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Communication

[*Comunicação*]

Caffeine levels in leaves of *Palicourea marcgravii* and *Palicourea hoffmannseggiana* (Rubiaceae)

[*Níveis de cafeína nas folhas de* Palicourea marcgravii *e* Palicourea hoffmannseggiana *(Rubiaceae)*]

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Several plants of the genus *Palicourea* (Rubiaceae Juss.) are known to contain monofluoroacetate (Lee *et al*., 2012; Cook *et al*., 2014), causing lethal poisoning in cattle, water buffaloes, sheep, and goats in Brazil (Oliveira Neto *et al*., 2017; Koether *et al*., 2019). *Palicourea marcgravii* A.St.-Hil. is considered the poisonous plant that causes most deaths in cattle in Brazil because of its wide distribution in the country and its high palatability and toxicity (Ubiali *et al*., 2020). *Palicourea hoffmannseggiana* (Schult.) Borhidi (syn. *Palicourea barbiflora* DC and *Psychotria barbiflora* DC.) is responsible for some cases of poisoning in cattle (Camargo, 1962; Schons, 2011; Pedroza, 2015).

Monofluoroacetate may be detected in human or animal samples by analytical methods such as high-performance liquid chromatographic (HPLC) with UV detection (Minnaar *et al*., 2000) or with fluorescence detection (Xie *et al*., 2007), and liquid (Hamelin *et al*., 2010) or gas chromatography coupled to mass spectrometry (Buchweitz *et al*., 2021). Due to limited access to analytical methodologies for measurement of the monofluoroacetate in animal samples, the diagnosis of *Palicourea* poisoning is often made by determining this toxin in the plant associated with the clinical and pathological changes (Oliveira Neto *et al*., 2017; Koether *et al*., 2019). Thus, determining another phytochemical compound in animal samples that could serve as a marker of plant consumption could facilitate diagnosis.

In a study using thin-layer chromatography (TLC), the presence of caffeine in the leaves of *P. marcgravii* was reported (Górniak *et al*., 1986). Caffeine is a well-known purine alkaloid found in plants such as coffee (*Coffea* spp.), tea plant (*Camellia sinensis*), cola (*Cola nitida*), cocoa (*Theobroma cacao*), maté (*Ilex paraguariensis*), and guaraná (*Paullinia cupana*) (Ashihara & Crozier, 2001). As the detection of caffeine is much simpler than monofluoroacetate, it can even be done by TLC after solid phase extraction (SPE), which has a low implementation cost. Fluoroacetate can also be determined by TLC (Castro da Cunha *et al*., 2012), but the sensitivity of the technique is insufficient to detect residues in animal samples in most cases of poisoning. In this way, caffeine could be used as a marker of the consumption of *Palicourea* in animal samples. However, the levels of caffeine present in *Palicourea* leaves are unknown. Therefore, the present study aimed to determine caffeine levels in leaves of *P. marcgravii* and P. *hoffmannseggiana*.

We collected young leaves of ten specimens of *P. marcgravii* (Fig. 1A) and ten specimens of *P. hoffmannseggiana* (Fig. 1B) in municipalities of Conselheiro Lafaiete and Belo Horizonte, Minas Gerais state, Brazil, respectively, in November 2023. Voucher specimens were deposited in the BHCB herbarium (Universidade Federal de Minas Gerais - UFMG, Belo Horizonte, MG, Brazil) under numbers BHCB216861 and BHCB216860, respectively. Full descriptions of the voucher specimens and digital images of preserved specimens are available online (https://specieslink.net/rec/220/216861 and https://specieslink.net/rec/220/216860).
Furthermore, young leaves of two coffee trees (*Coffea arabica*) cultivated in the municipality of Belo Horizonte were used as a positive control for caffeine.

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Figure 1. Leaves and flowers of plants used in the present study at local of collection. (A) *Palicourea marcgravii* A.St.-Hil. (B) *Palicourea hoffmannseggiana* (Schult.) Borhidi.

