

## Full Length Research Paper

## Secondary metabolites from endophytic fungus from *Lippia sidoides* Cham.

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Received 14 March, 2017; Accepted 18 April, 2017

*Lippia sidoides* Cham. (Verbenaceae) is a species native to the Brazilian northeast, widely used in popular medicine. Its leaves were used for the isolation of endophytic fungi and extraction of metabolites. Among them, three were selected according to fungitoxicity tests against the maize phytopathogenic fungus, *Curvularia lunata* (Wakker). However, the objective of this study was to identify the role of *L. sidoides* extracts associated with their endophytic fungi, necessary to reduce excess of fungicides applied on the maize crop. Metabolites were evaluated for antioxidant activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH), phenols, total flavonoids and one of it endophytic fungus were evaluated for synergism (*Verticillium* sp. and plant extracts). The endophytic fungi and plant extracts evaluated for phenolic content ranged from  $0.29 \pm 0.05$  to  $96.94 \pm 11.86$  mgEAG/g, the content of flavonoids from  $14.31 \pm 1.56$  to  $192.33 \pm 4.58$  mgER/g, and antioxidant activity could only be observed for the plant extract with  $EC_{50} 81 \pm 0.3\%$ . The secondary metabolites identified by HPLC in the plant extract were catechin, quercetin, gallic acid and naringin. Naringenin, catechin, epigallocatechin gallate and quercetin were identified in the extract of the fungi viz. *Verticillium* sp. and *Fusarium* sp. Synergistic analysis between a 1:1 proportion of plant and fungal extracts has shown more efficient (79.0%) inhibition of *C. lunata*. Thus, alternative control of phytopathogenic fungi can be accomplished using plant extracts associated with their endophytic fungi, reducing the excess of fungicides applied on the maize crop.

**Key words:** *Curvularia lunata*, endophytic fungal, HPLC, extract, *Verticillium* sp., *Fusarium* sp., *Colletotrichum* sp.

### INTRODUCTION

Endophytic fungi isolated from plants belonging to tropical regions produce more active secondary metabolites than those from temperate regions, because they are exposed to an environment of greater biodiversity (Bhardwaj et al., 2015). These fungi are recognized as

important new sources of bioactive compounds for applications in agriculture, medicine, and the food industry (Yadav et al., 2014b; Sadananda et al., 2011).

Over time, some endophytic fungi have developed the ability to produce the same or similar bioactive

substances to those produced by host plants (Sharma et al., 2016). This lends a great advantage to the study of the relationships between endophytes and their host plants (Fouda et al., 2015). Nevertheless, most of the time, these valuable bioactive compounds are scarce in nature; therefore, the development of a substitute approach for their efficient production is warranted (Kusari et al., 2013).

More recent estimates based on high-throughput sequencing methods suggest that as many as 5.1 million fungal species exist (Taylor et al., 2014). The estimate of known species has almost tripled in the period between 1943 (38000 described species) and the present, amounting to an increase of more than 60000 described species (Blackwell, 2011). Accordingly, endophytes are good sources of genetic diversity and new species belonging to a large group of fungi that colonize tissues of healthy plants without causing apparent symptoms (Fatima et al., 2016). They protect plants against herbivores, insect attack, or invading pathogens by entering this mutually beneficial relationship, according to host habitat conditions (Bhardwaj et al., 2015).

Bioactive products that can be produced by endophytes can be classified as saponins, phenols, flavonoids, steroids, tannins, alkaloids, anthraquinones, terpenoids, and others (Li et al., 2015). These compounds defend the plant against phytopathogenic fungi. For example, flavonoids are well-known substances, and their production involves a variety of processes such as cell signaling, plant growth, and reproduction (Garrido-Arandia et al., 2016).

*Lippia sidoides* Cham. (Verbenaceae), also known as "rosemary pepper" is an aromatic plant of popular medicinal use found in the Caatinga region of the Brazilian Northeast (Santos et al., 2016). It presents insecticidal activity against *Tenebrio molitor* (Lima et al., 2011) and larvae of *Aedes aegypti* (Lima et al., 2013) and acaricide against *Tetranychus urticae* Koch (Soares et al., 2016). When tested against different pathogenic bacteria, such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli* (Ratnaweera et al., 2015) and different fungi and yeasts, including *Candida albicans* (Premjanu et al., 2016), it demonstrated a strong antimicrobial action. Thus, compounds produced by endophytic fungi of plants with recognized biological activity for biotechnological purposes can be applied in the alternative control of urban and agricultural pests (Dutta et al., 2014), in place of the toxic chemicals sold on the commercial market.

On the other hand, the *Curvularia* spot, a disease caused by the fungus *Curvularia lunata* (Wakker) Boed., has a high incidence in maize (*Zea mays* L.). Since the

1990s, it has caused economic loss in maize crop productivity in China (Gao et al., 2015) and has been progressing in recent years in Brazil (Assunção et al., 2006). The pathogen mainly infects maize leaves, leaf sheaths, and husks and causes soaked or yellow necrotic spots in the early stages, which then expand to round, oval, spindle-shaped, or strip lesions (Hou et al., 2013). The green leaf area of maize is considered the main source of photoassimilates for the plant (Akram et al., 2014) and, according to Liu et al. (2016), a loss in this source may reflect the development of the plant and the production of grains.

In this paper, the objective was the isolation and identification of the endophytic fungus from *L. sidoides*. To this end, antioxidant activity, phenols and total flavonoids, and the evaluated metabolites were identified by HPLC. The extracts of endophytic fungi and plants were evaluated by the selective test of fungitoxicity and synergism against *C. lunata*.

