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Coumarin Contents in Young *Mikania glomerata* Plants (Guaco) under Different Radiation Levels and Photoperiod

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SUMMARY. *Mikania glomerata* Sprengel (Asteraceae) has its pharmacological activity ascribed to coumarin. It is a medicinal plant used extensively in popular medicine in Brazil to treat respiratory diseases. In the present work we present the results of an experiment performed with the objective of evaluating the effects of different radiation levels and photoperiod in the content of coumarin of *Mikania glomerata.* Sixty day-old seedlings obtained from cutting propagation, were submitted for 100 days to four light levels (0% or full sunlight, 30%, 50% and 70%) and four photoperiodS (8, 12, 16 and 20 h). Coumarin contents in leaves and stems dry were evaluated through high performance liquid chromatography (HPLC). The results obtained revealed that coumarin content in the leaves of young plants (100 days old), cultivated under the full sunlight, was twice larger when compared with the adult planted growing under the same radiation conditions. The upper part of the plants, both on the leaves and stems, presented greater coumarin content, at all levels of radiation. The photoperiod also influenced significantly the coumarin content in *Mikania glomerata* leaves and stems. The coumarin content was higher in 16 h light period.

RESUMEN. "Niveles de cumarina en plantas jóvenes de *Mikania glomerata* (guaco) sometidas a diferentes niveles de radiación y fotoperiodo". La actividad farmacológica de *Mikania glomerata* Sprengel (Asteraceae) se encuentra asociada a su contenido de cumarina. Es uma planta medicinal ampliamente utilizada en la medicina popular brasileña para el tratamiento de enfermedades respiratorias. En este trabajo se presentan los resultados de un experimento realizado con el objetivo de evaluar los efectos de diferentes intensidades de radiación y fotoperíodos en el contenido de cumarina de *Mikania glomerata.* Plántulas con 60 días de edad obtenidas por propagación vegetativa se sometieron durante 100 días a cuatro intensidades de luz (0% o pleno sol, 30%, 50% y 70%) y cuatro fotoperíodos (8, 12, 16 y 20 h). Los niveles de cumarina en las hojas y tallos secos fueron determinados por cromatografía líquida de alta resolución (CLAR). Los resultados obtenidos revelaron que en plantas jóvenes (100 días de edad), cultivadas a pleno sol, el nivel de cumarina en las hojas fue dos veces mayor cuando se compararon con plantas adultas crecidas en las mismas condiciones de radiación. En la parte superior de la planta, tanto las hojas como los tallos presentaron mayores niveles de cumarina para todas las radiaciones estudiadas. El fotoperíodo influenció significativamente en el nivel de cumarina em hojas y tallos de *Mikania glomerata.* El nivel de cumarina fue mayor em el fotoperíodo de 16 h de luz.

INTRODUCTION

Mikania glomerata Sprengel (Asteraceae), popularly known as guaco, is a medicinal plant extensively used in the Brazilian popular medicine to treat respiratory diseases. Pharmacological assessments have confirmed the folkloric action of guaco in respiratory pathologies such as asthma. Previous studies reported that the effects of guaco are related to a direct relaxation of the smooth respiratory muscle accompanied by anti-inflammatory and anti-allergic actions [1].

Guaco is present in many commercial phytotherapeutic preparations but little is known about its agronomic management. Furthermore, for regulation purposes, the quality of medicinal and aromatic plants and their products has to fulfill requirements of safety, efficacy and stability. In order to meet these demands, detailed knowledge about the agronomic parameters of

KEY WORDS: Coumarin, Light intensity, Ledicinal plant, *Mikania glomerata,* Photoperiod.
PALABRAS CLAVE: Cumarina, Fotoperiodo, Intensidad luminosa, *Mikania glomerata,* Planta medicinal.

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a species is required whether the plant comes from the wild or is cultivate.

Secondary metabolites biosynthesis is regulated genetically and by environmental factors. Among the environmental facts, the effect of light is very important as a consequence of its significant and complex mechanism of action on the metabolism, and influencing the accumulation and quality of the main ingredients [2,3].

