INFLUÊNCIA DA FERRUGEM (*Puccinia mikaniae*) NA PRODUÇÃO DE CUMARINA EM GUACO

INFLUENCE OF RUST (*Puccinia mikaniae*) IN THE PRODUCTION OF COUMARIN IN 'GUACO'

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Abstract

In this study, we investigated the role of a rust disease (*Puccinia mikaniae* H. S. Jacks. & Holw) by the production and accumulation of coumarin in *Mikania glomerata* Spreng. (Asteraceae). Known as "guaco," this plant is native to Brazil and its leaves are used for the treatment of respiratory disorders, and coumarin is its chemical marker. The coumarin content was analyzed in samples obtained from both healthy and stressed from rust disease leaves of *M. glomerata*, and from a healthy *M. laevigata*. The sample obtained from the healthy leaves of *M. glomerata*, collected as the plant was stressed by the fungus, showed 0.94% of coumarin, which was significantly higher than the other samples analyzed. Leaves from a healthy seedling of the same individual, obtained by cutting, showed 0.68% coumarin. The other samples, including the sample of *M. laevigata*, presented a content around 0.3%. This differentiated coumarin production seems to indicate a defense mechanism, a systemic resistance response, acquired due to the fungal attack. Thus, in order to obtain a product with high coumarin content, it is possible the use of healthy leaves from guaco plants with rust disease.
Keywords: *Mikania glomerata*. *Mikania laevigata*. Secondary metabolites. Rust disease.

Resumo

Neste estudo investigou-se o papel de uma doença fúngica (*Puccinia mikaniae* H. S. Jacks. & Holw) na produção e acumulação de cumarina em *Mikania glomerata* Spreng. (Asteraceae). Conhecida como "guaco", esta planta é nativa do Brasil e suas folhas são utilizadas no tratamento de desordens respiratórias, sendo a cumarina seu marcador químico. Avaliou-se o teor deste marcador em amostras obtidas de diferentes folhas de *M. glomerata* saudáveis ou estressadas pela doença fúngica e de um indivíduo saudável de *M. laevigata*. A amostra das folhas saudáveis de *M. glomerata*, coletada quando esta se encontrava estressada pelo fungo, apresentou 0,94% de

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cumarina, percentual significativamente maior do que todas as outras amostras analisadas. Folhas de uma muda saudável do mesmo indivíduo, obtida por estaquia, apresentou 0,68% de cumarina. As demais amostras, incluindo a amostra de *M. laevigata* saudável, apresentaram um teor em torno de 0,3%. Esta produção diferenciada de cumarina pode indicar um mecanismo de defesa, uma resposta de resistência sistêmica, adquirida devido ao ataque fúngico. Portanto, com o objetivo de obter um produto rico em cumarina, é possível utilizar folhas saudáveis de espécies de guaco que apresentam doença fúngica.
Palavras-chave: *Mikania glomerata*. *Mikania laevigata*. Metabólitos secundários. Doença fúngica.

1. INTRODUCTION

Popularly known as “guaco,” *Mikania glomerata* Spreng. and *Mikania laevigata* Sch. Bip. ex Baker (Asteraceae), are native to the Atlantic Forest, mainly in the south and southeast of Brazil (LORENZI; MATOS, 2008). The genus *Mikania* comprises about 450 species distributed mainly in the region Neotropical. It can be easily recognized by the capitula with four phyllaries, four florets surrounded by a subinvolucral bract (HONÓRIO *et al.*, 2019). The aqueous leaf extracts of these plants have been used by Brazilian inhabitants for ages, and Brazilian indigenous peoples have an ancient tradition of using “guaco” against snakebites. The pharmacological effects of this plant are generally attributed to the leaves. Presently, the syrup made with the extracts of this species has been freely provided by the Brazilian National Public Health System as medicine for the treatment of respiratory diseases, including asthma, cough, and bronchitis (NAPIMOGA; YATSUDA, 2010). Its therapeutic indication and safety are validated by the Simplified Register list (BRASIL, 2014) of the Brazilian Health Regulatory Agency (ANVISA). They are listed in the Brazilian Pharmacopeia (BRASIL, 2019), in the 1st Brazilian Phytotherapy Formulary (BRASIL, 2011), in the first supplement of this formulary (BRASIL, 2018) and in the 2nd Brazilian Phytotherapy Formulary (BRASIL, 2021).

