

Cultivation of Guaco (*Mikania laevigata* Sch. Bip. ex Baker) in the Lower Amazon River and monitoring of coumarin, its principal active constituent

Cultivo do Guaco (*Mikania laevigata* Sch. Bip. ex Baker) no Baixo Rio Amazonas e monitoramento de cumarina, seu principal constituinte ativo

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Resumo

Mikania laevigata (Asteraceae) é popularmente usada no tratamento de doenças respiratórias, incluindo asma, bronquite e tosse. No Brasil, extratos de guaco são usados como produtos medicinais autorizados pela Agência Nacional de Vigilância Sanitária (ANVISA). No estudo, avaliou-se a adaptação e influência da variação sazonal do guaco, em um cultivo mantido em Santarém, Estado do Pará, Brasil; além do monitoramento da cumarina, seu principal constituinte ativo. No cultivo do guaco, as variáveis climáticas e o teor de cumarina foram avaliados mensalmente. A cumarina foi analisada por CCD (Cromatografia em Camada Delgada) e quantificada por HPLC (Cromatografia Líquida de Alta Eficiência) com base nos extratos hidroalcoólico e aquoso das folhas desidratadas. Na estação chuvosa (janeiro a julho), o guaco teve um crescimento homogêneo. Na estação de estiagem (agosto a novembro) houve perda das plantas que cresciam a pleno sol. O teor de cumarina foi detectado durante o desenvolvimento da planta, com níveis que variaram de $0,470 \pm 0,01\%$ a $0,886 \pm 0,063\%$. O tempo da coleta não influenciou no teor de cumarina nas plantas cultivadas com 50% de sombra, tendo a média de 0,712% no extrato hidroalcoólico e 0,744% no extrato aquoso. Baseado nos resultados, em condições de sombreamento parcial, assegurou-se que o cultivo do guaco no Baixo Rio Amazonas pode ser replicado pelos pequenos agricultores, em seus Arranjos Produtivos Locais (APL) de plantas medicinais.

Palavras-chave: Guaco. Cumarina. Extratos farmacêuticos. Amazônia. Arranjos produtivos locais de plantas medicinais.

Abstract

Mikania laevigata (Asteraceae) is popularly used in the treatment of respiratory diseases, including asthma, bronchitis, and cough. In Brazil, guaco extracts are used as medicinal products authorized by the National Health Surveillance Agency (ANVISA). In the study, it was evaluated the adaptation and the influence of the seasonal variation of guaco, in a cultivation held in Santarém, state of Pará, Brazil, and a monitoring of coumarin, its primary active constituent. In the growing of guaco, the climate variables, and the coumarin content were evaluated monthly. The coumarin was analyzed by TLC (Thin Layer Chromatography) and quantified by HPLC (High Performance Liquid Chromatography) from hydroalcoholic and aqueous extracts of dried leaves. In the rainy season (january-july), the guaco had a homogeneous growth. In the dry season (august to november), there was loss of plants grown in full sun. The coumarin content was detected throughout the development of the plant, with levels that varied from $0.470 \pm 0.01\%$ to $0.886 \pm 0.063\%$. The time of collection does not influenced in the coumarin content of the plants grown with 50% shade, having the mean of 0.712% in the hydroalcoholic extract and 0.744% in the aqueous extract. Based on the results, in partial shade conditions, it was ensured that small farmers could replicate the cultivation of guaco in the Lower Amazon River, in their local productive arrangements of medicinal plants.

Keywords: Guaco. Coumarin. Pharmaceutical extracts. Amazon. Local productive arrangements of medicinal plants.

Introduction

Mikania laevigata Schultz Bip. ex Baker (Asteraceae), popularly known as Guaco, Guaco-de-cheiro or Guaco-do-mato, is a plant with real pharmacological importance and used for the country's phytotherapy programs. In Brazil, along with *M. glomerata*, this species is on the list of medicinal plants of national interest for utilization in the Unified Health System (SUS) (RENISUS, 2009), and in Brazilian official guides (FARMACOPÉIA BRASILEIRA, 2006; FORMULÁRIO NACIONAL DA FARMACOPÉIA BRASILEIRA, 2012).