Samples of 100 mg of crushed dry leaves were put in a 15mL polypropylene tube, and 5mL of deionized water was added. The mixture was homogenized and incubated in a sonicator bath for 40 minutes. After incubation, the supernatant was separated into another tube, and the leaves were again extracted with 5mL of deionized water in a sonicator bath for 40 minutes. The supernatants obtained from the two extractions were mixed. A volume of 5mL of the combined supernatant extracts was put on a SPE C8 cartridge (CHROMABOND C8, 45µm, 6mL/500mg, Macherey-Nagel, Düren, Germany) previously conditioned with 5mL of water, 5mL of methanol, and 5mL of water. Caffeine was eluted with 2 x 0.5mL of methanol:2% acetic acid in water solution (70:30, v:v). SPE was done using a vacuum manifold (CHROMABOND SPE vacuum manifold for 12 positions, Macherey-Nagel, Düren, Germany). The eluate was transferred to a vial and injected directly into the HPLC.

Chromatographic analyses were performed in an HPLC system (Shimadzu Prominence LC-20A) equipped with a diode array detector (SPD-M20A). Chromatographic separations were carried out on a Welch Welchrom C18 column (4.6 x 100 mm, 5µm). The injection volume was 20µL, and the mobile phase was methanol:water (25:75) at an isocratic flow of 1.0mL/min. The detection wavelength was 274 nm, and the spectra were recorded from 190 to 400 nm. The total chromatographic run time was 12 min. The standard calibration curve (0 to 500 µg/mL) was obtained using pure caffeine, and method validation data is presented in Table 1.

As the leaves of coffee trees are known to contain caffeine (Ashihara *et al*., 1996; Ashihara and Crozier, 2001), they were used as a positive control. In the present study, caffeine levels in coffee leaves were 6.4 and 7.7mg/g (Fig. 2A), which are similar to the values reported by Ashihara *et al*. (1996) (7.1±2.6mg/g). As the concentrations of caffeine are higher in young leaves than in mature ones in coffee trees (Ashihara *et al*., 1996; Ashihara and Crozier, 2001), we used young leaves in the present study. However, no caffeine was detected in all evaluated samples of *P. marcgravii* (Fig. 2B) and *P. hoffmannseggiana* (Fig. 2C) leaves. As the limit of detection of the used analytical method is 0.15µg/mL, caffeine levels in leaves of the two *Palicourea* species are lower than 3.0µg/mg, if present.

Table 1. Performance parameters of the chromatographic method for measurement of caffeine in leaves

Parameter		Result
Limit of detection		0.15 µg/mL
Limit of quantification		0.50 µg/mL
Recovery		
	20 µg/mL	91.1%
	50 µg/mL	88.3%