## MATERIALS AND METHODS

### Collection of the vegetal material and preparation of the vegetal extract

*L. sidoides* from Ceará was collected in Gurupi (11°44'48"S latitude, 49°02'55" Longitude O), Tocantins, Brazil. It was then identified and deposited at the Herbarium of the Federal University of São João Del Rei under the reference number 8303. The plant material was sent to the Laboratory Integrated Pest Management of the Gurupi Campus– UFT, where the extracts were dried and obtained according to methodology of Al-Marby et al. (2016). The leaves were dried in the shade at room temperature, subsequently cut and subjected to extraction with cold solvent. Thirty grams of plant were used for 1.5 L of methanol in each extraction for a period of 7 days. After this period, the mixture was filtered and evaporated under reduced pressure to obtain the extracts.

### Cultures of endophytic fungi

For isolation, fresh leaves of *L. sidoides* were surface washed in tap water and soap to remove impurities. As described by Banhos et al. (2014), leaves were successively immersed in 70% alcohol (1 min), 2.5% sodium hypochlorite (4 min), and 70% alcohol (30 s), followed by washing with autoclaved distilled water (6 min) and drying on sterile filter paper. After washing, 50 µL of the distilled water used in the asepsis of the plant material was removed, then this volume was added (inoculated) in a Petri dish with PDA culture medium (potato, dextrose, and agar) in order to verify the efficiency of the asepsis process. The leaves were fragmented and inoculated into 9-cm diameter Petri dishes containing PDA culture medium. To the culture medium were added 50 µg/mL tetracycline or 100 µg/mL chloramphenicol (antibiotics). For the isolation of pure cultures, sequential peaks were made in Petri dishes containing the same culture medium, using the streaking technique. Subsequently, they were stored in tubes for centrifugation with glycerol at -80°C for the

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preservation of pure cultures. For tentative identification, microscopic slides of each endophyte were prepared (Kohlmeyer and Kohlmeyer, 1972) and examined under a light microscope.

### ***Curvularia lunata* strain**

*C. lunata* fungus was obtained from Federal University of Tocantins, in our laboratory culture collection, previously identified based on morphological and molecular characters. The initial isolation of the fungus was obtained by growth on PDA medium (39 g L<sup>-1</sup>) supplemented with ampicillin (500 mg L<sup>-1</sup>) for 7 days. Petri dishes were monitored daily and fungal colonies that did not present contaminants were picked and transferred onto new plates with the same culture medium.

### **Extraction of secondary metabolites of endophytic fungi**

Two 7-mm diameter mycelial agar discs of the endophytes were inoculated into 200 mL of Czapek fermentation medium to which was added 100 µg mL<sup>-1</sup> chloramphenicol (Plotnikov et al., 2016). After 21 days of incubation under constant stirring (120 rpm) at 28 ± 1°C, the culture fluids were separated from the mycelial masses by vacuum filtration, and the extract was obtained following the methodology of Dhankhar et al. (2012). For extractions, organic solvents such as hexane and ethyl acetate were used in order to obtain the largest number of active substances.

### **Determination of total phenolic compounds**

The content of phenolic compounds of both the plant extract and three extracts of the endophytic fungi with the best results for fungitoxic activity was determined by the Folin-Ciocalteu method, according to Sánchez-Rangel et al. (2013) with modifications, where gallic acid was used as the standard. Methanol solutions of the extracts were prepared at a concentration of 1 mg mL<sup>-1</sup>. Then, in 15 mL vials, 0.5 mL of the extract, 5 mL of Milli-Q water, 0.5 mL of Folin-Ciocalteu reagent (1 M) and 0.5 mL of sodium carbonate were added (20%, m/v). The blank was prepared by placing 0.5 mL of distilled water in place of the Folin-Ciocalteu reagent. An analytical curve was also prepared for the standard gallic acid reagent at concentrations from 10 to 100 µg mL<sup>-1</sup>. For the blank (reference solution) of the curve, 50 µL of distilled water was added instead of gallic acid. All tubes were homogenized with the aid of the test tube agitator. All reactions were performed in triplicate and kept under dark incubation for 1 h. The readings were performed in a spectrophotometer (BioSpectro model SP-220) at 765 nm (Zhou et al., 2009). The results obtained by spectrophotometric analyses of total phenols were expressed as milligram equivalents of gallic acid per gram of extract (mgEAG/g).

### **Determination of total flavonoids**

The determination of the total flavonoid content was performed according to the methodology developed by Da Silva et al. (2015). The solutions of the plant extract and the three extracts of endophytic fungi with the best results in the fungitoxic activity were prepared using 1 mg mL<sup>-1</sup> of methanol as the solvent. Then, in a 15-mL vial filled with aluminum foil, 0.5 mL of the extract, 0.5 mL of aqueous acetic acid (60%, v/v), 2 mL of pyridine methanolic solution (20%, v/v), 1 mL of aluminum chloride (5%, w/v) and 6 mL of Milli-Q water were combined. The blank (reference solution) was made with all the above-mentioned reagents replacing the aluminum chloride with methanol. All reactions were carried out in triplicate and homogenized in a tube shaker. The reactions were

maintained for 1 h in the dark, and the absorbances were measured at 420 nm in a spectrophotometer (BioSpectro model SP-220). The total flavonoid contents were determined by means of an analytical curve containing standards at concentrations of 1 to 10 µg mL<sup>-1</sup> of rutin, and the results were expressed in microgram equivalents of rutin per milligram of dry extract (µgER/mg).

