Phenolic compounds and bioactive extract produced by endophytic fungus *Coriolopsis rigida*

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Abstract

Coriolopsis rigida was isolated as an endophytic fungus from the medicinal plant *Cochlospermum regium*, and their production of secondary metabolites has not yet been investigated. Thus, the endophyte was cultivated on rice solid media to evaluate its ability to produce bioactive compounds and then the chloroform extract was obtained. Two phenolic compounds, tyrosol (**1**) and a new natural product *p*-hydroxyphenylacetamide (**2**), were isolated from the extract. The structures of the compounds were elucidated mainly by NMR. The extract showed potent antioxidant activity with an efficient concentration (EC_{50}) value of 0.33 mg mL^{-1}. Additionally, demonstrated allelopathic activity inhibited the seedling growth of *Lactuca sativa* L. and *Raphanus sativus* L. by 63% and 55%, respectively.

Keywords: Antioxidant Activity, Allelopathic Activity, Endophyte, *Cochlospermum regium*.

Content

Table S1: Comparing NMR Spectroscopic Data (^{1}H 600 MHz, ^{13}C 150 MHz, MeOH-$_{d4}$) with literature for compounds **1** and **2**.

	1 MeOH-$_{d4}$		*Ref*(Christophoridou and Dais 2009) DMSO-$_{d6}$		**2** MeOH-$_{d4}$		*Ref*(NMR 2021)	
Position	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1	3.68 (t, 7.2)	63.3	3.53 (t, 10.6)	62.7	-	175.4	-	172.8
2	2.71 (t, 7.2)	38.0	2.61 (tt, 13.6, 7.2)	39.3	3.48 (br s)	40.2	3.24	41.4
3	-	129.5	-	129.5	-	125.8	-	126.5
4 and 8	7.03 (d, 8.3)	129.6	7.02 (d, 8.4)	129.7	7.09 (d, 8.4)	129.9	7.05	129.8
5 and 7	6.71 (d, 8.3)	115.2	6.68 (d, 8.4)	115.0	6.73 (d, 8.4)	114.9	6.69	114.9
6	-	155.4	-	155.5	-	155.8	-	155.7

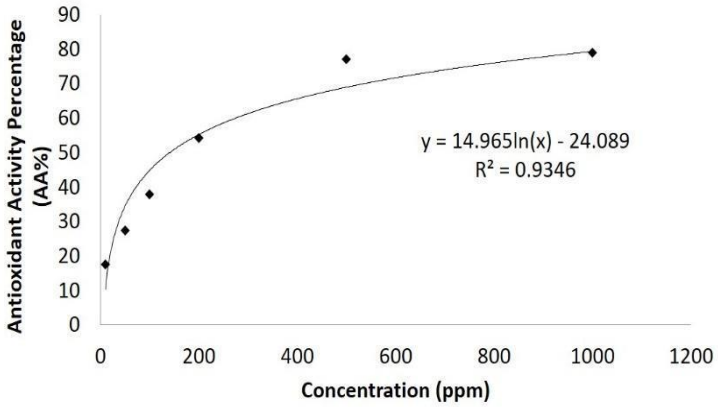

Figure S1: Representation of the Antioxidant Activity Percentage of crude extract determined by DPPH method.

