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Preparative HPLC Chromatographic Approach for the Rapid Isolation of Phytotoxins from the Fungus *Curvularia lunata* of *Spigelia anthelmia* Leaves

Abordagem Cromatográfica por CLAE Preparativo para o Rápido Isolamento de Fitotoxinas do Fungo Curvularia lunata *Associado às Folhas de* Spigelia anthelmia

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In the search for natural herbicides from phytopathogenic fungi, the *Curvularia lunata* fungal strain was isolated from a leaf spot of the *Spigelia anthelmia* leaves and then grown in a potato dextrose broth to obtain the initial crude extract. An efficient chromatography method was developed for demonstrating the use of reverse phase isocratic transfer of high-performance liquid chromatography (HPLC) analytical to a preparative scale. The rapid fractionation through preparative HPLC isolated two known compounds, radicinol **1** and radicinin **2**, reported in the literature as phytotoxic agents. Although, these substances did not show phytotoxic activity against *S. anthelmia* leaves when tested at a 100 µg/mL concentration.

Keywords: Phytotoxins; chromatography, preparative HPLC

1. Introduction

The *Spigelia anthelmia* (Loganiaceae), traditionally named "erva-lombrigueira", is a toxic plant popularly used in Brazil due to its anthelmintic properties. Besides the toxicity, their aerial parts are used in the medical field against chronic neuralgic pain and cardiac diseases. The genus *Spigelia* is well recognized as a weed plant, although several species displays bioactivity potential. Furthermore, most of the species do not belong to specified natural habitats, as they can easily be encountered growing in numerous different ecosystems, such as lawns fields, farms, and forests[1]. Regarding the chemical composition, several alkaloids have already been isolated from the *S. anthelmia*, such as spiganthine, actidin, choline, benzoylcholine, and 2,3-dimethylacrolylcholine, in addition to phenol carboxylic acids and flavonoids[1].

Weeds have a significant negative impact on agricultural productivity, and despite recent advances in weed control methods, losses in large crop production remain a topical issue. Thus, control measures, such as soil mobilization techniques and the use of chemical products with strong herbicidal properties, remain in high demand to minimize worldwide crop damage[2]. In addition, the recent decades, the widespread use of chemical herbicides to control innumerable types of invasive plants has led to the emergence of the first resistant weed populations. As a consequence, it leads to the indiscriminate use of hazardous chemical substances, which is responsible for causing severe environmental damage, such as the pollution of groundwater and rivers.[3] In this sense, it is crucial to search for alternative control systems that are more effective, economically viable, and less harmful to the environment, as the desirable characteristics for developing new herbicides.

On this basis, several plant species exhibit leaf damages caused by the presence of various pathogenic fungi, such as the species *Curvularia lunata*.[4] Also, when a filtrate from the culture of the pathogenic foliar fungus is inoculated into the leaves of a new host, it generally causes necrotic patches similar to the natural infection. This suggests that the damage is due to the presence of toxins produced by the inoculated pathogen.[6] Thus, secondary metabolites produced by fungi are a good alternative for searching innovative herbicides with bioactive natural product scaffolds.[5] These chemicals could act through different mechanisms and therefore have a greater chance of success, as they can target different enzymes or interfere with membrane structural layers, and thus achieving better efficacy in controlling weed resistance.[6]

Isolation of secondary metabolites is one of the most important steps in natural products research. Several methods developed to purify metabolites are time-consuming, and most of them are not suitable for large-scale production. Therefore, developing a suitable chromatographic method is a crucial step for the success of phytochemical studies. Herein, we presented the isolation of phytotoxic metabolites from crude fungal extracts of *Curvularia lunata* previously isolated from a leaf spot of *Spigelia anthelmia*. The metabolites were isolated using a rapid and efficient analytical high-performance liquid chromatography (HPLC) method, which was transferred to a preparative HPLC method. A rational way to predict the behavior of the isolated compounds in terms of retention time, resolution, and chromatogram appearance on a preparative scale is demonstrated. The preparative HPLC method allowed the simultaneous purification of radicinol **1** and radicinin **2** (constitutional isomers) isolated from the phytopathogenic fungal strain *Curvularia lunata*. The compounds isolated were then *in vitro* evaluated for their herbicidal potential.