Studies have demonstrated similarity in the chemical composition of leaves of *M. laevigata* and *M. glomerata*. The cinnamic acid derivatives, coumarin, and o-coumaric acid are the two primary constituents, apart from derivatives diterpenes of kaurane-type, as the cinnamoylgrandifloric acid and kaurenoic acid (BOLINA, GARCIA and DUARTE, 2009; NAPIMOGA and YATSUDA, 2010; GASPARETTO, FRANCISCO and PONTAROLO, 2013). Also, in *M. laevigata*, the coumarin is used as a chemical marker in the pharmaceutical formulations because of their bronquodilator and expectorant actions (GRAÇA et al., 2007; FERREIRA and OLIVEIRA, 2010; GASPARETTO et al., 2010). The guaco monograph in the Brazilian Pharmacopoeia (2006) has a validated method for identifying coumarin and quantification of the dried leaves of *M. laevigata*, with a minimum content established for the chemical marker (0.1%, w/w).

The use of medicinal plants on public health, as guaco, has been encouraged by the National Policy of Medicinal Plants and Herbal Medicines of the Ministry of Health of Brazil, which established standards for cultivation and processing of plants in order to produce quality herbal medicines (CZELUSNIAK et al., 2012). The growing of medicinal species is beneficial to the productive chain because it is simple, cheap, efficient

and promising technology, allowing the inclusion of small farmers by the diversification of their activities, with added value to the products of a given region (ANDRADE and CASALI, 2011). However, studies have shown that geographical origin, climate, and seasonality are important factors for obtaining the desired levels of coumarin in species/specimens cultivated of guaco (PEREIRA et al., 2000; CASTRO et al., 2006; BERTOLUCCI, et al., 2013; PASSARI, SCARMINIO and BRUNS, 2014). Although the information on the *M. laevigata* cultivation and monitoring of coumarin in the South/Southeast of the country is available, there are no reports in the literature for a cultivation of this species in Northern Brazil. Furthermore, nothing is known about the influence of seasonal variations and typical climatic factors of the Amazon biome on the content of coumarin in guaco leaves.

Therefore, in order to obtain information regarding the cultivation of *M. laevigata* in the Brazilian Amazon, whose processing could allow its replication by small farmers in Local Productive Arrangements (APL) with herbal medicines in the city of Santarém, Pará, Brazil (APLFITO-STM), the aim of this study was to evaluate the adaptation of this species to seasonal conditions (rainy and dry) and soil and climate of the Amazonian region, as well as their influence on the coumarin content of produced pharmaceutical extracts.

Materials and Methods

Installation and cultivation conditions

Seedlings of *M. laevigata* were provided by the Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas (CPQBA), of Universidade Estadual de Campinas (UNICAMP), São Paulo, Brazil, obtained from a plant matrix belonging to its collection of medicinal plants. Plant cuttings were propagated vegetatively based on healthy segments that measured 15 to 20 cm, previously treated with fungicide. The produced seedlings were kept in a greenhouse (at CPQBA) for two months to complete its roots and then transported (wrapped in damp paper and absence of light) by air to Santarém, Pará, Brazil. The seedlings were transplanted and cultivated in an experimental area of 240 m², at Alter do Chão, Santarém, Pará, Brazil, with coordinates 2° 31' S and 55° 00' W. As vegetative propagation was made of the same genotype, the cultivation was installed with plants of the same genetic basis. A plant voucher is deposited in the herbarium of UNICAMP, with the number UEC102046.

The experimental cultivation was done with two groups of plants. A group fully exposed to sunlight and another group with 50% shading. A hundred seedlings of the plant were used and transplanted into pits of 20 cm, spaced 1.0 m in rows and 2.5 m between the lines. The holes were previously fertilized with black earth and organic fertilizer (chicken manure), plus wood waste and dried leaves (ratio of 5: 1: 1). The driving of the plants was made in four-wire trellis (0.5, 1.0, 1.5 and 2.0 m high) and 14.0 m long. Cultural practices were continuously performed during plant development, as a frequency of irrigation, control of weeds, growth direction in the trellis, use of mulch, top dressing, and phytosanitary control. The experiment was conducted from January to December 2012.