### **Antioxidant activity**

The antioxidant capacity was measured by the DPPH method, as described by Soares et al. (2014) with modifications. The extracts obtained were prepared using methanol as the solvent at concentrations of 20 to 180 µg mL<sup>-1</sup>, w/v. In triplicate, 0.5 mL of the extract was added to a methanolic solution of DPPH (3 mL at 40 µg mL<sup>-1</sup>, w/v). White (reference solution) was made by replacing the DPPH with methanol in each reaction. All test tubes containing the reactions were stirred and held in the dark for 30 min. An analytical curve was performed for this activity using standard (positive controls) ascorbic acid and rutin (20-180 µg mL<sup>-1</sup>, w/v) also adding it to a methanolic solution of DPPH. Absorbance readings were taken at 517 nm in a spectrophotometer (BioSpectro model SP-220). The absorbance of the DPPH solution at 40 µg mL<sup>-1</sup> was also measured and used as a negative control. The antioxidant activity of free radical removal was expressed as the percentage of inhibition determined by the equation

$$\%AA = \left[ \frac{ABS_{nc} - (ABS_{sample} - ABS_{white})}{ABS_{nc}} \right] \times 100,$$

Where %AA is the percentage of antioxidant activity; ABS<sub>nc</sub> is the absorbance of the negative control; ABS<sub>sample</sub> is the absorbance of the sample; and ABS<sub>white</sub> is the absorbance of white.

Using calibration curves obtained by plotting the different concentrations in relation to the %AAs, the efficient concentration, the amount of sample required to decrease the initial concentration of DPPH by 50% (EC 50), which was expressed in µg mL<sup>-1</sup>.

### **Analysis by high-performance liquid chromatography (HPLC)**

High-performance liquid chromatography (HPLC) was developed in the Laboratory of Scientific Instrumentation, Federal University of Tocantins on a Shimadzu® LC-10 Series Avp Chromatograph, equipped with a pump (LC-10AD), degasser (DGU-14A), UV-VIS detector (SPD-10A) (CTO-10A), Rheodyne hand injector (20 µL loop), and CLASS integrator (LC-10A). Separation was performed by the gradient elution method using a Phenomenex Luna C18 5µ (2) (250 × 4.6 mm) reverse-phase column and pre-column Phenomenex C18 (4 × 3.0 mm) filled with material similar to that in the main column. Mobile phase A was 0.1% phosphoric acid in Milli-Q water and mobile phase B was 0.1% phosphoric acid in Milli-Q water/acetonitrile/methanol (54:35:11). Program gradient: 0 to 0.01 min, 0% B; 0.01-5 min, 0% B, 5-10 min, 30% B, 10-20 min 40% B, 20-29 min, 40% B, 29-30 min 50% B, 30-50 min 100% B, 50-80 min, 100% B. Flow rate: 1 ml/min; temperature: 22°C. UV detection was done at 280 nm. The compounds were identified by comparing the retention times of samples with the authentic standards, such as gallic acid, catechin, epigallocatechin gallate, naringin, quercetin, and naringenin (Sigma®). The quantities of the compounds were expressed in micrograms per milligram of extract (µg/mg) by correlating the area of the analyte with the calibration curve of standards built in concentrations of 4.5 to 18 µg mL<sup>-1</sup>.

### **Selection according to fungitoxicity against *C. lunata***

In order to select the endophytic fungi with the best antibiotic

**Table 1.** Mycelial inhibition screening of *Lippia sidoides* Cham. endophytic fungi against the plant pathogen *Curvularia lunata* Wakker.

Fungus	Extract from ethyl acetate	Extract from hexane
LS-1 (u.f.)	+	+++
Colletotrichum sp.	+	=
Verticillium sp.	+++	=
Alternaria sp.	+	=
LS-5 (u.f.)	+	+
LS-6 (u.f.)	+++	=
LS-7 (u.f.)	++	=
LS-8 (u.f.)	=	++
Colletotrichum sp.	+++	=
Phomopsis sp.	=	+
Fusarium sp.	=	++
Fusarium sp. 1	+++	=
Fusarium sp. 2	+++	=
LS-14 (u.f.)	+++	=
LS-15 (u.f.)	=	++

LS, Initials of the *L. sidoides* plant to identify the endophytic fungus in the isolation. u.f., Morphologically unidentified fungus. They were visually classified as mycelial diameter equal to the control (=), smaller than the control (+), much smaller than the control (++), and much, much smaller than the control (+++).

activity, the *in vitro* mycelial growth inhibition capacity of the phytopathogenic *C. lunata* fungus was previously isolated and identified. According to Carotenuto et al. (2015) with modifications, the evaluation of inhibition of mycelial growth of the phytopathogen was carried out by adding to the surface of the 9 cm diameter Petri dish containing approximately 20 ml of BDA culture medium already solidified, 100  $\mu$ L of the extracts of the endophytic fungi. The extracts were diluted in Tween 80 and water. Discs 7 mm in diameter containing the mycelia of phytopathogenic fungus from pure colonies with approximately twelve days of growth in PDA were placed in the center of the plates.

Plates were incubated in biochemical oxygen demand (BOD) at  $25 \pm 1^\circ\text{C}$  and a photoperiod of 12 h of light and 12 h of darkness. After growth, the presence or absence of zones of inhibition was observed.

The bioassays were performed in triplicate, and a plate containing only one disk of micelium agar of the phytopathogen without extract served as a positive control.

### Synergism evaluation

After selection of endophytic fungi by the *in vitro* diffusible metabolite test according to the methodology to Mutawila et al. (2015), only one sample was chosen for synergism evaluation. The extract of endophytic fungus was used together with extract of *L. sidoides*. In accordance with the methodology of Tadtong et al. (2014), five different proportions of the two substances were used: 0:1; 1:3; 1:1; 3:1, and 1:0. Thus, the concentration of each substance was fixed at  $7500 \mu\text{g mL}^{-1}$ . In Petri dishes with already solidified BDA culture medium were placed 100  $\mu$ L of each proportion, scattered with a handle Drigalsky. Then, a mycelium-agar disk of *C. lunata* fungus was placed in the center of the plaque.

