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Geographical variation of isoquinoline alkaloids of *Annona crassiflora* Mart. from cerrado, Brazil

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ABSTRACT

The total content and profile of alkaloids in foliar samples of *Annona crassiflora* from eight different Brazilian Cerrado regions were investigated. Alkaloids were quantified and identified by GC/FID and GC/MS, respectively. Significant quantitative differences were found in these samples. Total alkaloid concentration varied from 221.1 ± 17.14 ug/g in Mogi-Guaçu (SP) to 2986.89 ± 367.1 ug/g (dry mass basis) in Brasília (DF). The alkaloids anonaine, annoretine, romucosine and xylopine were detected at different concentrations across regions. Alkaloid concentration and profile varied in *A. crassiflora* populations, indicating extensive phenotypic plasticity of these individuals.

1. Introduction

The Brazilian Cerrado has recently been listed as one of the 25 biodiversity hotspots for conservation priorities (Myers et al., 2000). However, in the past 40 years extensive agricultural practices have caused changes in up to 60% of this ecosystem (Ratter et al., 2006). Distinct phytophysionomies and edaphic conditions present in this biome have been associated with phenotypic variations in some of the native species (Cardoso and Lomônaco, 2003). The Cerrado consists of a vegetation complex that ranges from grassland to woodland (locally called *campo limpo* and *cerradão*, respectively). The intermediate physionomies represented by shrub savannas, wooded savannas, and woodlands (*campo sujo*, *campo cerrado* and *cerrado sensu stricto*, in this order) should be considered as ecotones (Coutinho, 1978). Despite this considerable physionomic variation, the Cerrado can be characterized as a biome that has water sources, although poor levels of soil nutrients (especially nitrogen) and toxic levels of aluminum (Pivelo and Coutinho, 1996) have been reported.

Annonaceae is one of the families with the highest floristic richness in Cerrado areas of the Brazilian territory (Lugnani et al., 2007). This family is formed of about 120 genera, which are distributed mainly in tropical and subtropical regions of the world (Joly, 2002). Some species of *Annona* are of economic importance because of their edible fruits and medicinal properties, like *Annona squamosa* (sugar apple), *Annona muricata* (soursop), *Annona reticulata* (custard-apple) and *Annona cherimola* (cherimoya) (Corrêa, 1984). Although the above-mentioned species are exotic to the Brazilian Cerrado, there are wild species growing in the Cerrado that despite the lack of significant economic importance, are appreciated by local communities living in the biome.

Annona crassiflora Mart., also known as 'araticum', 'marolo' or 'pinha-do-cerrado' (Almeida et al., 1998), is a tree native to the Brazilian Cerrado, widely spread throughout the biome. The species spans across the states of São Paulo, Minas Gerais, Bahia, Mato Grosso do Sul, Mato Grosso and Tocantins (Lorenzi, 1998).

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One of the important characteristics of Annonaceae species is the presence of isoquinoline alkaloids (Cavé, 1985). Reticuline benzylisoquinoline (Chang et al., 2000a), asimilobine and anonaine aporphines (Chang et al., 2000b), and liriodenine oxoaporphine (Wu et al., 1993) have been detected in species of *Annona*. Recent studies describe the isolation of reticuline, asimilobine, and liriodenine in some species of *Annona*, such as *Annona foetida* Mart., *Annona pickelii* (Diels) H.Rainer, *Annona salzmannii* A.DC., and *Annona sericea* Dunal, all native to Brazil (Campos et al., 2008; Costa et al., 2011; Da Cruz et al., 2011; Dutra et al., 2012). These alkaloids were found in leaves and barks of *A. crassiflora* from the Guianas (Hocquemiller et al., 1982).

Published data on an association between geographic distribution and variation in alkaloid levels and profile are scarce, especially for Brazilian species. Despite the great number of papers on the composition of alkaloids in several species of Annonaceae (Cavé, 1985; Chen et al., 1996, 1999), this is the first study devised to investigate quantitative and/or qualitative variations of such metabolites in species of this family based on geographical distribution. In this paper we analysed the composition and concentration of alkaloids in samples of *A. crassiflora* collected from eight different Cerrado regions, in an effort to shed new light on differences in metabolite concentrations in terms of geographical distribution.