2. Experimental Section

2.1. Instrumentation

The GC-MS analyses were performed using a Shimadzu QP -2010 equipped with a dimethylpolysiloxane capillary column RTX-5MS (30 m x 0.25 mm i.d. x 0.25 µm film thickness), with helium as the carrier gas and a constant flow of 2.9 mL/min. The temperature was first programmed at 70 °C for 2 minutes, then increased to 150 °C at 25 °C/min, then increased to 200 °C at 3 °C/min, and finally increased to 280 °C at 8 °C/min for 10 minutes. The injection volume was 1 µL in split mode, and the injector temperature was 250 °C. MS parameters were: Electron impact ionization mode with (EIMS) 70 eV. Nuclear magnetic resonance (NMR) spectra were recorded using deuterated chloroform (CDCl3) in a Varian Inova 500 spectrometer. Tetramethylsilane (TMS) was used as a reference standard. The HPLC instrument used was a Varian Pro Star equipped with UV-Vis and diode array detectors (DAD). The analytical and preparative columns used were Phenomenex Luna -C18 (250 × 4.60 mm, 5 µm) and (250 × 20 mm, 10 µm), respectively.

2.2. Vegetal material and fungal strain isolation

The *C. lunata* fungal strain was isolated from the necrotic leaves of *S. anthelmia* weed, identified by Professor Ludwig H. Pfenning, and deposited in the mycological collection of the Lavras Laboratory of Systematics and Ecology of Fungi of the Federal University of Lavras, Brazil, under the code CML 3137. The collection of the plant material was registered in SisGen - National System for the Management of Genetic Heritage and Associated Traditional Knowledge (# A6068F5).

The necrotic leaves of *S. anthelmia* were cut into pieces, transferred to Petri dishes containing potato dextrose agar (PDA), and after the initial growth, the fungal colonies were spiked until they reached their pure lineage. Furthermore, the purity of the isolated fungal colonies was confirmed by the appearance of homogeneous patterns on the PDA medium plates. Once a strain was deemed pure, the isolate was transferred to 10-mL flasks containing sterile water, sealed, and kept at 25 °**C** for storage. This procedure resulted in the isolation of the *C. lunata* strain.

2.3. Fungal cultivation and crude extracts

The *C. lunata* isolate was transferred to a Petri dish containing sterilized solid PDA, autoclaved at 120 °C for 15 min, and incubated for seven days. The fungus was then inoculated into potato dextrose broth (PDB), a liquid culture medium (1 L), and kept at 25 °C for approximately 28 days. For ethyl acetate (EtOAc) extract, the broth was filtered off the mycelium and then extracted with EtOAc (3X, 400 mL) followed by further concentration on a rotary evaporator.

2.4. Chemical isolation

The EtOAc extract (58 mg) was diluted in 1.0 mL H_2O/MeOH (1:9, v/v) and applied to previously washed and preconditioned cartridges (Supelco C18, 500 mg) for solid-phase extraction (SPE). The entire eluate was then combined and concentrated. The eluate fraction was filtered and injected into the HPLC instrument. First, a gradient system of the mobile phase consisting of MeOH/H_2O (5% to 100% MeOH in 25 min) was applied at the analytical conditions. The flow rate was 1.0 mL.min-1 on a Phenomenex Luna C18 column (250 × 4.6 mm i.d., 5 µm)**,** the injected volume was 20.0 µL, and the detection wavelength was set to 235 nm. Calculations were then performed to find the best chromatographic condition (Section 3). Analytical chromatography was performed under isocratic conditions at a flow rate of 1.0 mL.min-1 with mobile phase MeOH/H_2O (1:1, v/v) for 25 min and then transferred to preparative HPLC under isocratic conditions with mobile phase MeOH/H_2O (1:1, v/v) at a flow rate of 10.0 mL/min for 25 min on a Phenomenex Luna C18 column (250 × 20 mm i.d., 10 µm). The wavelength of UV detection was set to 235 nm. The injection volume was 200 µL. Compound **1** was isolated at a retention time (rt) of 15.7 min (5.5 mg), and compound **2** at 13.2 min (3.0 mg) was isolated in this procedure.

2.5. Phytotoxicity assay

Phytotoxicity experiments were performed according to the method previously proposed by Mahoney et al. 2003.[7] The method requires the removal of 1 cm diameter slices from healthy *S. anthelmia* leaves. The slices were placed in Petri dishes with moistened philter paper. Then 5 µL of

100 µg/mL was dissolved in MeOH and placed on a single spot in the center of the disc. The presence of necrosis in the plant tissues was assessed after 24 and 48 hours. The MeOH was also used as a negative control.