An evaluation was performed after 10 days of incubation, and the mycelial diameter was measured for comparison with the control (water and Tween 80).

### Statistical analysis

The data obtained in the experiments were subjected to statistical analysis, such as analysis of variance (ANOVA) and Tukey's test, with a significance level of  $\alpha = 0.05$ , and linear regression. Calculations were performed using Sigmaplot® 12.0 and Assisat® software.

## RESULTS

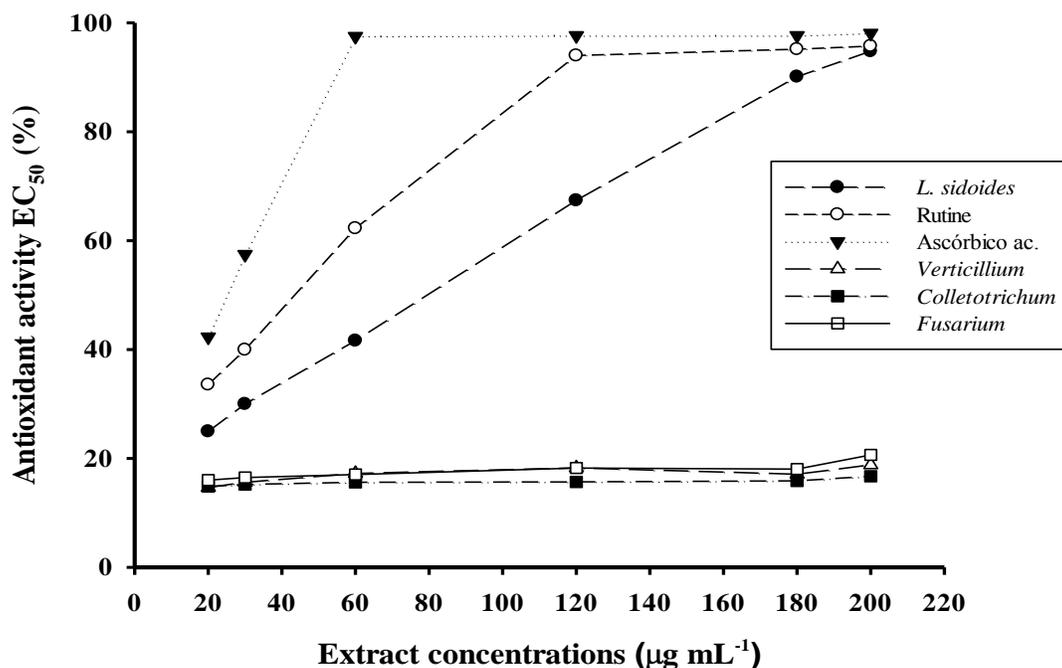
Fifteen morphologically distinct endophytic fungi were obtained from the fresh leaf fragments of *L. sidoides*. Biological tests were carried out using extracts made from both hexane and ethyl acetate against the phytopathogenic fungus of maize plants, *C. lunata*. The results of the selection according to fungitoxicity are presented in Table 1. Three extracts of different endophytes extracted with ethyl acetate were selected for the analysis of secondary metabolite synthesis. This analysis included the methanolic extract of *L. sidoides* leaves. These fungi were analyzed morphologically and identified at the genus level as *Verticillium* sp., *Colletotrichum* sp., and *Fusarium* sp. 1 (Table 1).

The three fungi with fungistatic capacity against *C. lunata* in this work were evaluated in order to identify the antioxidative profile of the respective fungal extracts. The results obtained by spectrophotometric analyzes of total phenols were expressed as milligrams of gallic acid equivalents per gram of extract (mgEAG/g). In this way, the total phenolic content for the plant extract, and extracts of the endophytic fungi *Verticillium* sp., *Colletotrichum* sp., and *Fusarium* sp. 1 were  $96.94 \pm$

**Table 2.** Mean values and standard deviations of total phenol content and flavonoids found in *Lippia sidoides* Cham., and endophytic fungi *Verticillium* sp., *Colletotrichum* sp. and *Fusarium* sp. 1.

Extracts	Total phenols (mgEAG/g)*	Total flavonoids (mgER/g)**
<i>L. sidoides</i>	96.94 ± 11.86 <sup>a</sup>	192.33 ± 4.58 <sup>a</sup>
<i>Verticillium</i> sp.	6.38 ± 1.27 <sup>b</sup>	21.56 ± 1.14 <sup>b</sup>
<i>Colletotrichum</i> sp.	0.29 ± 0.05 <sup>c</sup>	14.31 ± 1.56 <sup>c</sup>
<i>Fusarium</i> sp. 1	10.54 ± 1.69 <sup>b</sup>	64.59 ± 1.15 <sup>b</sup>

\*mgEAG/g, Milligram equivalents of gallic acid per gram of extract. \*\*mgER/g, milligram equivalents of rutine per gram of extract. Means followed by the same letter in the column do not differ statistically from each other by the Tukey test ( $p > 0.05$ ).

**Figure 1.** Percentage of antioxidant activity (%AA) of of *L. sidoides* endophytes fungi *Verticillium* sp., *Colletotrichum* sp., and *Fusarium* sp. 1, and positive controls (rutine and ascorbic acid) by the DPPH method.