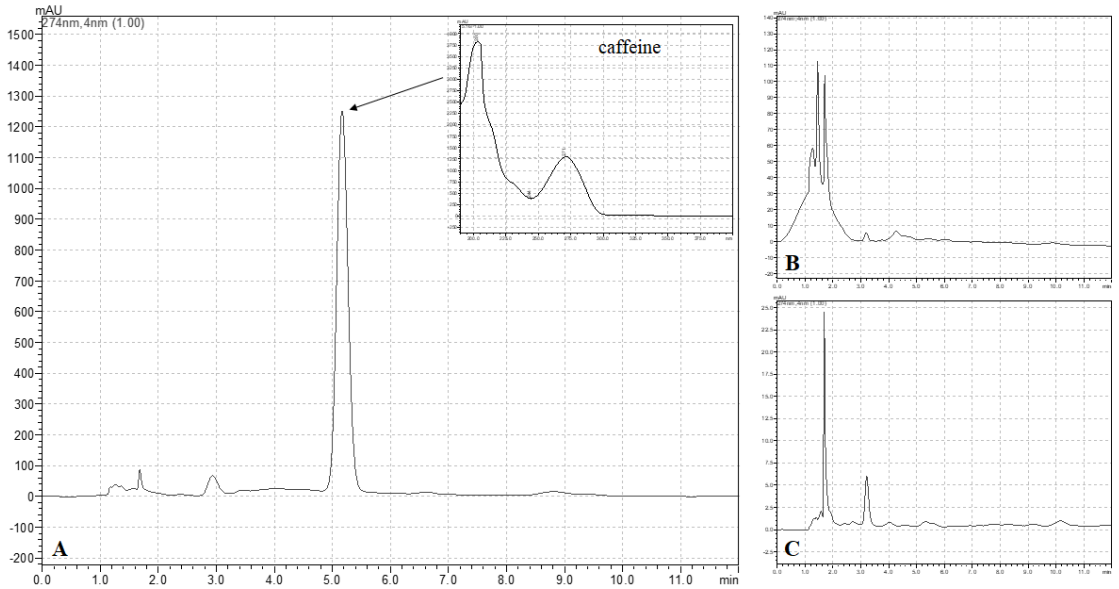

Figure 2. Chromatograms for measurement of caffeine in leaves. (A) Chromatogram of coffee trees (*Coffea arabica*) leaves extract showing caffeine peak at 5.16 min and its UV spectrum. (B) Chromatogram of *Palicourea marcgravii* leaves extract showing no caffeine peak. (C) No caffeine was found in *Palicourea hoffmannseggiana* leaves extract.

The previous report on the presence of caffeine in *P. marcgravii* leaves did not specify the quantity, as a qualitative methodology using TLC was used (Górniak *et al*., 1986). In this study, the SPE extraction of leave samples allowed to concentrate any caffeine in the final extract. Therefore, it is possible that the amount of caffeine present in these leaves should be lower than 3.0µg/g (0.003mg/g). Another possibility is the variation in caffeine levels due to genetic factors or related to the plant's place of growth. Even though there may be *Palicourea* leaves with detectable levels of caffeine, the existence of caffeine-free plants prevents using this alkaloid as an indicator of the plant's consumption.

In summary, no caffeine was detected in the evaluated *P. marcgravii* and *P. hoffmannseggiana* leaves. Therefore, the absence of caffeine in *Palicourea* leaves evaluated in our study makes it impossible to use this substance as a marker for the consumption of these plants.

Keywords: poisonous plants, plant poisoning, toxins, monofluoroacetate, ruminants

RESUMO

A presença de cafeína nas folhas de Palicourea marcgravii *foi relatada anteriormente, e esperava-se que a determinação desse composto em amostras animais servisse como marcador do consumo da planta. Porém, os níveis de cafeína presentes nas folhas de* Palicourea *são desconhecidos. Portanto, o presente*

estudo teve como objetivo determinar os teores de cafeína em folhas de Palicourea marcgravii *e* P. hoffmannseggiana. *Foram coletadas folhas jovens de 10 exemplares de* P. marcgravii *e 10 exemplares de* P. hoffmannseggiana. *Além disso, as folhas jovens de dois cafeeiros* (Coffea arabica) *foram utilizadas como controle positivo para cafeína. Essas amostras foram submetidas à medição de cafeína por cromatografia líquida de alta eficiência (CLAE). Os níveis de cafeína encontrados nas folhas de café foram de 6,4 e 7,7mg/g, mas a cafeína não foi detectada em todas as amostras avaliadas de folhas de* P. marcgravii *e* P. hoffmannseggiana. *Como o limite de detecção do método analítico utilizado é de 0,15µg/mL, os níveis de cafeína nas folhas das duas espécies de* Palicourea *são insignificantes (inferiores a 3,0µg/mg), se essa substância realmente estiver presente. A ausência de cafeína nas folhas de* Palicourea *avaliadas neste estudo impossibilita a utilização dessa substância como marcador do consumo dessas plantas.*

Palavras-chave: plantas venenosas, envenenamento por plantas, toxinas, monofluoroacetato, ruminantes

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