2. Material and methods

2.1. Plant material

Leaf samples of three individuals of *A. crassiflora* were collected from Cerrado areas in Luís Antonio and Mogi-Guaçú (state of São Paulo), Baldim, Belo Horizonte, Lagoa Santa and Santana do Riacho (state of Minas Gerais), Cristalina (state of Goiás), and Brasília (Brazilian capital) and analysed. Voucher specimens were deposited in the Herbarium of Departament of Botany, University of São Paulo (SPF) (Table 1).

2.2. Alkaloid isolation

Leaf samples were air-dried at 50 °C in a ventilation oven. Alkaloids were extracted from 20 g to 50 g of finely ground tissue of *A. crassiflora* with 250 mL of a slightly acid ethanol solution at room temperature for 24 h. The acid solution was basified with NH_4OH to pH 8 and extracted with 125 mL $CHCl_3$ three times (Suau et al., 2002). The organic solvent was evaporated and a brownish viscous residue was obtained. The crude alkaloid fraction was subjected to column chromatography over silica gel (230–400 mesh) and eluted with $CHCl_3$–MeOH 20:1 (Chang et al., 1998; Chen et al., 1999). Four fractions were obtained. The solvent was discarded and the sample was stored at room temperature until subsequent analysis. All fractions were submmited to gas chromatography. Alkaloids were detected only in fractions 3 and 4.

2.3. Identification and quantification of alkaloids

Papaverine (5 μg) was added to each sample as internal standard. They were solubilised in 1 mL of dichloromethane and analysed by gas chromatography.

Samples (1 μL in CH_2Cl_2) were analysed by GC/MS performed on an HP 5890 ser. II Plus GC coupled to an HP 5989B MS, with a 1:20 split ratio. A J&W Scientific DB-5HT capillary column (32 m × 0.32 mm × 0.1 μm film thickness) coated with (5%-Phenyl)-methylpolysiloxane was used as the stationary phase. Helium 1 mL/min was the carrier gas. The column temperature program was: 150°C/1 min, a rate of 10 °C/min to 280 °C, and then 280°C/10min. The injector temperature was 300 °C. MS were taken at 70 eV with 2.89 scans/s and fragments detection from 50 to 500 Da, source temperature 250 °C, quadrupole temperature 100 °C (Suau et al., 2002; modified).

Alkaloid content was determined by GC-FID using an HP 5890 ser. II Plus fitted with a flame ionization detector (FID) and an electronic integrator. The column type and temperature program, carrier gas and flow rate, split ratio and sample amounts were similar to those used in the GC/MS analyses. The injector and detector temperatures were adjusted to 300 °C.

2.4. Statistical analyses

Total alkaloid content and each component were submitted to One-Way analysis (ANOVA). In the event of significant variations ($p \leq 0.05$), data were submitted to the multiple comparison test through the Student Newman Keuls method. All statistical analyses were performed using software SigmaStat version 2.0 (Neter et al., 1996).

Alkaloid contents were previously auto-escalated and submitted to a cluster analysis by the UPGMA method and Euclidian Distance Index (Crisci and Armengol, 1983).

3. Results and discussion

The total alkaloid concentration (based on dry mass (DM)) in leaves of these plants varied among the samples. Values ranged from 221.1 ± 17.14 μg/g DM to 2986.89 ± 367.1 μg/g DM (Table 1). The total alkaloid content in *A. crassiflora* samples

Table 1
Location and alkaloid amounts (μg/g dried mass) in samples of *Annona crassiflora*. Values correspond to mean ± standard deviation. Voucher specimens were deposited at the SPF Herbarium.