Climatic Data

Climatic factors such as temperature (°C), solar radiation (W/m²), relative humidity (%) and rain precipitation (mm) were measured monthly from January to December 2012. Data were obtained from a meteorological station installed near the planting. The equipment used was: Datalogger Model CR1000 (Campbell, North

Logan, UT, USA) Thermo-hygrometer model HMP45C (Vaisala, Ventura, CA, USA) Pyranometer model LI200 (LI-Cor, Lincoln, NE, USA) and a Rain Gauge Model TR-525 (Texas Electronic, Dallas, TX, USA).

Plant material

The leaves of guaco, obtained from the unshaded crop and 50% of shading were collected from January to December 2012, from 8 to 10 am. The material came from the apical, intermediate and basal segments of several individuals. The samples were dried in an oven (Solab, Piracicaba, Brazil) with circulation and air exchange, for three days at 40 °C. Then, the material was crushed, conditioned at room temperature and protected from light and moisture for the extracts preparation.

Sample preparation

The dried plant material was prepared as a tincture (hydroalcoholic extract) and tea infusion (aqueous extract). The tinctures were made in the ratio of 10% (w/v) by maceration at 25 °C using an ethanol:water solution (70% v/v) for five days, with manual stirring twice a day. Then the solutions were filtered through filter paper, and the initial volume of the tinctures has been completed with the extraction liquid. Aliquots of each tincture were subjected to evaporation under reduced pressure at 50 °C, to obtaining hydroalcoholic extracts dried and kept under refrigeration at 5 °C. The aqueous extracts were prepared at the ratio of 2% (w/v) with hot water for 10 min to obtain the teas infusion. Subsequently, they were filtered, lyophilized, weighed and stored under refrigeration at 5 °C. All assays were performed in triplicate.

Phytochemical profile

The phytochemical profile of the hydroalcoholic and aqueous extracts (10 mg/mL in methanol, each) were obtained by thin layer chromatography (TLC) using aluminum plates covered with silica gel F₂₅₄ (M&N, Germany). Elution systems of different polarities, specific color reagents and standards were used to detect the following classes of secondary metabolites: coumarins, flavonoids, total phenolic, fatty acids and terpenoids (MARINI-BETTOLO, NICOLETTI and PATAMIA 1981). For the specific analysis of the chemical marker, a standard sample of coumarin (Sigma) was used in elution system with toluene:diethyl ether (1:1), saturated with acetic acid (10%) and revealed with ethanolic KOH (5%) on UV₃₆₅ light (WAGNER and BLADT, 1996). Retention factors (R_f) were calculated for the respective bright spots observed.

HPLC analysis

Analyzes were performed on a GE HPLC, Model AKTA Explorer 10S (GE Healthcare, USA), UV-VIS detectors and Unicorn software. An RP-18 column (150 x 4.6 mm, 5 µm, Whatman, USA) coupled to an RP-18 guard column (4.0 x 30.0 mm, 5 µm, Phenomenex, USA) was used. The elution was made in an isocratic mode with methanol:water (50:50), flow 1.0 mL/min and detection with UV₂₇₅ light, according to the Brazilian Pharmacopoeia (2005) and minor modifications. Hydroalcoholic and aqueous extracts were diluted with methanol (HPLC grade, Tedia, Brazil) and the samples filtered (0.22 µm) before being injected (10 µL).

The quantification of coumarin in the extracts was performed by calibration method with external standard. Coumarin with high purity was used as an external standard. Methanol solutions were prepared at concentrations of 5.0, 10.0, 25.0, 50.0, 75.0 and 100.0 µg/ml. The injections (10 µL) were made in triplicate (CELEGHINI, VILEGAS and LANÇAS, 2001). The concentration of coumarin in the extracts was determined

by regression analysis, using the ratio of the peak area (detector response) with concentrations of standard solutions. Linearity was established through the correlation coefficient r , where $r > 0.9999$. The final result was expressed as the percentage of coumarin in grams per 100 grams of dried plant material (% w/w), considering the biomass free of moisture.