3. Results and Discussion

Analysis of the ^{1}H NMR spectrum of the crude extract revealed two major compounds, which were then confirmed by the HPLC chromatogram obtained following the methodology described in Section 2.4 (Figure 1). In order to isolate and identify these compounds, the extract was subjected to pre-treatment using a 'clean-up' method (described in Section 2.4) and subjected to analytical and preparative HPLC. Moreover, the equivalence of selectivity between analytical and preparative scale must be carefully considered to achieve an efficient separation using the preparative HPLC scale. Therefore, some rules must be followed, such as selecting the same stationary phase in the analytical and preparative scales and the length of both columns, which should be the same. Concerning the column diameter, 4.6 mm for the analytical scale and 20.0 mm for the preparative scale are suitable for the method's correct implementation. In addition to 10-fold flow correction from analytical to preparative scale. Thus, for the preparative separation, the gradient mode was converted to the isocratic mode using 70% of the MeOH between the retention times of the two peaks in the gradient condition. The calculation was done according to Equation 1, a general formula used in the laboratory:

$$\%MeOH = 0.7[((\Delta\%MeOHgrad)/tgrad \times trpico) + \%IMeOHgrad] \quad (1)$$

Where $\%MeOH$ is the percentage of methanol or acetonitrile in the isocratic method used in the preparative column, $\Delta\%\ MeOHgrad$ indicates the variation in the gradient of the analytical column, and *tgrad* is the gradient time of the analytical column. In addition, *trpico* represents the retention time of the peak to be separated, and *%IMeOHgrad* represents the initial percentage of methanol and/or organic solvent in the analytical gradient.

The analytical flow was modified from 1 mL/min to 10 mL/min, and the injection volume was adjusted from 20 µL to 200 µL to achieve a successful transposition from the analytical to the preparative scale. The resolution (Rs) between the two peaks was set to 1.0, and peak projection was applied to determine the end of peak collection of compound **1** and the beginning of compound **2**. Thus, the preparative separation strategy employed resulted in the isolation of radicinol **1** at 13.2 min (3.0 mg) and radicinin **2** at 15.7 min (5.5 mg) (Figure 2). The applied method showed that the behaviour of compounds **1** and **2** concerning the chromatograms obtained at an analytical scale could be well predicted by using equation 1. This optimization of the transposition method from analytical to preparative scale was done using identical chemical stationary phases and flow correction. The identification of these components was based on comparing their spectroscopic data (^{1}H NMR) with values available in the literature.

Compound **1** was identified as radicinol by comparison with spectrometric data from the literature.[8,9] Mass

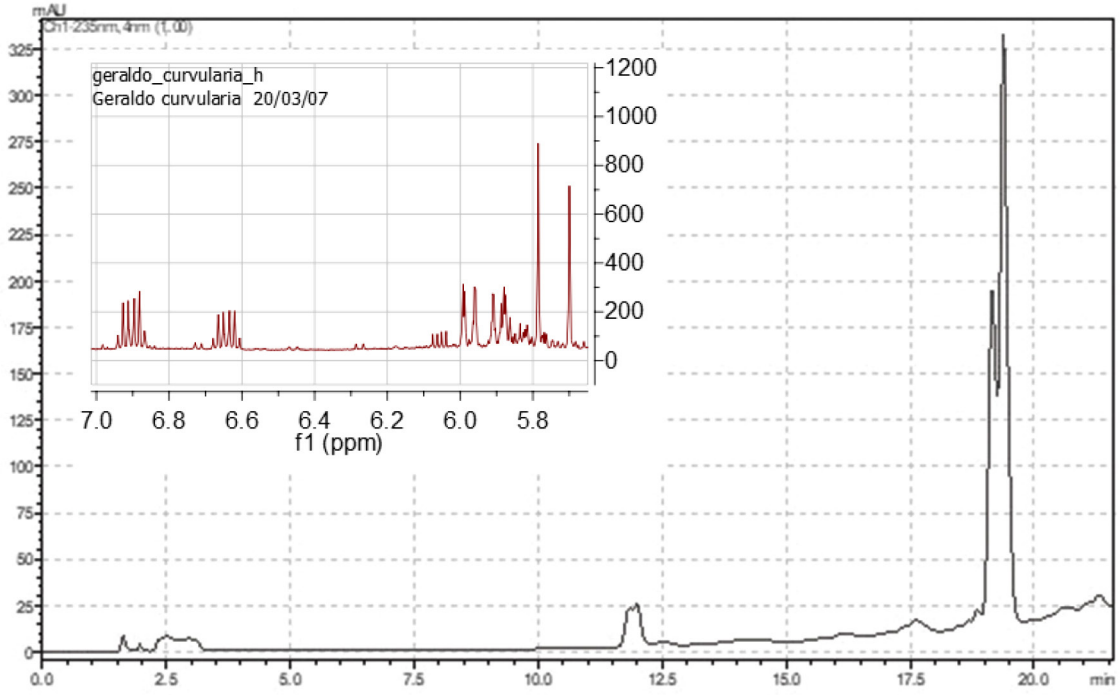

Figure 1. Analytical high-performance liquid chromatography (HPLC) chromatogram and ^{1}H NMR spectrum of crude extract of *Curvularia lunata*