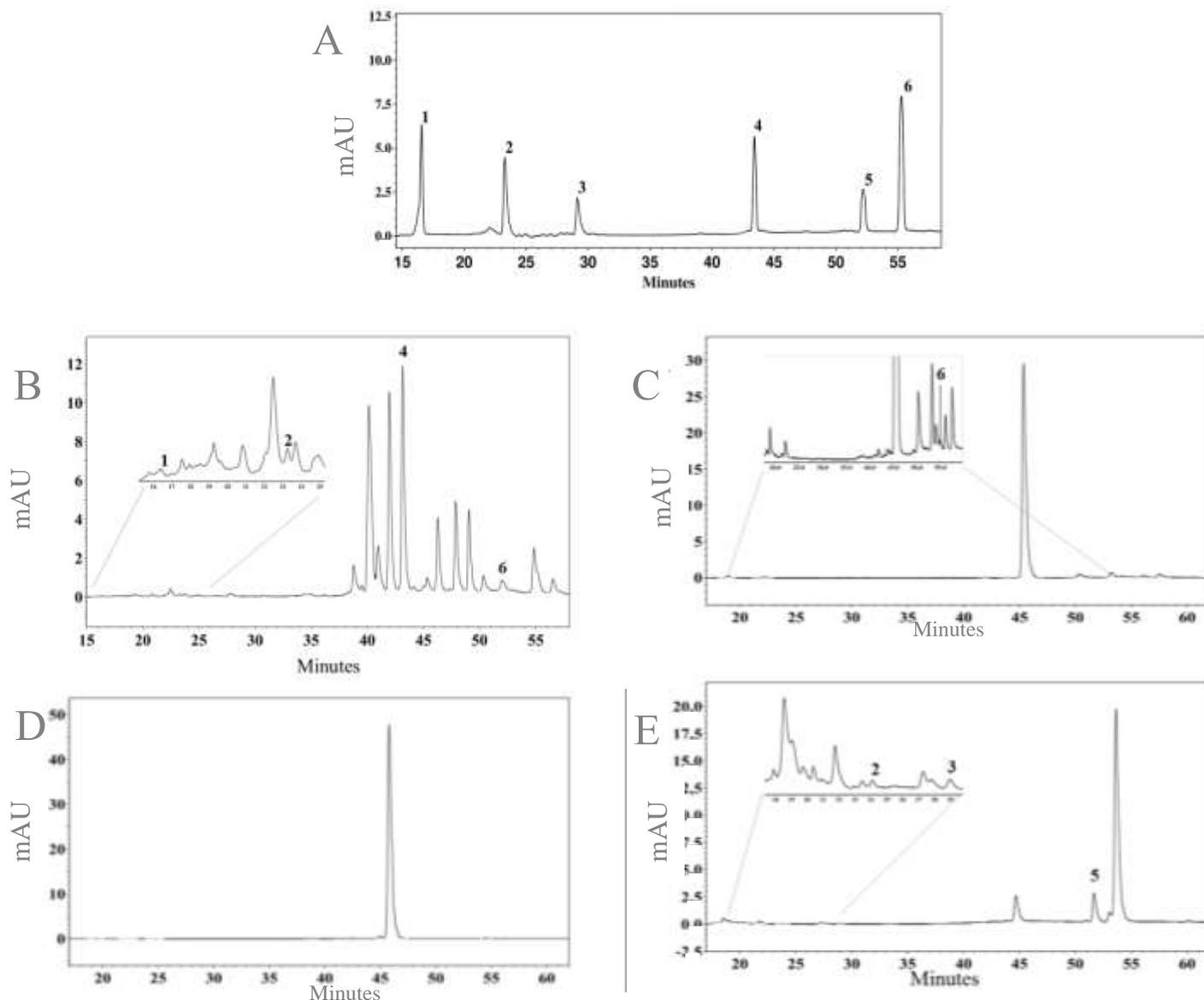
11.86, 6.38 ± 1.27, 0.299 ± 0.05 and 7.60 ± 1.69 mgEAG/g respectively (Table 2).

It was verified that the total flavonoid content of extracts of the fungi *Verticillium* sp., *Colletotrichum* sp., and *Fusarium* sp. 1 were 21.56 ± 1.14, 14.31 ± 1.56 and 64.59 ± 1.15 mgER/g, respectively (Table 2). These values are lower than that of the plant extract, which was 192.33 ± 4.58 mgER/g (Table 2).

The percentage of antioxidant activity (%AA) was also determined by the DPPH method. The curves are shown in Figure 1, indicating an increase only for the plant extract, with the increase of the amount of extract in the reaction medium. The results were higher than 65% from the concentration of 120 µg mL<sup>-1</sup>, reaching a maximum of 95% for the concentration of 200 µg mL<sup>-1</sup> for *L. sidoides* extract (Figure 1). The effective concentration (EC<sub>50</sub>), that

is, the amount of sample required to decrease the initial concentration of DPPH by 50% was also expressed in µg mL<sup>-1</sup>. The EC<sub>50</sub> for the plant extract was 81.03 µg mL<sup>-1</sup> (Figure 1). The %AA for the highest concentration (200 µg mL<sup>-1</sup>) for the fungi *Verticillium* sp., *Colletotrichum* sp., and *Fusarium* sp. was 19, 17 and 21%, respectively. Therefore, the EC<sub>50</sub> calculation could not be performed.

From the HPLC analyses performed for detection and quantification, fingerprints were obtained (Figure 2), which revealed matrices of phenolic compounds and flavonoids. In Figure 2B, the phenolic compound gallic acid 0.37 µg mL<sup>-1</sup> (Rt 16.4 min) was detected, along with the flavonoids catechin 1.67 µg mL<sup>-1</sup> (Rt 23.7 min), naringin 44.77 µg mL<sup>-1</sup> (Rt 43.2), and quercetin 3.08 µg mL<sup>-1</sup> (Rt 52.2 min). A high concentration of the compound naringin in the methanolic extract of the plant is



**Figure 2.** High-performance liquid chromatography (HPLC) fingerprints of authentic standards of phenolic compounds mixture (A), methanolic extract of the leaves of *L. sidoides* (B) and isolated endophytic fungi: *Verticillium* sp. (C), *Colletotrichum* sp. (D), *Fusarium* sp. (E) detected at 280 nm, as described in material and methods. Peak 1: Gallic acid; peak 2: Catechin; peak 3: Epigallocatechin gallate; peak 4: Naringin; peak 5: Quercetin; peak 6: Naringenin.

noteworthy.