In particular, the coumarin content in plants showed different responses to the variations in light intensity [5-8]. In addition to the light effect on the efficiency of a medicinal plant to produce secondary metabolites, the influence of the plant ontogenetic stage is also very important. Previous research conducted by our group demonstrated that the role of coumarin in the plant requires further clarification from the physiological point of view [5]. Moreover, important variations in the coumarin and umbelliferone contents have been detected in different plants depending on the plant organ and development stage, being the greatest contents found in the ripe leaves of *Justicia pectoralis* Jacq. [6]. However, our results demonstrated that *Mikania glomerata* showed higher levels of coumarin in young leaves when compared with adult ones [5].

Considering the lack of data in the evaluation of light requirements influencing economically important medicinal plant cultivation, the objective of the present study was to study the effect of light intensity and exposition time on coumarin production in *Mikania glomerata* Sprengel (guaco).

MATERIAL AND METHODS

The present study was carried in Lavras city, located in the southern region of Minas Gerais State, at 918m Altitude, Latitude 21° 14´S and 45° 00´W Gr. The plantlets used were produced by cutting propagation. The *Mikania glomerata* cuttings were removed from the plants, located in the Itumirim-MG city, by taking 15cm long cuttings from the mid thirds of the branches with one pair of leaves and placed on extruded polystyrene trays as 6.2 cm deep individual cells in a pyramid format. Each cell presented a 36 cm^3 volume. The trays dimension was 676 mm x 340 mm x 60 mm, with 122 cells per tray. Plantimax® substrate was used. The cuttings were kept in a greenhouse for 60 days with 75% relative humidity and 26 ± 2 °C temperature.

After this period, cuttings were placed in perforated 11 x 18 cm plastic bags with 6 kg capacity. The plastic bags were filled with vermiculite, cattle manure and sub-soil, in the proportion 20:30:50; the vermiculite was used as fine granules (apparent density, 125 kg/m^2) and the cattle manure was dried and sieved previous to its use. Dark Red Latosoil (DRL) was used as sub-soil material, at thirty centimeters below the plow able level. Then, the plastic bags with the cuttings were submitted to the experimental conditions in order to evaluate light intensities and exposition time effects.

Radiation levels

The *Mikania glomerata* plants were submitted to four light levels: 0% or full sunlight, 30% 50% and 70%. Black nylon screen was used to cover the upper and side parts of wooden frames shaded the plants. Throughout the experimental period the soil was kept close to field capacity. Stems and leaves were collected from the upper, mid and lower parts of 16 plants selected randomly per treatment at 100 days to extract and quantify the coumarin at different radiation levels.

The plant materials were ground in a grinder to a 48 mesh diameter. A factorial design was used among the three branch positions and four light levels (full sunlight, 30%, 50% and 70% shading).

Photoperiod effect

The propagated *Mikania glomerata* plants were transferred to growth chambers and submitted to the following light period treatments: 8, 12, 16 and 20 h. Sixteen plants were used in each light period. The chambers were mobile and equipped with an adjustable table illuminated with artificial light supplied by 75% fluorescent lamps (GE, 40W) and 25% Gro-Lux type lamps, which emitted radiation equivalent to 225 µmol quanta/m^2/sec. The radiation was determined by a quantometer coupled to a porometer (Steady State Porometer Licor 1600 M). In each chamber a curtain made of black-out' fabric was adapted and each mobile table was equipped with a temporizer system, with automatic stop and start. These conditions permitted us to work independently with the four photoperiods in the same growth room. The environmental temperature was controlled by an air conditioner, registering a mean temperature of 25° C and 75% relative air humidity.

Because of the climbing growth habit of this species, the plants were trained on bamboo stakes, remaining in the chamber conditions for 90 days. During this period, the plastic bags were irrigated in order to maintain the substrate moisture close to its field capacity.

To extract and quantify coumarin at different radiation levels, 8 plants were selected randomly per treatments at 100 days after transplanting and the branches were separated in the upper, mid and base parts of the stems and leaves. The dry matter was ground in a grinder to a 48 mesh diameter. A factorial design was used among the three branch positions and the four light period situations (8, 12, 16 and 20 h).