Region	Voucher	PHYS	Alkaloids					
			NI	Romucosine*	Xylopine	Annoretine	Anonaine	Total
Midwest								
Brasília (Brazilian capital) (15°57.045′S, 47°52.353′W)	A.P.M. Egydio 1	Woodland "cerradão"	178.83 ± 84.89*	300.69 ± 219.55	66.24 ± 13.34b	186.78 ± 11.09c	2254.33 ± 109.66a	2986.89 ± 367.11a
Cristalina (Goiás) (16°59.599′S, 47°45.843′W)	A.P.M. Egydio 2	Wooded savanna "campo cerrado"	0	2.5 ± 2.0	126.46 ± 21.41a	559.87 ± 27.86a	365.69 ± 20.11d	1067.04 ± 71.83b
Southeast								
Santana do Riacho (Minas Gerais) (19°20.234′S, 43°37.594′W)	A.P.M. Egydio 5	Woodland "cerradão"	43.23 ± 3.74b	13.63 ± 11.12	94.18 ± 14.42a	361.2 ± 95.18b	247.17 ± 65.6e	759.41 ± 25.23c
Baldim (Minas Gerais) (19°15.520′S, 43°57.028′W)	A.P.M. Egydio 6	Wooded savanna "campo cerrado"	28.73 ± 7.8b	240.18 ± 192.82	20.92 ± 1.04b	63.9 ± 16.67d	743.57 ± 62.85b	1097.3 ± 80.99b
Lagoa Santa (Minas Gerais) (19°33.831′S, 43°57565′W)	A.P.M. Egydio 4	Wooded savanna "campo cerrado"	6.52 ± 5.32*	4.07 ± 3.32	33.7 ± 10.61b	18.94 ± 15.47*	779.67 ± 12.71b	842.91 ± 12.6c
Belo Horizonte (Minas Gerais) (20°01.718′S, 44°00.596′W)	A.P.M. Egydio 3	Woodland "cerradão"	167.57 ± 23.69a	210.79 ± 2.92	0	130.31 ± 21.79c	442.83 ± 29.43c	791.32 ± 17.68c
Luis Antonio (São Paulo) (21°35.192′S, 47°46.692′W)	A.P.M. Egydio 8	Woodland "cerradão"	149.64 ± 37.5a	0	0	58.5 ± 20.25*	445.74 ± 114.5c	653.88 ± 106.88c
Mogi-Guaçu (São Paulo) (22°15.231′S, 47°09.386′W)	A.P.M. Egydio 7	Woodland "cerradão"	0	0	0	106.4 ± 2.14c	114.64 ± 15.97f	221.05 ± 17.14d

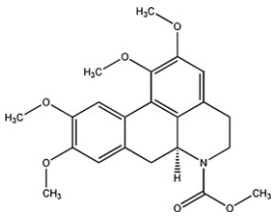
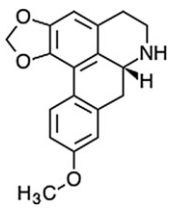
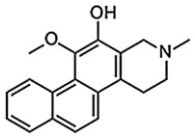
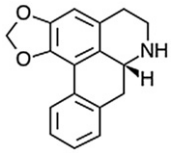
NI = not identified. PHYS = different phytophysionomies of Cerrado. Distinct lower case letters after alkaloid amounts indicate significant differences ($p \leq 0.05$) among samples. Asterisk represents values that were not submitted to analysis of variance (ANOVA).

collected in Baldim (Minas Gerais) (1097.3 ± 80.99 μg/g DM) and Cristalina (Goiás) (1067.04 ± 71.04 μg/g DM) are similar to the amount Hocquemiller et al. (1982) reported in leaves of *A. crassiflora* collected in the Guianas (1100 μg/g).

The total alkaloid concentration was significantly different among the samples obtained in eight different regions (ANOVA, $p < 0.05$). The largest amounts were detected in samples collected in Brasília (2986.89 ± 367.1 μg/g) and in the district of Cristalina (1067.04 ± 71.83 μg/g), located in the state of Goiás. Samples collected in Minas Gerais presented intermediate amounts, while samples from Luís Antonio and Mogi-Guaçú, in São Paulo, presented the lowest total alkaloid concentrations, 653.88 ± 106.87 μg/g and 221.1 ± 17.14 μg/g, respectively. In general, except for the Baldim sample, all other samples obtained in southeastern Cerrado regions had lower alkaloid levels, compared to samples collected in midwest Brazil (Table 1).