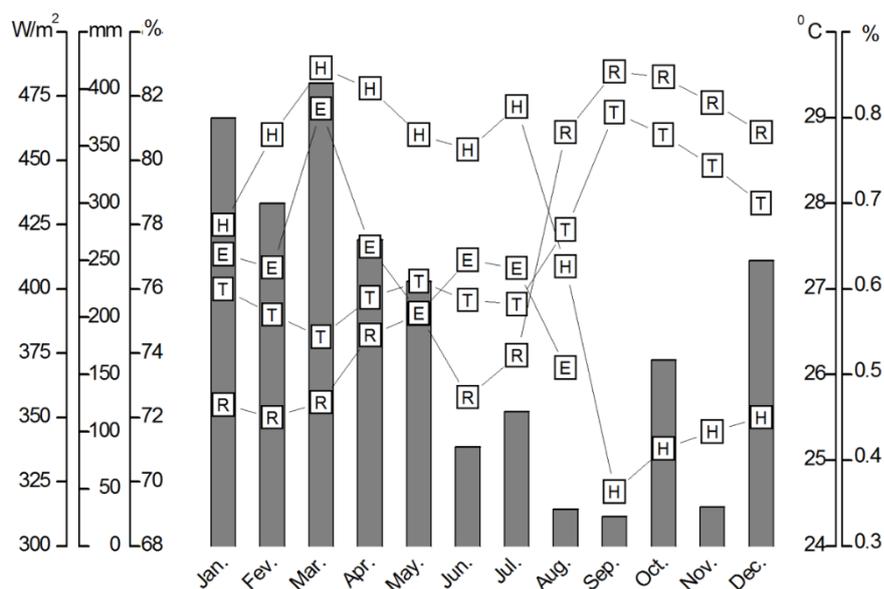
Statistical Analysis

All data were expressed as the mean \pm standard deviation (S.D), $n=3$. Statistical significance was evaluated using the Tukey test ($p < 0.05$ or $p < 0,001$) and Pearson correlation coefficients (r) were calculated to determine the relationship between the analyzed parameters (GraphPad Prism 5.0).

Results and Discussion

The guaco cultivation in the Lower Amazon River, in Santarém, Pará, Brazil, was carried out at reduced cost, to be replicated by small farmers in their local productive arrangements with medicinal plants. During the stabilization and growing of the plant, some climate variables were monitored to intending to its adaptation to soil and climate conditions of the region. These variables were rainfall precipitation, temperature, relative humidity and solar radiation, as seen in **FIGURE 1**.

FIGURE 1 - Climatic variables recorded during the guaco cultivation in Santarém, Pará, Brazil. Precipitation (mm), solar radiation (R, W/m²), humidity (H, %), temperature (T, °C), and coumarin content (E, %).



In general, the climate is characterized by two distinct seasons: the rainy season (February to May) and dry season (August to November). The months June-July and December-January are considered intermediaries between the two seasons. In 2012, the rainy season was from January to July, with a monthly precipitation above 70 mm. The highest cumulative rainfall was in March (405.3 mm) (Fig. 1). In the dry season, August, September and November showed the lowest precipitation, with a minimum of 26.2 mm in September. The temperature remained almost constant, with a low in March (26.4 °C) and maximum in September (29.1 °C).