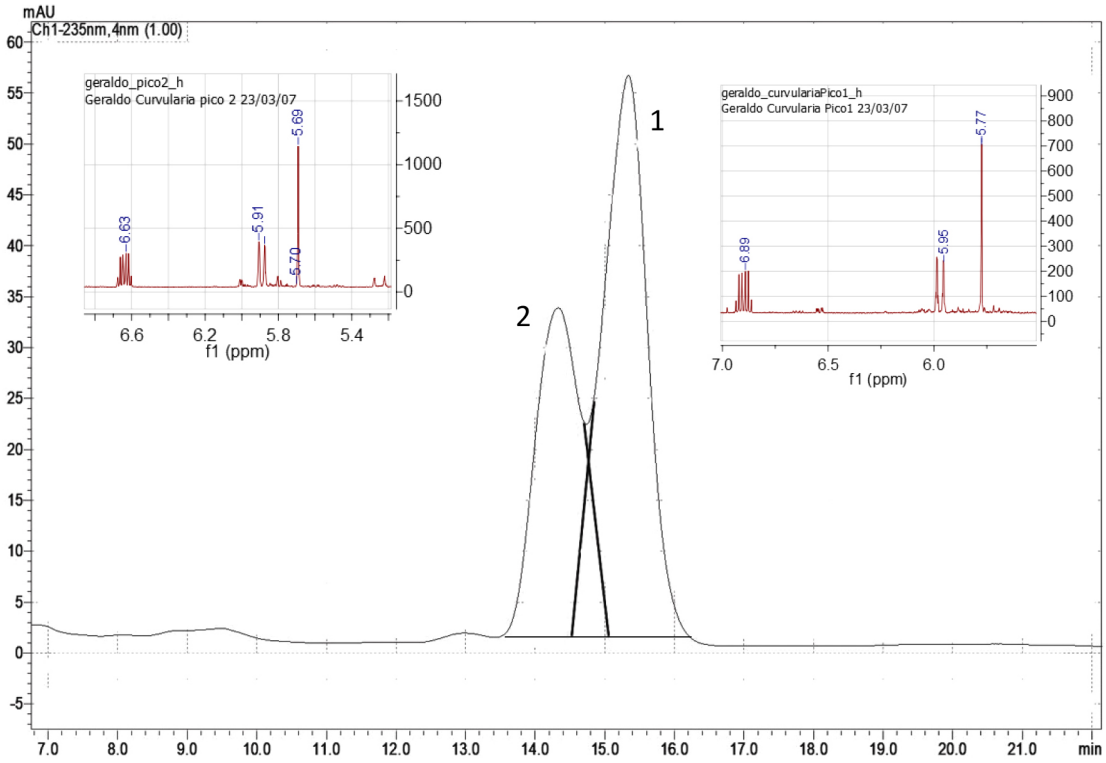

Figure 2. Preparative high-performance liquid chromatography (HPLC) chromatogram of crude extract of *Curvularia lunata* and ^{1}H NMR spectrum of isolated compounds **1** and **2**

spectrometry (EIMS) of 1 revealed a molecular ion $[M]^+$ of 238 (2) along with ions 189 (20), 181(100), 111 (45), and 69 (100), consistent with the molecular formula $C_{12}H_{12}O_5$. The ^{1}H NMR spectrum showed trans- double bond signals at δ_H 5.96 (dq, J = 16.0 and 1.5 Hz) and 6.89 (dq, J = 16.0 and 8.0 Hz) and a methyl group attached to a double bond at δ_H 1.88 (dd, J = 8.0 and 1.5 Hz), indicating possible branching. The ^{1}H NMR spectrum also registered a doublet at δ_H 1.58 (d, J = 6.0 Hz), indicating a methyl group on the carbinolic carbon, and three carbinolic signals at δ_H 3.58 (dd, J = 9.5 and 7.5 Hz), at δ_H 4.04 (dq, J = 9.5 and 6.0 Hz), and at δ_H 4.55 (d, J = 7.5 Hz), forming a system of spins. Moreover, a singlet at δ_H 5 and 6 indicated the presence of an aromatic pyrone. Moreover, the presence of these signals in the spectrum is consistent with literature data for radicinol8. The spatial correlation between H-2, 4.05 (dq, J = 9.5, 6.5 Hz) and H-4, 4.55 (d, J = 7.5) confirmed the structure determination of compound **1** (Figure 3, Table 1). Moreover, the spectrum of compound **2** was similar to **1**, differing only by the absence of a carbinolic H signal. Thus, this information indicated that one of the hydroxyl groups was likely oxidized, so compound **2** was identified as radicinin[8] by comparison with literature data (Figure 3, Table 1).