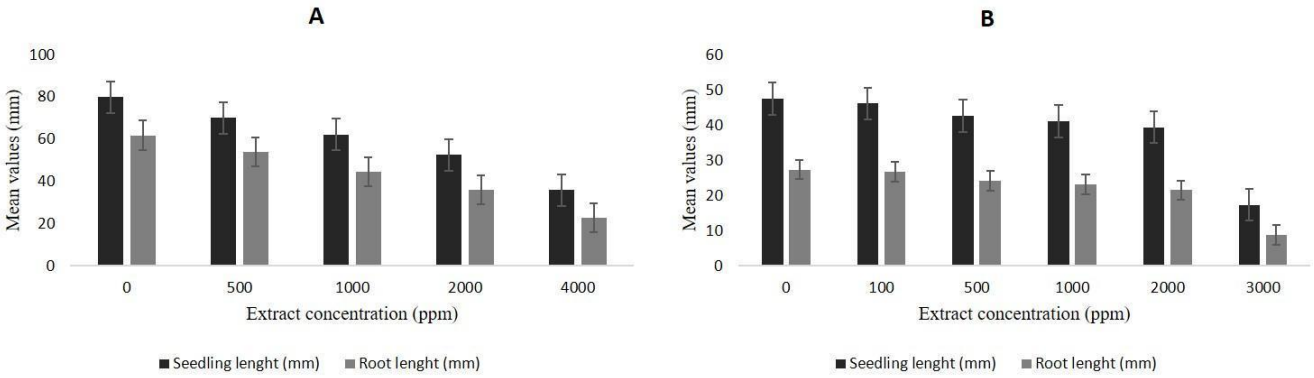

Figure S2: (A) Mean root and seedling length (mm) for *Raphanus sativus* with extract. (B) Mean root and seedling length (mm) for *Lactuca sativa* with chloroform extract.

Experimental

Identification of fungal species

The endophytic fungus isolated from *C. regium* was named Cr-1 until a taxonomic name could be assigned. The fungus was acquired from the fungi collection of the Federal University of Tocantins (Gurupi Campus), and it was identified using the CTAB (cationic hexadecyl trimethyl ammonium bromide) extraction protocol adapted from Venkateswarulu *et al*. (Venkateswarulu et al. 2018; Arruda et al. 2021), which utilized molecular analyses of the genomic DNA extracted from the fungal biomass, obtained after fifteen days of cultivation in Potato Dextrose Broth (PDB). The DNA was amplified using the Internal Transcribed Spacer Region (ITS)-1 and ITS-4 primers in pre-programmed thermocycler (Techne TC-5000), and the amplified product was purified using the Purilink PCR Purification Kit (Invitrogen). The amplified genomic region (ITS) was sequenced using capillary electrophoresis in an ABI3730 device, with POP7 polymer and BigDye v.3.1. The nucleotide sequence was analyzed using a database deposited at National Center for Biotechnology Information (NCBI) and nucleotide BLAST. Geneious® and MEGA® were used for aligning the genetic sequences and building the phylogenetic tree.

Growth of Coriolopsis rigida and preparation of the crude extract

C. rigida was grown on a Petri dish in PDA (Potato Dextrose Agar) medium for 7 days to obtain mycelium agar discs for fermentation. Ten polyethylene bags (500 g) containing 180 g rice and 160 mL distilled water were autoclaved twice for 25 minutes at 121 C. After the growth medium was sterilized, mycelia agar discs of the fungus were inoculated into the solid rice medium and then incubated in a BOD at 25 ± 1 °C for 20 days.

Following the growth period, the biomass was subjected to three consecutive solid/liquid extractions with ethyl acetate (EtOAc, Synth), at intervals of 15 hours. The solvent was then rotaevaporated to reduce the volume of ethyl acetate by a third. The concentrated extract was partitioned three times with distilled water. The acetate fraction was then solubilized in 80 % aqueous methanol (MeOH, Synth) and partitioned three times with hexane (Dinâmica). Methanol and hexane fractions were rotaevaporated to obtain the methanolic crude extract (MeOH extract, 2.3005 g) and hexane crude extracts, respectively. The methanolic crude extract was solubilized in 50 % aqueous methanol and partitioned three times with chloroform ($CHCl_3$, Dinâmica). The chloroform fractions were rotaevaporated to obtain the chloroform crude extract ($CHCl_3$ extract, 1.1458 g).

Evaluation of the presence of secondary metabolic class

A solution containing chloroform extract (7 mg) was prepared with 20 mL of distilled water. 3 mL of the solution was transferred to three test tubes, one was acidified to pH 3 with a 0.5 M hydrochloric acid solution (HCl), and the other two were alkalinized at pH 8.5 and 11 with 0.5 M sodium hydroxide solution (NaOH). The color change occurred in the solutions it was observed. The presence of the secondary metabolite classes flavones, flavonols and xanthones; flavanones; anthocyanins and anthocyanins; chalcones and auronas; flavanols were recorded as positive (+), when there was a change in color, and the absence of color and precipitation as negative (-) according Barbosa et al. (2004; Boaes et al. 2019). For the leucoanthocyanidin, tannins and flavones classes, the tubes containing the extract solution at pH 3 and 11 were heated (2-3 min) and the changes in color were observed.