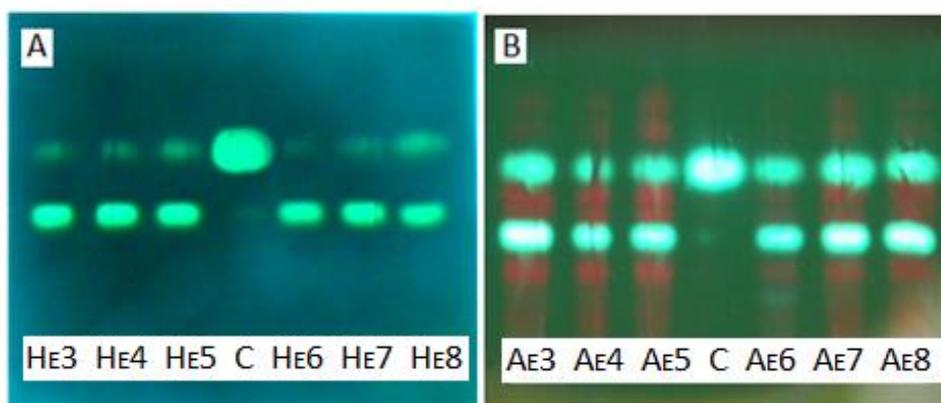
The average values of solar radiation ranged from February (355.9 W/m²) to September (484.6 W/m²) and the relative humidity varied from September (69.7%) to March (82.9%).

While growing in full sun, the guaco seedlings show a consistent leaf growth until the month of July, while it was raining. However, a reduction in the growth, leaf blight and total loss of the culture in the months of August and September were observed. The extreme conditions of high solar radiation, and water stress during the dry season, may have influenced the loss of plants to full sun. In this situation, it should be considered that sunlight and temperature are factors directly related to the photosynthetic activity of the plant, being decisive in the survival, growth and reproduction of tropical species (GONÇALVES, MARENCO and VIEIRA, 2001; TAIZ and ZEIGER, 2004). On the other hand, the high level of solar radiation and temperature can negatively interfere with photosynthesis, resulting in severe oxidative damage to the plants (KISLYUK et al., 2004). In cultivation with 50% of shade, the growth of the seedlings of guaco was stable throughout the year, indicating that partial interception of solar radiation favored its development in Santarém Pará, Brazil. In particular, the guaco grows naturally in forested areas with little sunlight, under the shade of tree-sized species (BERTOLUCCI, et al., 2013). Therefore, it was expected that the cultivation of guaco in full sun could present some problem related to the Amazon weather.

Under natural conditions or in the cultivation of medicinal plants, the soil and climatic factors can affect their growth and alter the production and content of secondary metabolites. The moment in which the plant is collected is fundamental since the amount and, often, the nature of the active constituents is not constant throughout the year (GOBBO-NETO and LOPES, 2007), which implicates in the biological effects measured in the plant. For the guaco that was grown in Santarém, coumarin as a chemical marker was present in all samples throughout the collection period of the leaves.

In hydroalcoholic and aqueous extracts of the leaves of guaco, grown in full sun and 50% of shading, the chromatographic profile (TLC) revealed a green fluorescent stain that eluted in the same pattern position of coumarin (R_f = 0.64), along with another greenish stain more polar, related to adsorption of ortho-coumaric acid (R_f = 0.44), as can be seen in **FIGURE 2**.

FIGURE 2 - Chromatographic profile (TLC) of coumarin detection (C, R_f=0.64) in the hydroalcoholic (EH) and aqueous extracts (EA) of guaco leaves, cultivated at full sun and with 50% shading. The numbers 3-8 represent the months from March to August 2012) (WAGNER and BLADT, 1996). In the plate, it is observed that the *ortho*-coumaric acid has eluted together (R_f = 0.44) with the coumarin.



In the phytochemical profile (TLC) of hydroalcoholic and aqueous extracts, in addition to the coumarin and ortho-coumaric acid, was detected the presence of terpenoids, flavonoids, and fatty acids. These results are in agreement with Bolina, Garcia and Duarte (2009) who observed the presence of triterpene, flavonoid glycosides and steroids in the ethanolic extracts of dried leaves of *Mikania glomerata* and *M. laevigata*. In this paper, the extracts of leaves growing with 50% shading showed the same metabolites related to leaf extracts cultivated under full sun, excepting the flavonoids. It is a justification that the insolation condition has considerable influence in producing these metabolites. It is evident that the presence and concentration of these metabolites are directly related to the therapeutic effectiveness of guaco extracts. Therefore, monitoring of the plant is a valuable tool for characterization and quality control (PASSARI, SCARMINIO and BRUNS, 2014). Also, as coumarin is a chemical marker of guaco and partly responsible for the observed bronchodilator effect, its determination is essential so that one can infer from the quality of the vegetable raw material used for the drug preparation and efficiency of the method employed in the extraction (ALVARENGA et al., 2009). The results for the coumarin content in guaco leaves for the period of plant collection (January to November 2012), are summarized in **TABLE 1**.