Coumarin extraction and extract clean up for quantification

Coumarin was extracted and quantified according Celeghini *et al.* [9]: Samples of 1 g of dried *M. glomerata* leaves and under sonication an ultra-sound bath for 40 min at room temperature. The ethanol solution was then filtered through paper and the solvent evaporated in a water bath at 60 °C. The residues were put in solution to a final volume of 5.0 mL with methanol (HPLC grade, LiChroSolv, Merck). The solutions were then filtered through a RP-18 cartridge (Merck) and centrifugated at 10,000 r.p.m. for 5 min. The supernatants were analyzed by HPLC.

HPLC conditions

The supernatants were analyzed by HPLC (Shimadzu LC-10 Ad). The stationary phase column (Supelcosil LC-18) was 4,6 mm i.d. x 250 mm and 5 µm particle size. The detection was carried out at 254 nm using a diode array detector (Shimadzu SPD-M10A). The solvent was CH3CN/H2O 40:60 isocratic at a flow rate of 1.0 mL/min; column temperature, 40 °C; sample injection, 20 µL. The analyses were performed by triplicate and each sample was injected in duplicate.

Calibration curve

Twenty µL of standard coumarin (Synth®) solutions at 0.05; 0.1; 0.2; 0.4; 0.6; 0.8 and 1.0 mg/mL were analysed by HPLC in triplicate. The calibration curve was constructed by plotting the amount of coumarin injected (µg) of the reference substance versus the peak area in the chromatogram.

RESULTS AND DISCUSSION

The chemical composition of M. glomerata has been related to the presence of coumarin (1,2-benzopyrone), lupeol, stigmast-22-en-3-ol, cinnamoylgrandifloric acid, *ent*-kaur-16(17)-en-19-oic acid and others kaurane-type diterpenes [10-13]. Among these compounds the coumarin has been reported as the main chemical marker for guaco extracts, due to its easy chromatographic evaluation and the availability of HPLC methods developed for its quantitative analysis [14,15].

The coumarin extraction and quantification methodology reported by Celeghini *et al.* [9] was used as it has been demonstrated to be a reproducible and sensitive technique in the quality control of *Mikania glomerata* hydroalcoholic extracts. The coumarin contents in the *Mikania glomerata* leaves and stems samples were calculated from the calibration curve, that presented a linear relationship between the peak area and the coumarin concentration in the different standard solutions tested (data not shown).

The presence of coumarin in the *Mikania glomerata* leaves and stems was confirmed by comparison of the chromatograms of the standard and the sample, where the coumarin showed a retention time of approximately 6.1 min in the experimental conditions indicated in Figure 1. Moreover, the increase in the coumarin peak area of the samples after the addition of the standard was indicative of its presence in the material analyzed. No significant differences in the coumarin contents were detected, either in the leaves or stems, for the different shading conditions at the level of 5% probability (Table 1).

Regarding the coumarin content in the different plant positions, the leaves and stems of the

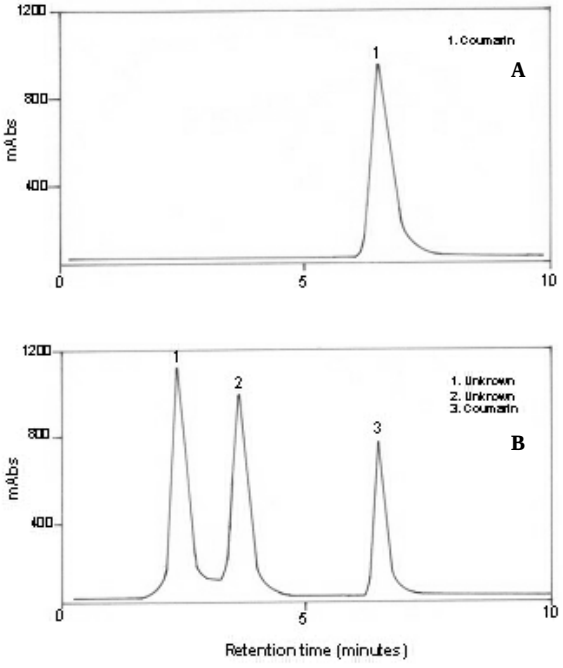

Figure 1. Standard coumarin chromatogram (**A**) and chromatogram obtained from hydro alcohol extract of *Mikania glomerata* plant material (**B**).

