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Curvularin produced by endophytic *Cochliobolus* sp. G2-20 isolated from *Sapindus saponaria* L. and evaluation of biological activity

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ABSTRACT

Endophytic fungi are organisms that live within intercellular tissues of healthy plants and are able to produce a huge variety of secondary metabolites with a broad spectrum of use. The endophytic fungus *Cochliobolus* sp. G2-20, still little known, and can effectively produce bioactive compounds. It was isolated from *Sapindus saponaria* L., a plant with antimicrobial effects. The aim of this study was to obtain secondary metabolites from the fungus *Cochliobolus* sp. G2-20 isolated from medicinal plant *S. saponaria* L., to characterize the main compounds of crude extract of secondary metabolites and to evaluate their antimicrobial activity. The endophytic fungus was grown in potato dextrose broth for 7 days without agitation at a temperature of 28°C. It was used for the filtration followed by centrifugation to the obtainment of broth without mycelium. The supernatant was partitioned with ethyl acetate resulting in an extract that was fractionated and used to identify metabolite compounds. After chromatographic procedures, the fractions were analyzed by ^{1}H (300 MHz) and ^{13}C (75.5 MHz) nuclear magnetic resonance for identification of the chemical components. Curvularin was identified from fraction D being the main substance produced by *Cochliobolus* sp. G2-20, and had an inhibitory effect on growth of the fungi *Moniliophthora perniciosa*, *Didymella bryoniae*, and *Fusarium solani* forma specialis (f. sp.) *glycines*, and bacteria *Micrococcus luteus, Xanthomonas axonopodis* pv. *phaseoli, Staphylococcus aureus, Escherichia coli*, and *Enterococcus hirae*. The extract of the endophytic fungus *Cochliobolus* sp. showed antibacterial and antifungal activities, thus confirming its potential for biotechnological applications.

INTRODUCTION

There are several interactions types between fungal and plant host as epiphytic, endophytic, pathogenic, and saprophytic (Manamgoda *et al*., 2011). Hence, it could be observed in the *Cochliobolus* species (as so Anamorphs), the endophytic association to various plant hosts as *Triticum aestivum* (Larran *et al*., 2007), *Piptadenia adiantoides* (Campos *et al*., 2008), *Hevea brasiliensis* (Gazis and Chaverria, 2010), *Luehea divaricata* (Bernardi-Wenzel *et al*., 2010), *S. saponaria* (Garcia *et al*., 2012a), and *Piper hispidum* (Orlandelli *et al*., 2012) with isolation frequency less than 10%, which is lower than the emergence of endophytes in others vegetables (Suryanarayanan *et al*., 2002; Thongkantha *et al*., 2008).

The bioactive compounds as antimicrobial metabolites produced by *Cochliobolus* species are poorly described. However, the combination of plant and endophytic microorganisms has different metabolites in response to pathogens than the plant without endophytes or the endophytes alone. Therefore, antibacterial and antifungal activities tests are routinely conducted to identify the biological potential of this endophyte in controlling the known pathogens from plants and humans (Chagas *et al*., 2015).

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†In memoriam.

Endophytic microorganisms are fungi and bacteria that inhabit inter- or intracellular spaces of tissues or plant-organs at least one phase of plant life cycle. These plant–endophyte interactions needs of a complex establishment of metabolic and genetic factors to provide simbiotics and/or mutualistic relationships between organisms (Kusari *et al*., 2012). This balanced interaction could produce several compounds that can be protective effects in the hosts against insects, pathogens, can reduce the herbivory, and others biotic and abiotic factors (Arnold *et al*., 2003; Azevedo *et al*., 2000; Firáková *et al*., 2007; Kaneko *et al*., 2010; Mejía *et al*., 2008; Terhonen *et al*., 2016). Thus, endophytic microorganisms have an economic and ecological potential as exploration sources of bioactive compounds with greater viability when compared with biocompounds from plants (Romão-Dumaresq *et al*., 2016; Schulz *et al*., 2002; Strobel *et al*., 2004; Yan *et al*., 2017).

This study was isolated, identified the chemical components, and evaluated the antifungal and antibacterial activity of metabolic fractions of an extract from *Cochliobolus* sp. G2-20 isolated from *S. saponaria*.