TABLE 1 - Coumarin content in hydroalcoholic and aqueous extracts of the leaves of guaco, grown in full sun and 50% shading (January-November 2012).

Month	Coumarin content (%)			
	Full sun cultivation		Shading cultivation (50%)	
	HE	AE	HE	AE
January	0.640 ± 0.048 aA	0.698 ± 0.100 aA	0.695 ± 0.027 aA	0.722 ± 0.030 aA
February	0.627 ± 0.031 aA	0.642 ± 0.054 aA	0.704 ± 0.013 aB	0.740 ± 0.002 aB
March	0.810 ± 0.024 bA	0.886 ± 0.063 bA	0.739 ± 0.031 aB	0.803 ± 0.034 aAB
April	0.649 ± 0.007 aA	0.706 ± 0.037 aA	0.729 ± 0.031 aB	0.784 ± 0.032 aB
May	0.572 ± 0.016 acA	0.501 ± 0.045 acA	0.701 ± 0.008 aB	0.720 ± 0.005 aB
June	0.634 ± 0.029 aA	0.634 ± 0.015 acA	0.653 ± 0.014 aA	0.673 ± 0.014 aA
July	0.624 ± 0.016 aA	0.593 ± 0.032 acA	0.653 ± 0.013 aAB	0.730 ± 0.039 aB
August	0.509 ± 0.003 cA	0.470 ± 0.010 cA	0.668 ± 0.011 aB	0.707 ± 0.064 aB
September	-	-	0.710 ± 0.016 aA	0.762 ± 0.075 aA
October	-	-	0.807 ± 0.008 aA	0.849 ± 0.041 aA
November	-	-	0.781 ± 0.133 aA	0.698 ± 0.028 aA

HE = Hydroalcoholic extract; AE = Aqueous extract; Mean ± standard deviation (% w/w, n=3). Different small letters within the columns (a-c) and distinct capital letters within the lines (A-B) are significantly at the $p < 0.05$ or $p < 0.001$ (Tukey test).

The coumarin contents in the leaves of guaco, extracted in the form of tincture (hydroalcoholic extracts), ranged from $0.509 \pm 0.003\%$ to $0.810 \pm 0.024\%$ in the unshaded crop, and from $0.653 \pm 0.014\%$ to $0.807 \pm 0.008\%$ in growing with 50% shadow. In the leaves of guaco extracted in the form of tea infusion (aqueous extracts), the coumarin contents were $0.470 \pm 0.010\%$ to $0.886 \pm 0.063\%$ in the extensive sun cultivation, and $0.673 \pm 0.014\%$ to $0.849 \pm 0.041\%$ in the growing with 50% shade. These values are above that required by the Brazilian Pharmacopoeia (minimum of 0.1%, w/w) (FARMACOPÉIA BRASILEIRA, 2006), demonstrating that the cultivation of guaco in the Amazon region can provide vegetable raw material of quality. Also, it became apparent that the extractive methods were efficient.

Comparing the coumarin content in the leaves of guaco grown in full sun and with 50% shading significant differences were observed, where the highest percentages were obtained in cultivation with 50% shade (see **TABLE 1**). In the growing of guaco with 50% shading, a significant variation was observed in the coumarin content of the leaves sampled during all the collection period (January to November). Also, it was noted that in the dry season (extreme weather conditions) the coumarin level remained constant, reaching the peak in October (aqueous extract: $0.849 \pm 0.041\%$ and hydroalcoholic extract: $0.807 \pm 0.008\%$) (**TABLE 1**). October was considered atypical in 2012, with high precipitation to the dry period (163.2 mm), explaining in part the meaningful content of coumarin in the cultivation with 50% shade, in both extracts.