Shading levels (%)	Leaf	Stem
Full sunlight	5.39 [a]	2.18 [a]
30% of shading	5.45 [a]	2.93 [a]
50% of shading	4.38 [a]	1.96 [a]
70% of shading	5.98 [a]	1.42 [a]

Table 1. Coumarin contents in *Mikania glomerata* leaves and stems under different shading levels in 100 day-old young plants after transplant (mean of the three branch positions). *Means followed by the same letter in the column do not differ significantly by the Scott-Knott test at 5% level of probability.

Plant position	Leaf	Stem
Upper region	6.95 [a]	3.70 [a]
Median region	4.23 [b]	1.29 [b]
Basal region	2.97 [b]	1.22 [b]

Table 2. Coumarin contents in *Mikania glomerata* leaves and stems within each plant position (mean of all the treatments). *Means followed by the same letter in the column do not differ significantly by the Scott Knott test at 5% probability.

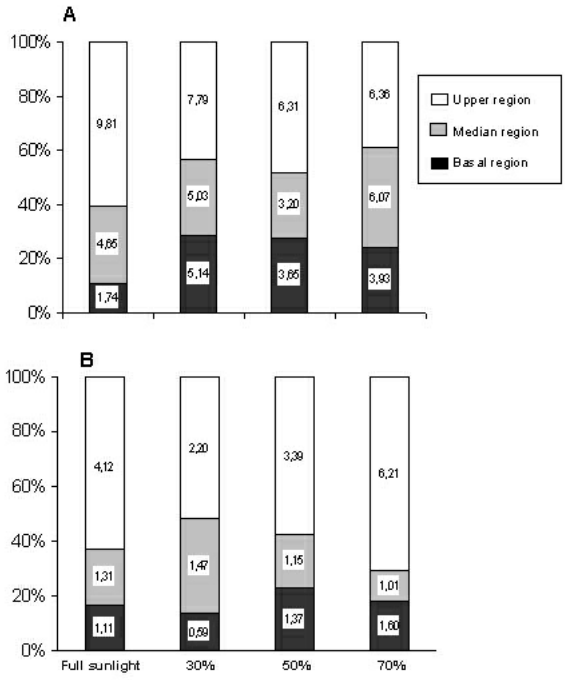

Figure 2. Coumarin contents distribution in leaves (**A**) and ítems (**B**) located in different regions of the Branco (upper, median and basal) Under different levels of shading (30, 50 and 70% using black nylon screen). * The numbers inside of column meaning mg of coumarin per g dry matter of Guaco with percentage correspondent of each region.

base and mid regions presented lower coumarin contents than those of the upper region (Table 2). The relatively high value of the coumarin concentration in the stems (3.70 mg) suggested to use the upper part of the stem and the leaves for extraction purposes.

The higher values found in the upper part, both in the leaves and the stems, could indicate that coumarin is involved in the growth and development process of the plant. These results (Table 3) are important, from phytotherapeutic medication production point of view because the low phytomass volume together with higher coumarin contents, make the end product economically more viable for the pharmaceutical industry. According to literature [5] coumarin is present in all the *Mikania glomerata* organs at different concentrations. The leaves have the highest coumarin content (5.20 mg/g dry matter) followed by flowers (1.04 mg/g dry matter), stems (1.05 mg/g dry matter) and roots (0.11 mg/g dry matter).

Previous research [5] also detected variations in the coumarin contents in young *Mikania glomerata* leaves (5.91 mg/g dry matter) when compared with adult leaves (2.15 mg/g dry matter). According to Pereira *et al.* [16], the higher concentrations of coumarin observed in leaves near the apical bud of *Mikania glomerata* plant suggest that meristematic tissues may be the site of synthesis of coumarin and that coumarin is translocated to different parts of the plant.

Moreover, the evaluation of closed coumarin derivatives in *Peucedanum palustra* showed that their greatest accumulation occurred during plant growth phase [5]. Similarly with *Mikania glomerata* coumarin contents was detected about twice as high in young plants than old in *Justicia pectoralis* Jack. Var. Stienothylla Leon [6] plants.