MATERIALS AND METHODS

Fungal material

The endophytic fungus *Cochliobolus* sp., strain G2-20 (ITS1-5,8S-ITS2 Genbank accession number: GQ461566.1) was isolated from inside the leaf tissue of *S. saponaria* by Garcia *et al*. (2012a). This strain belongs to the Laboratory of Microbial Biotechnology, State University of Maringá, Paraná, Brazil.

Fermentation and compound extraction

In accordance with the previous results of *Cochliobolus* sp. G2-20 (Garcia *et al*., 2012a), the endophyte grown in petri dishes with potato-dextrose agar (Himedia, Mumbai, India) at 28°C, approximately 7 days before transferal to broth. To isolate secondary metabolites, the method used by Rukachaisirikul *et al*. (2008) and Flores *et al*. (2013) was followed with some modifications as: the incubation time was 7 days at 28°C under stationary conditions; after remove the cellular debris by centrifugation step, the supernatant was partitioned twice with ethyl acetate at a ratio of 1:1 (100 ml of solvent in 100 ml of fermented medium). Therefore, the solvent was evaporated in the rotary evaporator (Marconi MA 120) at 40°C, resulting in the crude extract.

Purification and identification of chemical constituents

The procedures of chemical analyzes were adapted from Specian *et al*. (2012) and Flores *et al*. (2013). The chemical profile of extract was evaluated by thin layer chromatography (TLC). Thereafter, a liquid chromatographic column was used to separate compounds using a column of silica gel LH-20 as the stationary phase, and methanol (MeOH) as the mobile phase. This process resulted in 80 fractions (numbered 1–80). These fractions were also analyzed by TLC for grouping by similarity resulting in 11 final fractions with different weight: A (6.9 mg), B (4.6 mg), $C_{crystal}$ (7.2 mg), C_s (101.1 mg), D_s (62.5 mg), D (7.7 mg), EF (43.5 mg), H (42.8 mg), I (15.1 mg), and JK (10.2 mg). The $C_{crystal}$, C_s, and D_s terminologies are due to the formation of crystals or of a supernatant when resuspended with ethyl acetate. Fractions $C_{crystal}$, D, EF, and I were chosen for analysis by nuclear magnetic resonance (NMR) of ^{1}H (300 MHz) and ^{13}C (75.5 MHz) for elucidating the chemical structures present in the fractions. These fractions were chosen, because they had greater purity when analyzed by TLC.

Antibacterial assay

Human pathogenic bacteria used were *Micrococcus luteus* (ATCC 9341), *Salmonella typhi* (ATCC 19430), *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), and *Enterococcus hirae* (ATCC 1227). The phytopathogenic bacterium used was *Xanthomonas axonopodis* pv. *phaseoli* from the collection of pathogenic microorganisms of Cenargen – Embrapa Genetic Resources and Biotechnology.

The bacteria grew for 24 hours in Luria–Bertani (LB) broth at 37ºC, except for *X. axonopodis* that grown at 28ºC. The cultures were adjusted to a concentration of 10^6 cells/ml, as described by Sambrook and Russell (2001). The antibacterial assay of subfractions $C_{crystal}$, D, EF, and I was performed in accordance with Flores *et al*., (2013). Tetracycline (Sigma) at 50 µg ml^{-1} was used as positive control.

Antifungal assay

The antifungal test was performed according to Gomes-Figueiredo *et al*. (2007) with some modifications described by Flores *et al*. (2013). The pathogenic fungi used were *Sclerotinia sclerotiorum*, *Fusarium solani* f. sp. *glycines, Moniliophthora perniciosa*, *Colletotrichum gloeosporioides*, and *Didymella bryoniae* which belong to the collection of microorganisms at the Microbial Biotechnology Laboratory, State University of Maringá, Paraná, Brazil, and they were tested the subfractions $C_{crystal}$, D, EF, and I. The negative controls were water and MeOH and the positive control used were the fungicide Derosal plus® (carbendazim + thiram, Bayer) at a dilution of 10^{-1}.

Statistical analysis

All experiments testing the antimicrobial activity were performed in triplicate and analyzed using a completely randomized design. The results were evaluated by the analysis of variance followed by Tukey's ($p < 0.05$) test for comparison of means. Sisvar v.5.3 was used for the analysis (Ferreira, 2011).