Mikania glomerata and *Mikania laevigata* are similar in morphology, and often used without distinction. Their chemical composition, however, seems to be different (DELLA PASQUA *et al.*, 2019). Coumarin, o-coumaric acid, and kaurenoic acid are considered the main metabolites of *Mikania* species and their pharmacological effects have been attributed mainly to the presence of these compounds (GASPARETTO *et al.*, 2015). Despite the therapeutic relevance of o-coumaric and kaurenoic acids, coumarin (1,2-benzopyrone) is the main pharmacological substance and used as a biomarker for “guaco” (PASSARI *et al.*, 2014).

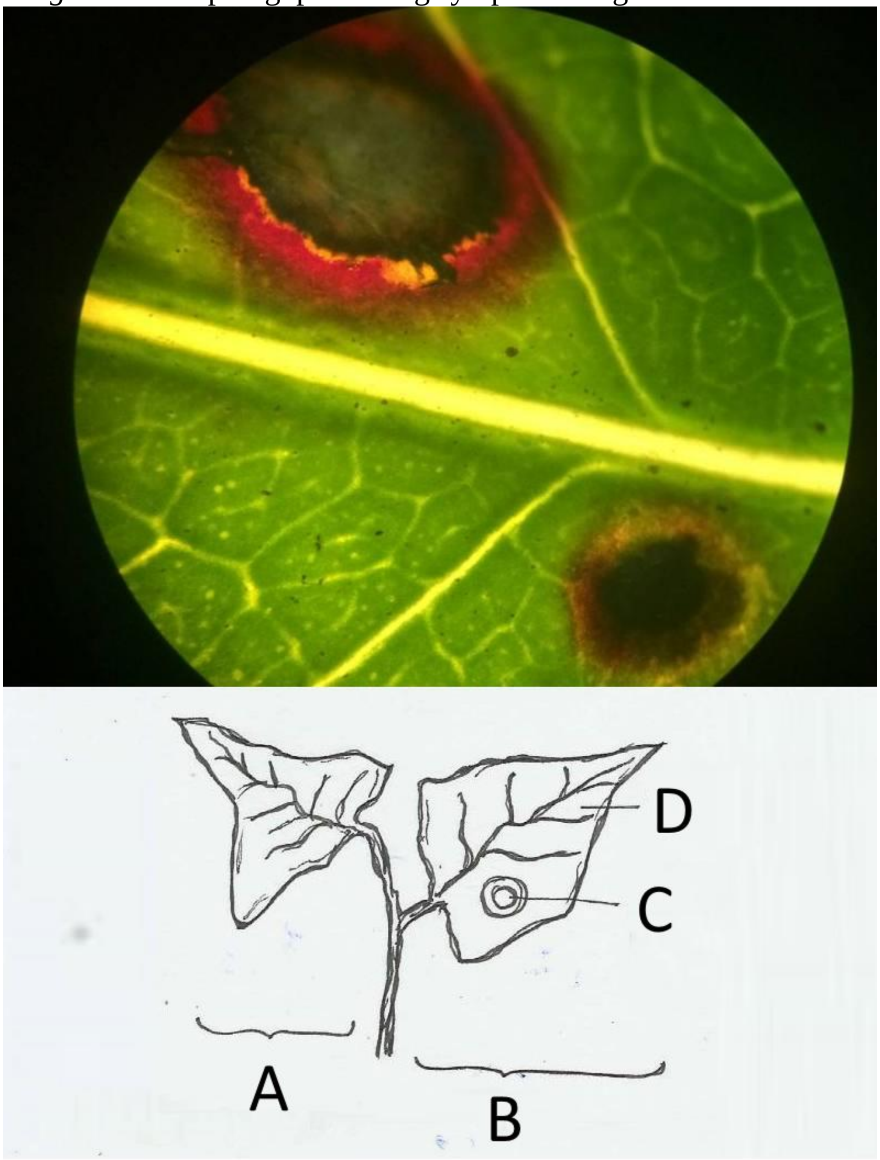
In order to analyze the influence of rust disease on coumarin production, we determined its content in different samples of guaco leaves. Four samples were obtained from a plant *M. glomerata* as it was stressed from rust disease and one after it was already recovered. Another sample was obtained from an unstressed *M. laevigata*, without disease.

2. MATERIAL AND METHODS

Leaves of a *M. glomerata* presenting symptoms of guaco rust (*Puccinia mikaniae* H. S. Jacks. & Holw) were collected in Passo Fundo, Brazil (28° 15’ 46’’ S and 52° 24’ 24’’ O; voucher number RSPF 14436), at the end of fall, in 2014 (samples A to D). This same plant was propagated by cuttings, obtaining rooted seedlings. From the visually healthy leaves, without the fungus, of one of these seedlings, grown for four years, was obtained another sample in December 2021 (sample E). After the collection, the samples were sun dried at room temperature for one

week, crushed manually through maceration with mortar and pistil, and grouped as follows (Figure 1):
Sample A: healthy leaves (not affected by the fungus) of the stressed plant.
Sample B: whole leaves with parts stressed from fungal disease.
Sample C: leaves stressed from fungal disease, using only the part of the leaf affected by the fungus.
Sample D: leaves stressed from fungal disease, using only the part of the leaf not affected by the fungus.
Sample E: leaves from the healthy seedling.