Four alkaloids were partialy identified based on MS spectra data (Table 2): two aporphines (anonaine and xylopine), a phenanthrene (annoretine) and a benzylisoquinoline (romucosine). The occurrence of anonaine was expected, since it has already been reported in leaves of *A. crassiflora* from Guianas (Hocquemiller et al., 1982). Xylopine and anonaine were also detected in *A. squamosa* (Bhaumik et al., 1979), which has similar medicinal potential to that of *A. crassiflora*, as previously published (Lorenzi and Matos, 2002). These alkaloids were recently isolated from the bark of *A. salzmannii* (Da Cruz et al., 2011). Romucosine has been detected in several *Annona* species, e.g., *Annona purpurea* (Chang et al., 2000b) and *A. cherimola* (Chen et al., 2001). Besides the alkaloids, two steroids were also found: β-sitosterol and stigmasterol (data not shown). These steroids were previously reported in other species of *Annona*, like *Annona montana* (Wu et al., 1993) and *Annona glabra* (Chang et al., 2000a). β-Sitosterol was recently isolated from the bark of *A. salzmannii* (Da Cruz et al., 2011) and leaves of *A. pickelii* (Dutra et al., 2012). Additionally, a mixture of β-sitosterol and stigmasterol was isolated from the stem of *Annona amazonica* R.E.Fr. (Pinheiro et al., 2009).

Table 2
Mass spectrometry data for the four partialy identified alkaloids from *Annona crassiflora*. RT: retention time. Reference column shows an example of a paper used for data comparison.

RT (min)	Compound (Type)	*m/z* (relative intensity, %)	Reference
13.15	Romucosine (Benzylisoquinoline) 	55 (26), 69 (31), 81 (29), 102 (33), 109 (26), 123 (19), 139 (20), 163 (38), 165 (60), 179 (14), 193 (13), 208 (24), 235 (16), 251 (9,27), 266 (96), 278 (18), 293 (26), 308 (100)	Chen et al., 1996
13.23	Xylopine (Aporphine) 	294 (100), 295 (53)	Bhaumik et al., 1979
15.28	Annoretine (Phenantrene) 	293 (100)	Wu et al., 1993
15.44	Anonaine (Aporphine) 	265 (100)	Hsieh et al., 1999

Anonaine was the main alkaloid detected in nearly all samples of *A. crassiflora*. Only the samples collected in Cristalina (state of Goiás) and Santana do Riacho (state of Minas Gerais) did not present this alkaloid as the main constituent. The sample collected in Brasília presented the highest value (2254.33 ± 109.67 μg/g) (Anova $p \leq 0.05$). Annoretine was the most representative compound in plants obtained in Cristalina (state of Goiás) and Santana do Riacho (state of Minas Gerais). These samples contained the highest annoretine concentration (559.87 ± 27.86 μg/g) (Anova $p \leq 0.05$) (Table 1). Xylopine and romucosine, which were also detected in *A. crassiflora* samples, were widely variable among regions and individual samples. Although xylopine was not been detected in samples collected in Belo Horizonte, Luís Antônio and Mogi-Guaçú, the highest variability in alkaloid profile was observed in romucosine content. Even among individuals collected in the same region, this compound was not uniformly present in samples. Such variation is corroborated by the high standard deviation values (Table 1, Fig. 1).

A cluster analysis was carried out with the mean concentrations of the four alkaloids identified in samples of *A. crassiflora* from different regions. Two clusters were created based on alkaloid content. Cluster I grouped the samples obtained in Mogi-Guaçú, Luís Antonio (São Paulo), and Belo Horizonte (Minas Gerais), while cluster II put together samples originating from Santana do Riacho, Lagoa Santa and Baldim (Minas Gerais), Cristalina (Goiás), and Brasília (Fig. 2). Xylopine was the determining factor in clustering samples, since it is absent in samples forming cluster I (Table 1, Fig. 1). Inside each group, the similarity relationship was based on quantitative variation, not on qualitative profile.

