	49.85 d	14.58 d	34.44 b	51.07 b	51.84 c	42.04
18	30.78 b	46.45 c	48.98 d	14.79 d	32.52 b	48.05 c	57.93 b	41.76
19	35.28 a	24.81 f	102.83 c	33.98 a	23.42 e	41.09 d	43.39 d	50.64
20	21.14 c	54.77 b	91.14 c	22.52 b	23.63 e	44.05 c	63.01 b	30.38 b

Means followed by the same letters within each column did not significantly differ by Scott–Knott Test at P < 0.05. CV: coefficient of variation.

concentration and biotic and abiotic factors.

RESULTS AND DISCUSSION

The quantification of loganin contents in *P. rigida* plants from the investigated populations was discrepant, the assessions collected in Luiziania, GO, were the most productive (101.63 mg/g of d.w.) and plants collected in Jaguara, MG, presented the lowest yields of iridoid (21.09 mg/g of d.w.). The variation on the loganin production was observed inside and among populations (Tables 3 and 4). Obtained results indicate that the concentration of iridoids in *P. rigida* plants are influenced by both genetic and environmental variability.

A positive correlation was observed between loganin accumulation and the longitude (r = 0.43) and a negative correlation between loganin concentration and latitude (r = -0.59). Studies carried out with other iridoid-producer species reported that the production of iridoids differ as a

Table 5. Correlation between loganin content and soil macro and micro nutrients measured by Pearson's coefficient.

Parameter	Pearson's coefficient (r)
Organic matter	0.10
pH	0.09
Macro-nutrients	
Phosphorus (P)	-0.16
Potassium (K)	-0.63
Calcium (Ca)	-0.66
Magnesium (Mg)	0
Sulfur (S-So_4)	-0.30
Micro- nutrients	
Iron (Fe)	-0.02
Manganese (Mn)	-0.47
Zinc (Zn)	-0.28
Boron (B)	-0.17
Copper (Cu)	-0.32

result of seasonal variation (Hogedal and Molgaard, 2000) and that both water stress and absence of UV light incidence promote iridoid production (Martz et al., 2009; Wang et al., 2010). So far, no studies have reported the influence of latitude and longitude on loganin accumulation.

In the present work, no correlation between iridoid yields and the altitude was observed. However, Darrow and Bowers (1997) reported that the production of the Aucubin and Catalpol in *Plantago lanceolata* varied according the altitude, higher levels of those iridoids are produced in mountain plants compared with plants from areas of lower altitudes.

Though the correlation between loganin content and biometric parameters were r = -0.3 for plant height, and r = -0.29 for diameter, it is premature to affirm that younger plants produce higher yields of loganin. More specific studies should be carried out on *P. rigida* ontogenetic stages to confirm that finding.

In all the collection sites, the soil pH was low, ranging from 3.7 to 4.3. Except for Magnesium, all the soil macro and micronutrients showed negative correlation with the loganin concentration (Table 5) and a more expressive correlation was observed with potassium (r = -0.50) and calcium (r = -0.51). Those results corroborate others obtained with *Plantago landeolata*, which showed that the yields of the iridoids Aucubin and Catalpol were higher when the assessions were collected in nutrient-poor soils (Darrow and Bowers, 1999). Also, work carried out with *Centella asiatica* described by Devkota et al. (2010) reported that terpene production was higher in plants from poor and sand rich soils when compared with plants cultivated in fertilized soils (Devkota et al., 2010). Generally, there is a consensus, in that low nutritional conditions have a direct impact on the production of the secondary metabolites, especially in the production of tannins and terpenoids (Jacobson et al., 2005). According to Bryant et al. (1983), the deficiency of nutrients limit the growth of plants more than photosynthesis, and the absorbed carbon is allocated to the production of defensive compounds instead of being destined to the production of primary metabolites for plant (Coley et al., 1985; Herms and Matton, 1992; Kytö et al., 1996).

Conclusions

A significant variation was determined on loganin content in natural populations of *Palicourea rigida* HBK, indicating the necessity of conducting new approaches to investigate the genetic variability within the studied populations. Based on our findings, there is an evidence that *P. rigida* plants from areas with latitude lower than 16° 21' and longitude higher than 47° 50' accumulate higher concentrations of loganin. Moreover, it was observed that plants grown on low fertile and acidic soil presented higher concentration of iridoids, indicating that Cerrado areas that are not convenient for the development of cultures that demand high levels of nutrients should be destined for harvesting iridoid-producer species like *P. rigida.*

Considering the fact that iridoids represent an important class of chemical markers for the superorders Corniflorae, Gentianiflorae, Loasiflorae e Lamiiflorae, obtained results will contribute to further studies on the phylogeny and chemosystematics of plant species.

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