Figure 1. *Mikania glomerata* Spreng. presenting symptoms of guaco rust.



(A) leaf without the fungus; (B) leaf with the fungus (whole); (C) leaf with the fungus (only the part containing the fungus); and (D) leaf with the fungus (only the part without the fungus).

Leaves from an unstressed *M. laevigata* (without rust disease), collected in Porto Alegre, Brazil (30º 02’ 13.6’’ S and 51º 11’ 00.5’’ W; voucher number ICN 159187) was also analyzed (sample F).

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Species registration was carried out in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SISGEN) with the number A4260E3.

For determination of coumarin content in "guaco" leaves, the method described by Osório and Martins (2004), with some adaptations, was used.

A calibration curve was initially built with 1,2-benzopyrone (Sigma Aldrich, batch 030M1441V) – the reference substance. Approximately 0.1 g of the reference substance were accurately weighed, dissolved into 5 mL of methanol in a 50.0-mL volumetric flask and distilled water was added until the volume reached the marked line, yielding a stock solution of 2 mg mL^{-1}. Aliquots of 1.0, 2.0, 3.0, 4.0, and 5.0 mL were transferred to 100.0-mL volumetric flasks. Around 20 mL of distilled water and 5 mL of 5% lead acetate were added to each flask, making up the volume with distilled water. The solution was filtered, and 10.0 mL of the filtrate was transferred to a 50.0-mL volumetric flask. The volume was made up with 0.1 M hydrochloric acid (final concentration of 4 to 20 µg mL^{-1} of coumarin). Absorbance was read in a UV-Vis (Perkin Elmer Lambda 20) spectrophotometer at 320 nm, using the 0.1 M hydrochloric acid solution as a blank. Based on the results, a calibration curve was built. The analyses were performed in triplicate.

The samples were prepared from 1 g of dried plant material, subjected to ultrasonic cleaning for 20 min with 10 mL of ethanol:water (1:1). After filtering, an aliquot of 5.0 mL was transferred to a 50.0-mL volumetric flask. Approximately 20 mL of distilled water and 5 mL of aqueous solution of 5% lead acetate were added, following the same procedure used for the preparation of 1,2-benzopyrone.

The coumarin contents were calculated based on the absorbance values in the calibration curve. The results were expressed in percentage of coumarin equivalents in the plant raw material. The analyses were performed in triplicate.

The data obtained were assessed by analysis of variance (F test) and the means were compared by Tukey's test at a 5% significance level.

3. RESULTS AND DISCUSSION

The coumarin contents are shown in Figure 2. Sample A (healthy leaves from the stressed *M. glomerata*) stands out for the high coumarin content (0.94%), differing significantly from the others. Sample E, obtained from the leaves of *M. glomerata* recovered from the rust disease, showed a coumarin content of 0.68%, significantly greater than samples B, C, D and F. No significant differences were observed in those leaves affected by the fungus, regardless of whether the part affected by the fungus (sample C, 0.37%), the leaf part without the fungus (sample D, 0.34%) or the whole leaf (sample B, 0.26%) was assessed. No significant differences were observed by comparing the leaf obtained from *M. laevigata* without rust disease (sample F, 0.37%) with the parts (affected and not affected by the fungus) collected from the stressed *M. glomerata* (samples B, C and D).

There are controversies regarding the concentration of coumarin when comparing the species *M. glomerata* and *M. laevigata*. Bolina *et al*. (2009) report a coumarin content of 0.30% for *M. glomerata* and 0.43% for *M. laevigata*, and suggest that the two species can be used interchangeably. Bertolucci *et al*. (2009) found an average coumarin content of 0.37% in *M. laevigata* and undetectable amounts of this marker in *M. glomerata*. These results were confirmed by De Melo and Sawaya (2015), and Almeida *et al*. (2017), who found significant amounts of coumarin in *M. laevigata*, while in *M. glomerata* its content was very low or even undetectable. Costa *et al*. (2018) also found the presence of coumarin in leaves of *M. laevigata*, but not in *M. glomerata*. Ueno *et al*. (2019) studied the volatile compounds of the plant and concluded that only