It was also verified by HPLC in this study which flavonoids could be synthesized by the endophytic fungi and which could be identified and quantified according to the standards in the laboratory where the analysis was performed. According to Figure 2C, it can be seen that the extract of the endophytic fungus *Verticillium* sp. synthesized a compound identified as naringenin  $0.61 \mu\text{g mL}^{-1}$  (Rt 55.0 min) in addition to other compounds that could not be identified.

The fungus *Colletotrichum* sp. also produced secondary metabolites of the flavonoid class, yet it was not possible

to compare any peak with laboratory standards. As shown in Figure 2D, the visualization of many uniform peaks indicated that many compounds were synthesized. The fungus *Fusarium* sp. synthesized (Figure 2E) three substances identified as catechin  $0.31 \mu\text{g mL}^{-1}$  (Rt 23.3), epigallocatechin gallate, and quercetin  $19.73 \mu\text{g mL}^{-1}$  (Rt 29.1 min).

A synergism test was also performed between *L. sidoides* extract and the endophytic extract of *Verticillium* sp. (Table 3). The curve was fitted to a 2<sup>nd</sup> degree equation with  $R^2 = 0.9999$ . The highest inhibitory effect was in the proportion of 50% essential oil and 50% fungal

**Table 3.** Effect of synergism between the *Lippia sidoides* (Cham.) plant extract and the endophytic extract of *Verticillium* sp. against mycelial growth of *Curvularia lunata* (Wakker) *in vitro*.

Treatment (proportions of extracts)	Inhibition (%)
Control	0 ± 0.0
<i>L. sidoides</i> + <i>Verticillium</i> sp. (0:1)	54.8 ± 1.2
<i>L. sidoides</i> + <i>Verticillium</i> sp. (1:3)	43.5 ± 1.2
<i>L. sidoides</i> + <i>Verticillium</i> sp. (1:1)	79.0 ± 1.6
<i>L. sidoides</i> + <i>Verticillium</i> sp. (3:1)	71.3 ± 2.7
<i>L. sidoides</i> + <i>Verticillium</i> sp. (1:0)	22.4 ± 4.6

extract, both of which were at a concentration of 7500 µg mL<sup>-1</sup>. Proportions of 0:1, 1:3, 1:1, 3:1 and 1:0 (v/v) inhibited 54.8, 43.5, 79.0, 71.3 and 22.4% of the mycelial growth of *C. lunata in vitro* (Table 3).

## DISCUSSION

Some previous works found the same endophytic fungi. You et al. (2009) isolated the endophytic fungus *Verticillium* sp. from the roots of *Rehmannia glutinosa* by identifying two compounds produced by this fungus: 2,6-dihydroxy-2-methyl-7-(prop-1E-enyl)-1-benzofuran-3(2H)-one, reported for the first time, and the ergosterol peroxide, which inhibited the growth of pathogenic fungi. Suradkar et al. (2014) were able to isolate the endophyte *Verticillium albo-atrum* from *Withania somnifera* (L.) and *Ocimum sanctum* L.

The fungi *Colletotrichum* sp. and *Fusarium* sp. were also found in other studies isolating endophytic fungi. *Alternaria alternata*, *Colletotrichum gloeosporioides*, *Drechslera dematioidea*, *Guignardia bidwelli*, *Fusarium lateritium*, and *Phomopsis archeri* were endophytic fungi isolated from *L. sidoides* and identified by Siqueira (2011). In this work, the author isolated 15 endophytic fungi from *L. sidoides*, the same number of isolates in the present work. Similar results were verified, where the fungi *Fusarium*, *Colletotrichum*, *Phomopsis*, and *Alternaria* were also present in the work of Siqueira (2011).

Among the metabolites produced by *Colletotrichum* sp.: fusarentine 6,7-dimethyl ether, monocerin, and colletotrialide (Tianpanich et al., 2011), stigmaterol, sitostenone, squalene, ergosterol and ergosterol peroxide (Carvalho et al., 2016) were identified. *Colletotrichum* species have been identified to produce a variety of secondary metabolites genes, including flavones, peptides and terpenes (Crouch et al., 2014) and many other metabolites as Jayawardena et al. (2016) shows.

The genus *Fusarium* is also widely known as a producer of a variety of chemical compounds derived from its secondary metabolism (Nongalleima et al., 2013). Wang et al. (2011) reported anticancer activity against human PC-3 (prostate), PANC-1 (pancreas) and

A549 (lung) cells from a secondary metabolite called beauvericin isolated from the endophytic *Fusarium oxysporum* of the *Cinnamomum kanehirae* plant. This same endophyte, *F. oxysporum*, nonpathogenic strains, isolated by Kundu et al. (2016), produced , bikaverin (1), 3-O-methyl-8-O-methyl fusarubin (2), 8-O-methyl fusarubin (3), anhydrofusarubin (4) and fusarubin (5).

In addition, the methanolic extract of *Fusarium proliferatum* prepared by Mohana et al. (2012), endophytic of *Dysoxylum binectariferum* demonstrated cytotoxic activity in HCT-116 (colon) and MCF-7 (breast) human cancer cells.