The harvest season significantly affected the coumarin content in the leaves of guaco grown in full sun. The coumarin content reached the highest rate in plants collected between January and April, with its peak in March (aqueous extract: $0.886 \pm 0.063\%$ and hydroalcoholic extract: $0.810 \pm 0.024\%$). March also had the highest rainfall (405.3 mm) and higher relative humidity (82.9%) (**FIGURE 1**). On the other hand, in August, the precipitation and relative moisture have showed low rates (31.9 mm and 76.7%, respectively), which added to the high values of temperature ($27.7\text{ }^{\circ}\text{C}$) and the solar radiation (461 W/m^2), have contributed to low coumarin content (aqueous extract: $0.470 \pm 0.010\%$ and hydroalcoholic extract: $0.509 \pm 0.003\%$), throughout the growing period.

Significant correlations ($p \leq 0.05$) were observed when comparing the coumarin levels in guaco leaves grown in full sun, with edaphoclimatic factors (precipitation, humidity relative, solar radiation, and temperature) measured during the harvest period (see **TABLE 2**).

TABLE 2 - Correlation between the content of coumarin in the extracts, against the edaphoclimatic factors measured during the cultivation of guaco in Santarém, PA, Brazil.

Edaphoclimatic Factors	Correlation Coefficient (R)	
	Hydroalcoholic extracts	Aqueous extracts
Temperature ($^{\circ}\text{C}$)	-0.871*	-0.805*
Air relative humidity (%)	0.695*	0.581
Solar radiation (W/m^2)	-0.683*	-0.665*
Precipitation (mm)	0.707*	0.757*

* Significant at $p \leq 0.05$.

These results show that solar radiation and temperature can influence the content of coumarin negatively while higher precipitation and humidity rates can maximize the content of this chemical marker in the leaves of guaco, when grown in full sun.

Some methods for preparation of hydroalcoholic extracts of *M. glomerata* leaves were analyzed by using maceration, maceration under sonication, infusion, and supercritical fluid extraction. The best result was maceration under sonication, considering the ratio extraction yield/extraction time (CELEGHINI, VILEGAS and LANÇAS, 2001). The same authors have also described an HPLC method to determine coumarin content in these extracts, as well as, they found most content of coumarin in the aerial parts of *M. glomerata* with twelve months old, when collected in January (PEREIRA et al., 2000; CELEGHINI, VILEGAS and LANÇAS, 2001). Another study showed that a highest coumarin content was obtained from the leaves of *M. glomerata* when the plant was cultivated in full sun and 30% shading (CASTRO et al., 2006). Bertolucci and

collaborators (2013) evaluated the influence of seasonality and solar radiation in the coumarin content of the leaves of *M. laevigata* when cultivated at different levels of interception of sunlight (0%, 40%, and 80%). These authors concluded that the plant developed in the summer, with 80% of shading, maximizes the content of coumarin.

The studies mentioned above (PEREIRA et al., 2000; CELEGHINI, VILEGAS and LANÇAS, 2001; CASTRO et al., 2006; BERTOLUCCI et al., 2013) were performed in experimental plantation in southeastern Brazil, where the summer season (December to February) is characterized as hot and humid, with precipitation levels ranging on 227 mm. Considering our study here in the Amazon biome, high levels of solar radiation and temperature during the dry season may have been the determining factors for the guaco not survived, when grown in full sun. Also, there was the reduction of coumarin content. On the other hand, the protection of the photosynthetic system of guaco seedlings with partial shading, reducing these harsh conditions of the dry period in the Amazon, enabled the development of the plant with a constant content of coumarin throughout the cultivation and plant collection.

In conclusion, this paper recommends that the best cultivation condition for guaco in the Lower Amazon River is 50% shading, which gives a regular content of coumarin throughout the year. Partial interception of solar radiation was an important factor in the development and adaptation of guaco at the Amazonian climate. Accordingly, the cultivation of the plant can be replicated by farmers in their Local Productive Arrangements of Medicinal Plants, or even in orchards and backyards, using a partial shade condition, with good screens or large tree species.

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