RESULTS AND DISCUSSION

Endophytic fungi and bacteria are a new source of bioactive molecules, producing different kinds of metabolites as

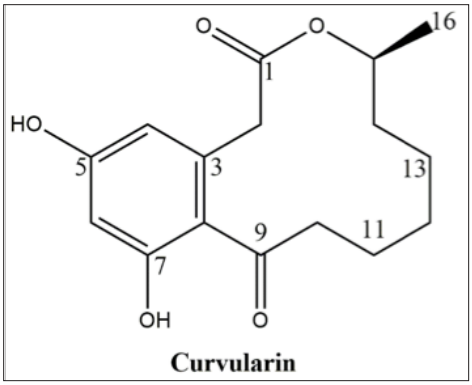

Figure 1. The molecular structure of curvularin, $C_{16}H_{20}O_5$.

potential pharmaceutical drugs as those produced by plants and marine organisms (Firáková *et al*., 2007; Ramasamy *et al*., 2010).

Fraction D revealed a characteristic ^{1}H NMR spectrum. The NMR spectra of ^{1}H and ^{13}C were compared with the published data (Dong *et al*., 2014), allowing the identification of the major compound as curvularin (Fig. 1; Fig. 2A–F; and Table 1).

The curvularin extracted from fraction D present an ^{13}C NMR spectrum of δ 209.91 (C, C-9), 172.90 (C, C-1), 161.41 (C, C-5), 159.69 (C, C-7), 137.40 (C, C-3), 121.01 (C, C-8), 112.38 (CH, C-4), 102.84 (CH, C-6), 73.94 (CH, C-15), 44.79 (CH_2, C-10), 40.86 (CH_2, C-2), 32.13 (CH_2, C-14), 27.87 (CH_2, C-12), 25.80 (CH_2, C-13), 24.00 (CH_2, C-11), and 20.64 (CH_3, CH_3-15), very similar to the compound curvularin (Dong *et al*., 2014; Ye *et al*., 2015) (Table 1).

Curvularin is a macrolide antibiotic produced by a large number of species of fungi from several genera, among which