The results confirm that both the plant and endophytic fungi produce compounds of a phenolic nature. Some studies corroborate our results, even though some previously reported fungal extracts contained more total phenols than ours. Yadav et al. (2014a) verified the phenolic compound content of endophytic fungi extracts obtained from ethyl acetate. The highest concentration of phenols was observed in the extract of *Chaetomium* sp. (60.13 ± 0.41 mgEAG/g), followed by that of *Aspergillus niger*. The total phenol concentration values of the 21 endophytic fungi studied ranged from 4.20 to 60.13 mgEAG/g. Srinivasan et al. (2010) obtained values of 18.33 ± 0.68 mgEAG/g of total phenols from the extract of the endophytic fungus *Phyllosticta* sp.

In order to confirm the synthesis of total flavonoids by the endophytic fungi present in the *L. sidoides* and plant extract (Table 2), extracts were obtained with the solvent ethyl acetate (AcOEt) to extract compounds with recognized antimicrobial activity, such as phenols and flavonoids (Baba and Malik, 2015).

However, even if they are smaller, it can be verified that the endophytic fungi contribute to the plant in the production and synthesis of secondary metabolites for its protection. Hence, an appreciable amount of flavonoids produced by the endophytic fungi may be present in the quantity verified in the plant; moreover, they contributed to its synthesis.

Huang et al. (2007) isolated 42 endophytic fungi from *Nerium oleander* and found flavonoids among the main bioactive compounds of some cultures. They also clarified that some phenolic compounds are produced so

that certain endophytic fungi survive along with their host plants. Qiu et al. (2010) found flavonoid contents of  $0.01162 \pm 0.0014$  and  $0.01256 \pm 0.00378$  mgER/mL in extracts of *Aspergillus nidulans*, *Aspergillus oryzae*, and *Ginkgo biloba* endophytes, respectively. Their values were lower than those of the present work; however, different fungi were used.

The data obtained in the determination of this bioactivity is significant when compared with others obtained in the work of Almeida et al. (2010). At a concentration of  $100 \mu\text{g mL}^{-1}$  (or  $1 \text{ mg mL}^{-1}$  as reported), there was an antioxidative activity of 99.5% and an  $\text{EC}_{50}$  of  $16.3 \mu\text{g mL}^{-1}$  in the ethanolic extract of *L. sidoides*. The results obtained in the present study were lower; however, the solvent used for the extraction may have been the source of this difference. They also assayed the DPPH activity of plant-isolated substances such as tecomaquinone and naringenin. At a concentration of  $1 \text{ mg mL}^{-1}$ , %AA was 64.7% and the  $\text{EC}_{50}$  was  $720 \mu\text{g mL}^{-1}$  for the flavonoid naringenin. The compound tecomaquinone did not display any activity.

As for the antioxidant activity of the extracts of endophytic fungi, they showed a low capacity to sequester free radicals, as there was no negative effect on the measured absorbance. Initial values remained essentially unchanged at all concentrations tested. It can be said that there was activity; however, compared with the results obtained from the plant extract, it was much lower than expected. It is believed that perhaps the cultivation conditions used did not promote the biosynthesis of antioxidant molecules that are detectable by this method. In addition, there was no change in the amount of free radicals as the concentration of fungal extract varied.

Observing the work of Devi and Singh (2015), they also did not obtain positive results of a significant amount of free radical sequestration by DPPH in extracts of *Verticillium*. However, they reported an antioxidative activity 95% in *Alternaria* sp. 2 made from ethyl acetate. Yadav et al. (2014a) carried out tests with extracts of endophytic fungi, including the genus *Fusarium* sp. They identified antioxidant activity by DPPH of *Aspergillus peyronelii* and *Aspergillus niger* of 71 and 72%, respectively, but identified low activity in extracts of *Fusarium* sp. Nath et al. (2014) also reported a strong activity in the sequestration of free radicals in ethanolic extracts of *Colletotrichum gloeosporioides* of  $0.67 \pm 0.05$ ,  $47.89 \pm 0.06$ ,  $73.84 \pm 0.08$ ,  $60.09 \pm 0.08$ , and  $52.77\% \pm 0.06\%$  at concentrations of 10, 25, 50, 75 and  $100 \mu\text{g mL}^{-1}$ , respectively.

Endophytic fungi and plants can produce flavonoids together, varying their biological activities, such as the genus *Bauhinia variegata* (cow's foot), which had antidiabetic activity confirmed by the metabolic profiles of both the plant and the associated endophytic fungi (Costa, 2005).

These analyzes by HPLC allowed us to identify

phenolic constituents described in the literature as important in several biological functions. Phenolic acid has a strong anti-inflammatory, antimutagenic, and antitumor action, inhibiting genes related to the cell cycle, metastasis, angiogenesis, and apoptosis (Verma et al., 2013). The detection of three types of flavonoids, catechin, naringin, and quercetin, in the *L. sidoides* plant (Figure 2B) also allows presenting and confirm the possibility of harnessing its leaves for biological applications. Several studies have already demonstrated the potential and ability of these metabolites to act to reduce the risks associated with pathologies such as intestinal inflammation, bacterial infections, diabetes, cardiovascular diseases, and cancer, among others (Clemensen et al., 2017; Macheleidt et al., 2016). In the present study, however, antifungal activities of the extracts are related to the antioxidative activity of flavonoids present in considerable amounts (Funari et al., 2012).

Many studies report the capacity of flavonoid production by plants, such as those found in the leaves of *Euphorbia nerifolia* (pencil-tree), where Sharma et al. (2014) identified a flavonoid as quercetin by HPLC. This substance and other flavonoids are credited with antioxidative and anticarcinogenic activity. Naringenin is a flavonoid of the flavanone subclass, found mainly in citrus fruits (Dou et al., 2013), with a beneficial antioxidative and neuroprotective effect (Raza et al., 2013).