Figure 2 shows the coumarin contents found for the different regions of the plant at four radiation levels. The results indicated that the upper region of the plant, both for leaves and stem, presented the highest coumarin content at all the radiation levels. The coumarin contents in buds located at different positions of the *Mikania glomerata* branch were different, presenting higher values in the second bud (the apical bud was excluded) [5], indicating that the meristem region collude be the site of coumarin synthesis in *Mikania glomerata* and that later, coumarin should be translocated to other parts of the plant as previously reported [17]. This hypothesis is strengthened with data of Matern [17] who demonstrated that the biosynthetic pathway for coumarins is in the plastids.

Regarding coumarin synthesis in *Mikania*

glomerata, sunlight may favor the accumulation of secondary metabolites. Comparing only the leaves of young plants cultivated in the different radiation treatments of the upper part of the plant, it was observed that the greatest coumarin contents were obtained in full sunlight and 30% shade, respectively. This probably occurred because of morphophysiological alterations in the *Mikania glomerata* leaf under different radiation levels. The direct relationship between anatomic adaptation and efficiency in physiological processes was already study [18]. These results also elucidated the relationships between the coumarin content and light intensity, but the relationship between the chloroplast size and structure and their possible physiological implications, involving light quality, duration and quantity should be analyzed to confirm this hypothesis, once the biosynthesis site of the coumarin are in the plastids.

The involvement of plastids in phenolic metabolism were studied in *Petunia*. Feeding experiments with [^{14}C] phenylalanine demonstrated in these isolated organelles: the synthesis of cinnamate and benzoate derivatives which, with the exception of the first term of each serie, are *o*-hydroxylated (*o*-coumaric acid, coumarin and salicylic and gentisic acids); the lack of formation of labelled *p*-hydroxylated derivatives and flavanoids. The results are in agreement with the characterization, in the plastids of specific enzymes (PAL, cinnamate-2-hydroxylase) and the absence of cinnamate-4-hydroxylase [19].

These results are correlated [5] with the accumulation of determined secondary metabolites requires complex structures and thus they are found in greater quantity in mature parts of the plant, and other types are biosynthesized and accumulated in young tissues, because the internal growth phase favors their biosynthetic routes. This question should be investigated in all the medicinal species, because there is no rule that can be applied to a type of substance class or plant family.

There was a close relationship between light intensity and secondary metabolite production because all the substances produced by the plant were involved directly or indirectly with photosynthesis.

Regarding the light periods assessed, it was ascertained that the different light periods studied influenced the coumarin contents significantly in the *Mikania glomerata* leaves and stems (Table 3). Lower coumarin content was observed in the leaves and stems in the shorter light periods.

Photoperiod	Leaf	Stem
8 hours	1.046 [a]	0.796 [a]
12 hours	2.033 [a]	0.696 [a]
16 hours	6.216 [c]	1.176 [b]
20 hours	3.710 [b]	1.093 [b]

Table 3. Coumarin contents in *Mikania glomerata* leaves under different light periods. *Means followed by the same letter do not differ significantly by the Scott-Knott test, at the level of 5% probability.

Table 3 also shows that the coumarin contents in the leaves were significantly higher in 16-h light periods and fell considerably when the light period was extended from 16 to 20 h. This behaviour at 16 h may be a consequence of the light period duration and the ratio of secondary metabolite production, as all the plants components are involved directly or indirectly with photosynthesis and consequently with the anatomic, physiologic and chemical alterations that occur in the entire plant [18]. Although no data was found in the literature on the effect of light period and coumarin production, similar examples regarding the effect of light period on secondary metabolite synthesis and accumulation have been reported for other species, such as *Symphytum officinale* L. [20], *Spinacia oleracea* L. and *Agrosthena githago* L. [21], *Perilla ocymoides* [22], *Gomphrena macrocephala* [23], *Oryganum syriacum* [24].

In brief, the study of the factors influencing secondary metabolites formation can maximize their production, improving the quality of medication without increasing the costs of the productive process [20,25]. As demonstrated in this work, it is also possible to use the knowledgement of the factors influencing the chemical composition of the medicinal plants to make them to produce selected components with beneficial actions, in greater quantities and more accessible to be extracted, standardized and used.

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