Several papers have been published on the link between alkaloid variability and abiotic factors, e.g. humidity and nitrogen availability (Saenz et al., 1993; Hunt et al., 2005), temperature (Salminen et al., 2005), light intensity and light quality (Vásquez-Flota and Deluca, 1998). However, this relationship is still controversal. Although wide variation in total alkaloid content has been detected in *A. crassiflora* leaves, no significant correlation was observed between alkaloid content and mean temperature (°C) or relative humidity (%) (data not shown). All samples were obtained between March and April 2007, with little or no variation in temperature and/or humidity. Further studies addressing the role played by soil parameters such as soil humidity and nutrient availability in alkaloid production of *A. crassiflora* are ongoing.

Although many authors have demonstrated the Cerrado phytophysiognomy as a determining aspect for phenotypic variation in some species (Cardoso and Lomônaco, 2003), no correlation among alkaloid levels and specific Cerrado physiognomic patterns has been verified. In cluster I, the samples obtained in Luis Antonio (SP) and Belo Horizonte (MG) were harvested in a *cerrado sensu stricto*. The samples collected in Baldim (MG) and Lagoa Santa (MG), cluster II, were obtained in a *campo cerrado*. Oppositely, samples obtained in Brasília and Mogi-Guaçú (SP), which presented quite different alkaloid concentrations, were both collected in a *cerradão* physiognomy. Despite the similar alkaloid concentrations, the Cristalina (GO) samples were collected in a *campo cerrado*, and the samples of Santana do Riacho (MG) were collected in a *cerradão*.

Besides ecological aspects, the alkaloid profile of *A. crassiflora* suggests that the species has potential medicinal applications. Anonaine, romucosine and annoretine have already been shown to exert significant antibacterial activity (Paulo et al., 1992). These compounds also inhibit platelet aggregation (Chen et al., 2001), and are cytotoxic (Chang et al., 1998). Therefore, samples collected in Brasília and Cristalina (GO), which presented the highest concentrations of anonaine and annoretine, should have their potential medicinal use thoroughly evaluated.

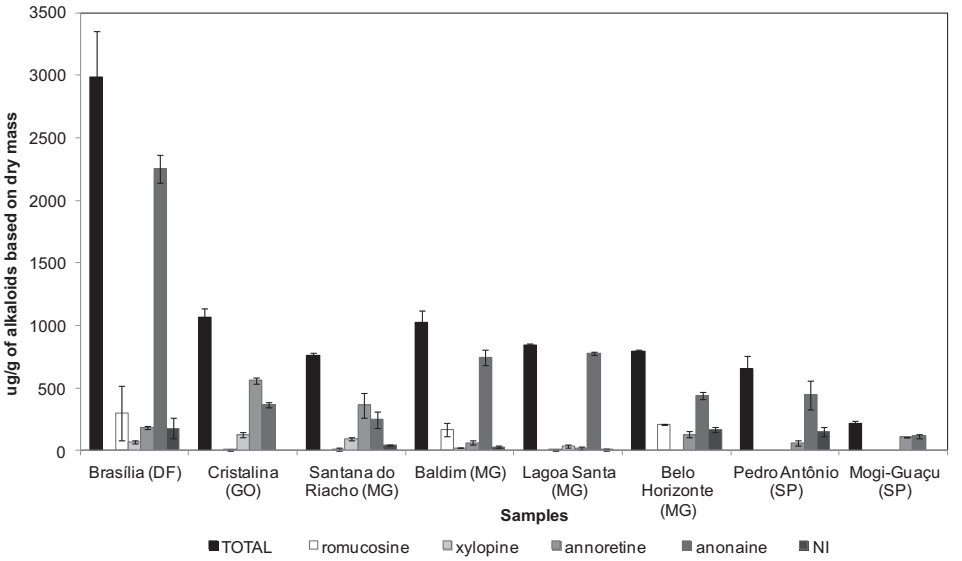


Fig. 1. Alkaloid distribution among samples of *Annona crassiflora*. NI = not identified. DF = Brazilian capital, GO = state of Goias, MG = state of Minas Gerais, SP = state of São Paulo.