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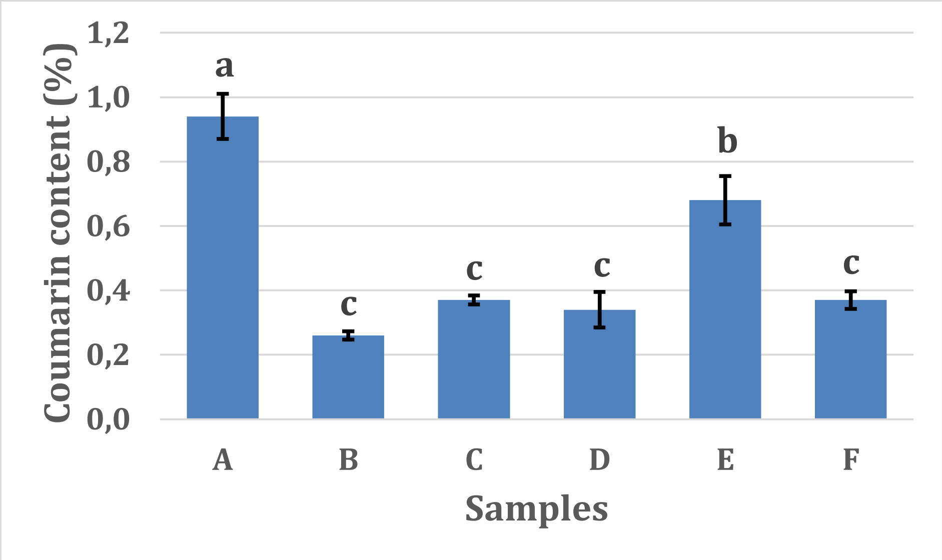
M. laevigata contained coumarin. Therefore, recent studies indicate higher levels of coumarin in *M. laevigata* than in *M. glomerata*. However, in the present work, we found a significantly higher coumarin content in *M. glomerata* (0.94%) than in *M. laevigata* (0.37%), and the presence of the fungus was essential to obtain this result. Coumarin and its derivatives act in the control of pests, weeds and other pathogens (HUSSAIN *et al*., 2018). According to Sousa *et al*. (2022), coumarins may have their synthesis increased in a plant when it comes into contact with pathogenic fungi. Furthermore, the antifungal activity of essential oil of another species of *Mikania* (*M. cordata*) has been evaluated against four phytopathogenic fungi (*Alternaria alternata*, *Botrytis cinerea*, *Fusarium oxysporum* and *Penicillium expansum*). The essential oil inhibits the growth of all tested fungi (TRIPATHI *et al*., 2016).

Figure 2. Coumarin contents (% of dry matter) in samples of guaco leaves.



Samples (A) to (D): *M. glomerata* presenting symptoms of guaco rust. (A) leaves without the fungus; (B) leaves with the fungus (whole); (C) leaves with the fungus (only the part containing the fungus); (D) leaves with the fungus (only the part without the fungus); Sample (E): leaves from recovered *M. glomerata*. Sample (F): leaves from *M. laevigata*. Means followed by the same letter are not statistically different from each other according to Tukey's test, at a 5% significance level.

Even after the plant recovered from the fungal infection, it still had a significantly higher coumarin content (0.68%) than the species *M. laevigata*. The maintenance of this high content of coumarin in the *M. glomerata* species may be also explained by the fact that it is in an organic production environment. Santos *et al*. (2019) showed that the organic culture system favored a greater production of coumarin in *M. glomerata* species than the conventional system. According to the authors, the defense system of *M. glomerata*, when grown in an organic system, produces double the concentration of coumarin. This may be related to the action of organic fertilizers in the metabolism.

In higher plants, systemic acquired resistance is a general response that is triggered after exposure to a pathogen, allowing them to resist diseases. This resistance can be induced by a wide variety of pathogenic agents, especially by those that cause necrosis, such as guaco rust (*Puccinia mikaniae* H. S. Jacks. & Holw). In this *M. glomerata* and *P. mikaniae* pathosystem, the pathogen induced resistance by favoring priming (GOELLNER; CONRATH, 2008; BALMER *et al*., 2015), which represents plant defense response or sensitization, prompting the youngest leaves to increase coumarin levels so as to protect them from the etiologic agent of necrosis.

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4. CONCLUSIONS

As a result of our research, we found high coumarin content in healthy leaves collected from *M. glomerata* stressed by the rust disease. In the specific case of guaco, in which coumarin is deemed to be directly accountable for its therapeutic effects, the fact that high content of coumarin were found in healthy leaves of a plant infected by rust is of paramount importance. Thus, to increase the active principles in a medicinal plant or in a phytotherapeutic product, it is recommended that healthy leaves of stressed plants be used.

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