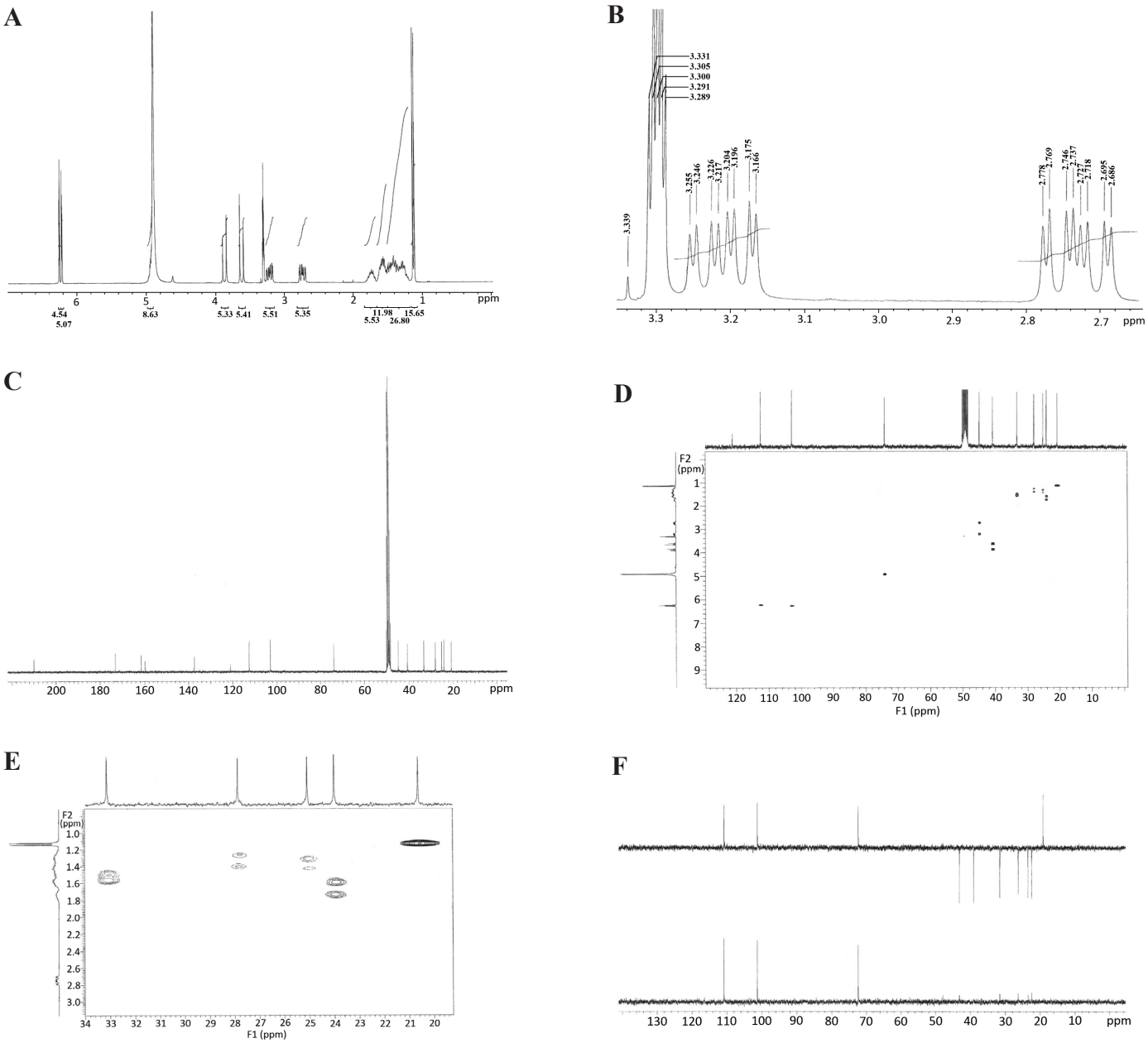

Figure. 2. (A) NMR spectrogram analyses based on curvularin identification in fraction D from an ethyl acetate extract obtained from *Cochliobolus* sp. G2-20: NMR spectrogram of 1H (δCD^3OD; 300MHz); (B) NMR spectrogram analyses based on curvularin identification in fraction D from an ethyl acetate extract obtained from *Cochliobolus* sp. G2-20: expansion of ^{1}H Region 2.50–3.50 ppm; (C) NMR spectrogram analyses based on curvularin identification in fraction D from an ethyl acetate extract obtained from *Cochliobolus* sp. G2-20: spectrum of ^{13}C (δCD^3OD; 75.5 mHz); (D) NMR spectrogram analyses based on curvularin identification in fraction D from an ethyl acetate extract obtained from *Cochliobolus* sp. G2-20: spectrum of heteronuclear single quantum coherence (HSQC) (δCD^3OD); (E) NMR spectrogram analyses based on curvularin identification in fraction D from an ethyl acetate extract obtained from *Cochliobolus* sp. G2-20: expansion of spectrum by HSQC (region of 20–34 ppm in F1); (F) NMR spectrogram analyses based on curvularin identification in fraction D from an ethyl acetate extract obtained from *Cochliobolus* sp. G2-20: spectrum of distortionless enhancement by polarization transfer (DEPT) (δCD^3OD).

Curvularia, *Penicillium*, and *Alternaria*. It has been identified as protein 90 (HSP90) inhibitors. HSP90 is a chaperone that is involved in cell signaling, proliferation, and survival (Aly *et al*., 2010). Thus, curvularin represents a promising treatment for cancer, as well as a potential antibiotic and herbicide (Jiang *et al*., 2007; Tilley and Walker 2002).

Initially, this compound was identified as produced by *Curvularia* sp. and later from *Penicillium steckii* (Vesonder *et al*., 1976). Jiang *et al*. (2007) isolated a similar compound called β-dehydrocurvularin from the endophytic fungus *Curvularia eragrostidis*, of interest due to its potential as biological control of the weed *Digitaria sanguinalis* showing significant inhibitory effect on seed germinations, in addition to causing extensive necrosis in leaves of many known weeds while corn and soybeans remained unaffected; therefore, β-dehydrocurvularin was considered as a natural bioherbicide (Jiang *et al*., 2007).

This is the first report in which curvularin was isolated from *Cochliobolus* endophytic strains. It has already been detected in *Curvularia* and it has been identified in soil *Cochliobolus* (Ghisalberti and Rowland 1993).

Five humans pathogenic and one phytopathogenic bacteria were used to test the antibacterial effect of the extracts produced from *Cochliobolus* sp. G2-20. All metabolic fractions showed some activity against all bacteria. There was significant inhibition of the phytopathogenic bacterium *Xanthomonas axonopodis* pv. *phaseoli* and pathogenic bacteria *S. typhi, M. luteus, S. aureus* and *E. coli* (Table 2).