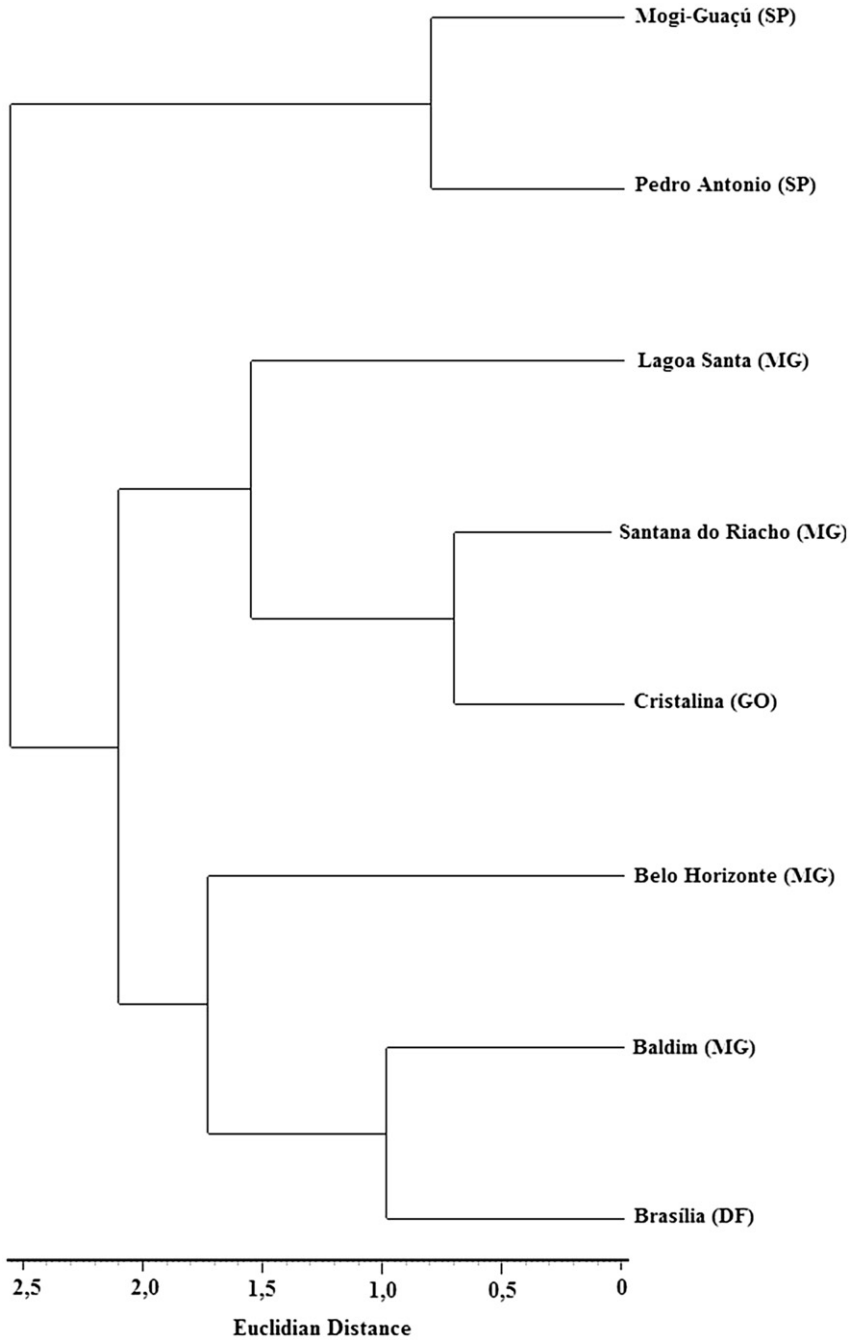


Fig. 2. Cluster analyses of samples of *Annona crassiflora* from different regions based on mean concentration of xylopine, anonaine, annoretine, romucosine and total alkaloid content using Euclidian Distance indice and UPGMA. DF = Brazilian capital, GO = state of Goias, MG = state of Minas Gerais, SP = state of São Paulo.

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