To evaluate the presence of steroids and triterpenoids, 7 mg of chloroform extract were dissolved in 10 mL of chloroform. Afterwards, it was filtered with cotton and transferred to a dry test tube. 1 mL of acetic anhydride was added and stirred gently, then 3 drops of concentrated sulfuric acid (H_2SO_4) were added. Then it stirred gently again. The change in color was observed. For the anthraquinone class, 7 mg of chloroform extract were dissolved in 5 mL of toluene, 2 mL of 10% NH_4OH solution was added, stirring gently. Observing the appearance of a pink, red or violet color in the aqueous phase, which indicates a positive reaction. For the presence of alkaloids, 10 mg of the crude extract was dissolved in 2 mL of 5% HCl solution. Then, a few drops of Bouchardat reactive were added, the appearance of a reddish orange precipitate indicates the presence of alkaloids (Boaes et al. 2019). The Bouchardat reagent was prepared using 1.0 g iodine (Dinâmica) and 2.0 g potassium iodide (Alphatec) in 100 mL of distilled water.

Isolation and identification of chemical constituents

The $CHCl_3$ extract (0.500 g) was subjected to chromatographic fractionation in a glass column. Sephadex (LH-20) was used as the stationary phase, and a 100:0, 50:50 and 0:100 acetone:methanol gradient was used for elution. A total of 34 fractions were obtained and pooled according to their thin layer chromatography (TLC) profile, using aluminum silica gel 60 F254 precoated plates (Filter-Bio, Nantong, China) and eluted with $CHCl_3$:MeOH (9:1 v/v), the plates were revealed in λ=254 nm and iodine vapors. Fractions 11–14 (22.3 mg) were subjected to preparative TLC using silica gel 60 F254 plates (Filter-Bio) and a 9:1 $CHCl_3$:MeOH elution, which yielded compounds **1**+**2** (3.9 mg). The isolated substances were subjected to nuclear magnetic resonance analysis (^{1}H NMR at 600 MHz, HMBC, HMQC, and COSY) in a Bruker Avance III HD 600 spectrophotometer (Washington, USA) using a non-deuterated residual solvent signal as reference.

Antioxidant assay

Chloroform extract methanol solutions were prepared at six different concentrations (10, 50, 100, 200, 500, and 1000 μg mL^{-1}). A 2.7 mL aliquot of 2,2 diphenyl-1-picrylhydrazyl (DPPH, 40 μg mL^{-1}, Sigma-aldrich) methanol solution was added to 0.3 mL of the chloroform extracts at the different concentrations. The samples were left undisturbed, protected from light for 30 min, and the absorbance was then measured at 517 nm using a UV-340G Gehaka spectrophotometer (São Paulo, Brazil). A mixture of methanol (2.7 mL) and methanolic extracts (0.3 mL) was used as the blank, and a mixture of 2.7 mL of DPPH and 0.3 mL of methanol was used as the negative control. The percentage of antioxidant activity (% AA) was calculated using the equation: $\% AA = 100 \times (A_{control} - A_{sample})/ A_{control}$; where, A_{sample} is the absorbance of the radical in the presence of the extract, and $A_{control}$ is the absorbance of the control. The assay was performed in triplicates and mean absorbance was calculated for each concentration. The efficient concentration (EC_{50}) was calculated using calibration curves that were obtained by plotting the different concentrations with % AA, and it represented the concentration of the sample required to sequester 50 % of the DPPH radicals (Soares et al. 2014).

Germination test (allelopathy)

The allelopathic effect was assessed using lettuce (*Lactuca sativa* L., Feltrin Sementes) and radish (*Raphanus sativus* L., Feltrin Sementes) seed germination tests, with modifications to the protocol described by Gatti *et al*. (2004). Petri dishes were used for the germination tests. They were padded with a sheet of filter paper and moistened with 1 mL of $CHCl_3$ extract methanol solution at concentrations of 100, 500, 1000, 2000, and 3000 mg L^{-1} for lettuce, or 500, 1000, 2000, and 4000 mg L^{-1} for radish. Distilled water was used as the control. After evaporating the solvent (methanol), 15 seeds and 5 mL distilled water were added to each plate. The seeds were incubated at 25 ± 5 C (with a 12-hour photoperiod) for 4 days. The assay was performed in triplicates. After the incubation period, the percentage of germinated seeds was determined, and both the size of the radicle and that of the radicle to the stem were measured. The values were then subjected to variance and regression analysis using SISVAR 5.6 (Ferreira 2014).

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