Garcia *et al*. (2012b) observed a significantly positive inhibitory action of the crude metabolic extracts produced by the endophytic *Cochliobolus* G2-20 against the bacteria *M. luteus* (ATCC 9341), *S. aureus* (ATCC 25923), *E. coli* (ATCC 25922), *S. typhi* (ATCC 19430), and *E. hirae* (ATCC 1227). However, in this study, the metabolic fractions isolated from this endophyte did not have an inhibitory effect on *E. hirae.*

Hormazabal and Piontelli (2009) reports that the metabolite produced by *Curvularia protubera*, an endophyte from Chilean native gymnosperms, had the greatest inhibitory activity on *Bacillus subtilis*, *M. luteus*, and *S. aureus*, with inhibition zone diameters of 12, 9, and 16 mm, respectively. The metabolites tested by these authors do not present activity against *E. coli*, in contrast to our results.

The metabolic fractions $C_{crystal}$, D, EF, and I had significant activity against pathogenic fungi *M. perniciosa*, *F. solani* f. sp. *Glycines*, and *D. bryonae* (Table 3). There was no inhibition of *S. sclerotiorum* and *C. gloeosporioides*; there was mycelial growth throughout the plate in all treatments except the positive control.

According to Cappelletty and Rybak (1996) and Corning (2000), it is important to pay attention to toxins that are not usual, to be present as isolates in the natural environment. Hence, about the assay of fractions of extracts of fungi, the metabolites could have synergistic effects, with higher action than those produced by each metabolite separately. It is important considering divergent effect of fungal extracts and its metabolic fractions. *Cochliobolus* species

Table 1. ^{1}H (300 MHz) and ^{13}C (75.5 MHz) NRM (δCD_3OD) spectrum data of fraction D and of curvularin (Dong *et al*., 2014).

		Fraction D		Curvularin	
H/C	DEPT	δ_H (mult.; J in Hz)	δ_C	δ_H (mult.; J in Hz)	δ_C
1	C_0	-	172.9	-	171.5
2	CH_2	3.86 (d; 15.6 Hz) 3.61 (d; 15.9 Hz)	40.86	3.62 (d; 15,8 Hz) 3.62 (d; 15.8 Hz)	39.1
3	C_0	-	137.40	-	135.8
4	CH	6.10 (d; 2.1 Hz)	112.38	6.24 (d; 2.2 Hz)	110.9
5	C_0	-	161.41	-	159,7
6	CH	6.24 (d; 2.4 Hz)	102.84	6.69 (d; 2.2 Hz)	101.4
7	C_0	-	159.69	-	158.2
8	C_0	-	121.01	-	119,5
9	C_0	-	209.91	-	208.5
10	CH_2	1.58 (m) 1.45 (m)	44.79		43.3
11	CH_2	3.23 (dd; 2.7 Hz) 3.18 (dd; 2.5 Hz)	24.00	3.18 (ddd; 2.7, 9.0, 15.3) 2,75 (ddd; 2.6, 9.6; 15.2)	22.5
12	CH_2	1.30 (m) 1.20 (m)	27.87	1.74 (m) 1.55 (m)	26.3
13	CH_2	1.42 (m) 1.28 (m)	25.80	1.32 (m) 1.44 (m)	23.5
14	CH_2	1.56 (m) 1.46 (m)	33.13	1.59 (m) 1.45 (m)	31.6
15	CH	4.82 (m)	73.94	4.91 (m)	72.2
CH_3-15	CH_3	1.12 (d; 6.3 Hz)	20.64	1.12 (d; 6.4 Hz)	19.1

Table 2. Size of inhibition zone (mm) generated by fractions of metabolites of endophytic *Cochliobolus* sp. G2-20 on phytopathogenic and human pathogenic bacteria. The experiment was performed in triplicate. Data are mean ± standard deviation.