Among these, the flavonoids catechin and quercetin were also found in plant extracts. Thus, it can be confirmed that these two metabolites were probably synthesized by the endophytic fungi in conformation with the plant-fungus mutualistic interactions. In addition, as endophytic fungi produce the same compounds present in the plant, it can be inferred that fungi produce these compounds for the plant as a way of assisting them in their defense. These substances contribute greatly to the antioxidative activities credited to *L. sidoides* plants (Funari et al., 2012).

Kumar et al. (2013) characterized the vinblastine alkaloid from the extract of the endophytic fungus *F. oxysporium*, isolated from *Catharanthus roseus*. By HPLC analyzes, they obtained  $76 \mu\text{g L}^{-1}$  of the compound. Also Zaiyou et al. (2015) found in the extract of the endophyte *Fusarium* sp. The compound paclitaxel at the concentration of  $0.0153 \text{ mg L}^{-1}$ .

Besides, Chapla et al. (2014) also identified eight compounds synthesized by the endophyte *Colletotrichum gloeosporioides*, a new compound 2-phenylethyl 1H-indol-3-yl-acetate (1) and seven other known compounds: Uracil (2), cyclo-(S\*(S)-Pro-S\*-Val) (4), 2-(2-aminophenyl) acetic acid (5), 2-(4-hydroxyphenyl) acetic acid (6), 4-hydroxy-benzamide (7), and 2-(2-hydroxyphenyl) acetic acid (8). Table 4 shows the compounds identified by HPLC from extracts of both the plant *L. sidoides* and the endophytic fungi isolated from it.

**Table 4.** Identified compounds from samples of plant extracts and endophytic fungi identified and analyzed by HPLC with their respective peaks, concentrations, and retention times.

Sample (extract)	Compound identified	Peak	Concentration (mg ml <sup>-1</sup> )	Retention time (min)
<i>L. sidoides</i>	Gallic acid	2	0.37	16.4
	Catechin	8	1.67	23.7
	Naringin	21	44.77	43.2
	Quercetin	27	3.08	52.0
<i>Verticillium</i> sp.	Naringenin	11	0.61	55.0
<i>Colletotrichum</i> sp.	N.I. *	-	-	-
<i>Fusarium</i> sp. 1	Catechin	8	0.31	23.7
	Epigallocatechin gallate	10	0.94	28.9
	Quercetin	17	19.73	52.2

N.I.\* = compound not identified

The production of compounds that possess biological activity by endophytic fungi can be stimulated in the plant, by the host plant extract, and in this case, by the extracts of fermented fungi without contact with any part or extract of the plant (Dos Santos et al., 2015). When grown *in vitro*, fungi continued to produce metabolites. Further studies must be carried out to find out if the fungi would cease production for some time or if, in the presence of the plant extract or any part of it, the synthesis would continue, that is, what factors could encourage this production. No reports were found in the scientific literature on the inhibition of mycelial growth of phytopathogenic fungi using extracts of endophytic fungi and of plants with synergistic effects. However, in the work of Oliveira et al. (2014), synergistic activity was found between a butanolic fraction of *Lippia alba* extract with commercial antifungal agents against *Candida glomerata*. The minimum inhibitory concentration was 0.062 µg mL<sup>-1</sup>. This may be a potential alternative in the treatment for candidemia caused by yeast species.

The synergistic effect of the endophytic fungal extracts of *Aspergillus awamori*, *Penicillium* sp., and *C. gloeosporioides* with standard antibiotics was studied against the four test bacterial strains viz. *Streptococcus pyogenes*, *Escherichia coli*, *Enterococcus faecalis*, and *Salmonella enterica ser paratyphi*. A significant increase in the diameter of inhibition zones was observed when the fungal extracts in combination with the antibiotic Norfloxacin was used against the test pathogens. Crude extract of *A. awamori* in combination with Norfloxacin showed antimicrobial activity (25 and 31-mm inhibition zones) against *E. coli* and *E. faecalis*, respectively (Nath et al., 2013).

The secondary metabolites act on the fungus through cytoplasmic granulation, disorganization of cellular contents, and inactivation of enzymes, which inhibits germination, germinative tube elongation, and mycelial growth (Lo et al., 1996). Thus, it can be inferred that the best result of mycelial inhibition was determined by the

joint action of the extract of *L. sidoides* and extract of the endophytic fungus *Verticillium* sp. However, it was only a preliminary test, and other tests should be performed in order to obtain results on the best concentration of the metabolites, test the efficiency of the other fungi along with the plant extract, and add another type of extract.

Recent discoveries, as exemplified in this work, have associated endophytic fungi with medicinal plants, important sources of secondary metabolites of pharmaceutical, agricultural, and industrial interest. Brazil has a wide potential source of plants, due to the varied biomes, and a study of plant-endophytic interactions is necessary. The discovery of metabolites with recognized bioactive action produced by endophytes can help to stop the excessive exploitation of plants and consequently, the whole set of endophytes associated with it (Pusztahelyi et al., 2015). Thus, the study of fungal endophytes is becoming a great necessity, because, as in the present work, they assist their host plants in the synthesis of metabolites with recognized bioactivity. This property of endophytes will help to diminish the exploitation of plant biodiversity for drug extraction. Consequently, conservation strategies will be more efficient.

## Conclusions

The analyses of the study showed the presence of phenols and flavonoids in all extracts and high antioxidant activity was only observed in the plant extract. Secondary metabolites were identified and quantified by HPLC. The best result for mycelial inhibition by the synergism test was determined by the joint action of the extract of *L. sidoides* and that of the endophytic fungus *Verticillium* sp. against *C. lunata*.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

## ACKNOWLEDGEMENTS

This work was supported by the University Federal of Tocantins, Graduate Agronomy and Biotechnology, and Bioprocess Engineering Departments.

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