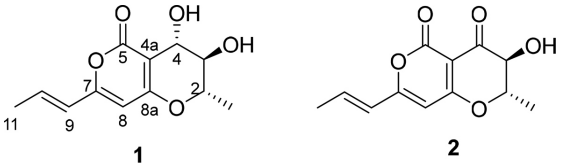

Figure 3. Radicinol **1** and radicinin **2** chemical structures

Table 1. ^{1}H NMR shifts of radicinol **1** and radicinin **2**

	Radicinol 1 $CDCl_3$ 500 MHz Experimental data	**Radicinol[8] $CDCl_3$ 270 MHz Literature data**	**Radicinin[9] $CDCl_3$ 500 MHz Literature data**	**Radicinin 2 $CDCl_3$ 500 MHz Experimental data**
2	4.05 dq (9.5, 6.5)	4.23 dq (8.0 6.5)	4.31 dq (12.5, 6.0)	4.36 dq (12.4, 6.3)
3	3.60 dd (9.5, 7.5)	3.76 dd (8.0, 6.5)	3.9 d (12.5)	3.98 d (12.4)
4	4.55 d (7.5)	4.70 d (6.5)	-	-
8	5.69 brs	5.85 brs	-	-
9	5.90 dq (16.0, 2.0)	6.02 dq (16.2)	5.99 dq (15.5, 1.5)	6.03dq (15.5, 1.8)
10	6.66 dq (16.0, 7.0)	6.72 dq (16.7)	6.93 dq (15.5, 7.0)	6.95 dq (15.5, 7.0)
11	1.84 dd (7.0, 2.0)	1.92 dd (7.2)	1.90 dd (7.0, 1.5)	1.95 dd (7.0, 1.8)
12	1.45 d (6.5)	1.52 d (6.5)	1.59 d (6.0)	1.64 d (6.3)

The *C. lunata* was isolated from necrotic the leaves of *S. anthelmia*, which a similar result was obtained by the phytochemical studies of Amadi and co-workers (2001).[10] Also, the isolated constituents, radicinol **1** and radicinin **2,** were isolated from *C. lunata* in another recent study by Srivastava et al. (2021).[11] However, as far as we know, this is the first study of a preparative HPLC method on isolating the metabolites radicinol **1** and radicinin **2**. The methods previously used in the literature to isolate these compounds have been described as involving multiple chromatographic steps, which has increased working time and probably required more significant amounts of organic solvents for the final isolation and purification of these compounds.

Thus, **1** and **2** were subjected to a phytotoxicity test at 100 µg/ml on the leaves of *S. anthelmia* and showed no phytotoxicity at the concentration tested. Therefore, before harvest no necropsied tissues were observed after the experiment, suggesting that the two isolated compounds were not the main culprits of the necrotic lesions observed on *S. anthelmia* leaves. However, this result suggests that these compounds may have selectivity for phytotoxicity since previous literature studies reported the phytotoxic effect of compound **1** isolated from *Alternaria radicina*, a carrot pathogen.[8,9] The authors reported that radicinol **1** caused leaf scorch and inhibited seed root germination. Phytotoxicity of the compounds was also found in *Coix lachrymal-jobi* by causing necrotic lesions on leaves at a concentration of 0.3 µg/leaf.[8, 9] In addition, compound **2** has already been isolated from other pathogenic fungal species, such as the phytopathogen of *Bipolaris coicis* (*Coix lacryma-jobi*), the *A. chrysanthemi* (*Chrysanthemum*), and the *A. radicina* (carrots). The presence of these compounds is considered relevant to the occurrence of common necrotic lesions in their respective hosts.[8]

4. Conclusion

Although compounds **1** and **2** showed no phytotoxic activity on *S. anthelmia* leaves at 100 µg/ml, previous studies have already reported their phytotoxic potential against other plant species, indicating the possibility that these components act as specific plant herbicides. Moreover, the developed preparative HPLC method proved to be efficient in isolating the similar compounds, radicinol **1** and radicinin **2**, and therefore might also be applied in other laboratories for this purpose.

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