Treatment	*X. ax.*pv. *phaseoli*	*S. typhi*	*M. luteus*	*S.aureus*	*E.coli*	*E.hirae*
Fraction $C_{crystal}$	0.00 ± 0.00c*	1.17 ± 2.02bc*	1.25 ± 2.17b*	1.17 ± 2.02bc*	1.17 ± 2.02c*	0.00 ± 0.00b*
Fraction I	1.33 ± 2.31c*	4.83 ± 3.09b	1.17 ± 1.04b*	0.00 ± 0.00c*	0.00 ± 0.00c*	0.00 ± 0.00b*
Fraction EF	3.08 ± 2.74bc	0.00 ± 0.00c*	2.58 ± 0.00b	4.00 ± 1.73b	0.00 ± 0.00c*	0.92 ± 1.59b*
Fraction D	6.75 ± 0.87b	0.00 ± 0.00c*	2.83 ± 0.00b	1.33 ± 1.38bc*	6.80 ± 2.02b	2.42 ± 2.27b
Tetracycline	25.41 ± 1.46a	36.75 ± 1.75a	34.67 ± 0.00a	34.92 ± 0.29a	35.50 ± 0.25a	33.75 ± 1.25a
Water	0.00 ± 0.00c	0.00 ± 0.00c	0.00 ± 0.00c	0.00 ± 0.00c	0.00 ± 0.00c	0.00 ± 0.00b
MeOH	0.00 ± 0.00c	0.00 ± 0.00c	0.00 ± 0.00c	0.00 ± 0.00c	0.00 ± 0.00c	0.00 ± 0.00b

Means followed by different letters (a, b, and c) in the column are significantly different (Tukey's test; $p < 0.05$). Water and MeOH: negative control, Tetracycline: positive control. *= *p*-value > 0.05 compared with negative controls.

Table 3. Antifungal activity of metabolites of the endophyte *Cochliobolus* sp. G2-20. Data are the average mycelial growth (mm) measured on day 7 of fungal pathogens treated with fractions of metabolites. The experiment was performed in triplicate. Data are mean ± standard deviation.

Treatment	*C. gloeosporiodes*	*M. perniciosa*	*D. bryonae*	*F. solani* f. sp. *glycines*	*S. sclerotiorum*
Water	4.50 ± 0.66a	5.00 ± 0.00a	4.37 ± 0.14a	4.10 ± 0.10a	4.63 ± 0.15a
MeOH	4.40 ± 0.60a	5.00 ± 0.00a	4.30 ± 0.00a	4.20 ± 0.00a	4.60 ± 0.00a
Derosal	3.10 ± 0.25b	3.47 ± 0.12d	3.00 ± 0.10b	2.47 ± 0.15c	3.00 ± 0.10b
Fraction D	4.40 ± 0.36a$^{\#}$	4.40 ± 0.17bc	2.97 ± 1.14b*$^{\#\%}$	2.73 ± 0.64bc*$^{\#\%}$	4.50 ± 0.10a*$^{\#}$
Fraction EF	4.23 ± 0.25a$^{\#}$	4.23 ± 0.07c	1.63 ± 0.07c	3.03 ± 0.25bc	4.50 ± 0.00a*$^{\#}$
Fraction I	4.50 ± 0.00a*$^{\#}$	4.40 ± 0.17bc	1.33 ± 0.07c	2.83 ± 0.12bc	4.60 ± 0.10a*$^{\#}$
Fraction $C_{crystal}$	4.47 ± 0.06a*$^{\#}$	4.50 ± 0.00b	1.57 ± 0.15c	2.70 ± 0.00c$^{\%}$	4.57 ± 0.06a*$^{\#}$
CV(%)	26.51	1.99	15.84	9.14	2.55

Means followed by different letters (a, b, and c) in a column are significantly different (Tukey's test; $p < 0.05$). Water and MeOH: negative control, Derosal: positive control. CV(%): coefficient of variation. *= *p*-value > 0.05 compared with Water control, $^{\#}$= *p*-value > 0.05 compared to MeOH control, $^{\%}$= *p*-value > 0.05 compared with Derosal control.

have been used for the biological control of plague grasses, such as crabgrass. Where the fungus was applied, grasses displayed typical disease symptoms like death or reduced growth. This fungus can be applied directly to the leaves, and the inclusion of additives such as glucose-based surfactants or starch may improve the action of the fungus (Tilley and Walker 2002).

CONCLUSION

The endophytic fungus *Cochliobolus* sp. G2-20, isolated from leaves of the plant *S. saponaria* produces metabolites, largely curvularin, a plant and fungal compound also produced by endophytic fungi of the genus *Curvularia*. The fraction C and D that contained curvularin has showed antifungal and antibacterial properties.

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