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ORIGINAL PAPER

Endophytic fungi from *Passiflora incarnata*: an antioxidant compound source

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Abstract

Endophytes are considered one of the most important microbial resources for obtaining biomolecules of therapeutic use. *Passiflora incarnata,* widely employed by the pharmaceutical industry, shows therapeutic effects on anxiety, nervousness, constipation, dyspepsia and insomnia based on their antioxidant compounds. In this study, from 315 endophytic fungi isolated from *P. incarnata* leaves, 60 were selected to determinate presence of chemical constituents related with antioxidant activity, based on their production of soluble pigments. The promising fungi were evaluated specifically on their potential to produce phenolic compounds, flavonoids and for antioxidant activity. Five isolates significantly produced flavonoids and phenolic compounds in the ethyl acetate and n-Butanol extracts, also saponins and high antioxidant activity against the DPPH (2.2-diphenyl-1-picrylhydrazyl) free radical. A strain of *Aspergillus nidulans* var. *dentatus* (former *Emericella dentata*) was able to produce tannins as well; its butanolic extract was very similar than the BHT (butylated hydroxytoluene) (94.3% × 94.32%) and Rutin (95.8%) reference substances in the DPPH radical scavenging. Similarly, a *Chaetomium* strain exhibited 93.6% and 94.7% of antioxidant activity in their ethyl acetate and butanolic fractions, respectively. The chromatographic analysis of the ethyl acetate fraction from the *Aspergillus* strain revealed the production of orcinol (3.19%). Four-methoxymethylphenol (4.79%), sorbicillin (33.59%) and ergosterol (23.08%) was produced by *Trichoderma longibrachiatum* and isopropenyl-1,4-dimethyl-1,2,3,3a,4,5,6,7-octahydroazulene were found in two *Fusarium oxysporum* strains. The phytochemical screening showed that all analyzed fungi were able to produce a kind of secondary metabolite (phenols, flavonoids, tannins and/or saponins). The study shows a great underexplored potential for industrial application of *P. incarnata* endophytes.

Keywords Antioxidant · DPPH radical scavenging activity · Phenolic compounds · Secondary metabolites · Endophytes

Introduction

Passiflora incarnata L. (Passifloracea) is a tropical perennial plant with climbing or trailing trunks. This species is originally from South America and its culture occurs mainly in Brazil and some other tropical regions of America, Asia and Australia (Kim et al. 2017). Unlike to the most known *Passiflora* species used in food industry, *Passiflora incarnata* have been largely exploited in traditional herbal medicine and, therefore, included in pharmacopeias from Germany, Switzerland and France (Dhawan et al. 2004). Leaves of *Passiflora incarnata* are widely used in pharmacology as sedatives, tranquillizers and antispasmodics (Dhawan et al. 2001). The main bioactive substances found in this plant are phenolic compounds, specifically flavonoids, C-glycosides derived from apigenin and luteolin (vitexin, isovitexin,

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orientin and isoorientin), besides alkaloids (Müller 2005). These compounds are termed antioxidants, because they have the capability for delaying, preventing, or removing oxidative damage by inhibiting or quenching-free radicals and reactive-oxygen species. The antioxidants synthetized in human body are insufficient to counteract the oxidative stress, thus exogenous sources rich in antioxidant are necessary for maintaining health. Several studies have showed that one of the main plant sources are medicinal plants (Xu et al. 2017).

Herbal medicines, as natural medicinal agents, are among the world's best-selling products. In this decade, this market has already moved billions of dollars worldwide and remains a big business to be explored (Ekor 2014). However, the extraction of antioxidant compounds from plants include the exploitation of large cultivation zones, ecologically unfriendly agricultural practices and expensive industrial process (Trantas et al. 2015). Other difficult for pharmaceutical industry is the low concentration recovered of target secondary metabolites, since they can occur with structurally similar molecules (Du et al. 2010). The microbial systems offer diverse advantages from easy manipulation to economical large-scale production. The industrial demand counts on an expressive part of the microbial exploitation: 45% from actinobacteria; 38% from fungi and 17% from bacteria. The market for microbial products, mainly to attend healthcare molecules demand is expected to reach $ 250.3 billion by the end of 2023 (Azevedo et al. 2000; Market Research Future 2019). The search for new products for biotechnology is an ongoing process and there is an expanding scenario for endophytes in this context (Rajamanikyam et al. 2017).

Scientific evidences point that all plant tissues are inhabited by a microbial community of endophytes (Kusari et al. 2012). They actively or passive enter to plant endosphere and can reside it as commensal, potentially pathogenic or beneficial organisms. The evolutionary adaptation of these microbes is attributed to critical roles that they may play in plant health. These include bioavailability of essential nutrients, attenuation of pathogens attack and synthesis of plant compounds. The frequent reports on their ability to produce the metabolites of their host plants has aroused interest in their study for application in the pharmaceutical, agronomic, food and chemical industries (Nicoletti and Fiorentino 2015). One can say that most endophytic fungi have not yet been investigated or even known, although they represent an abundant and reliable source of novel bioactive compounds with significant potential for exploitation in human society (Rajamanikyam et al. 2017).

In the last years, endophytic fungi were studied as antiviral, anticancer, antidiabetic and antimicrobial agents (Selvi and Balagengatharathilagam 2014; Yadav et al. 2014; Zhao et al. 2014; Market Research Future 2019), but very few studies have shown their antioxidant activity and estimation of phenolic compounds and flavonoids (Strobel et al. 2002; Pan et al. 2017). Considering the exploitation of natural substrates is the most successful strategy for the discovery of molecules for industrial and biotechnological application, the aim of this study was to perform a phytochemical screening of endophytic fungi isolated from *Passiflora incarnata* leaves.

Materials and methods

Collection of plant material

Leaves of *P. incarnata* were randomly sampled from mature, healthy-looking plants grown in the Centroflora Group agricultural fields, Botucatu, São Paulo, Brazil (22°56′18.40"S, 48°34′04.20"W). Voucher specimens were deposited at the Herbarium "Coleção de Plantas Medicinais e Aromáticas" (CPMA) under the identification number CPMA 1791. They were placed in individual plastic bags, labeled and kept refrigerated until lab processing.

Isolation and selection of endophytic fungi

Leaves were washed thoroughly in running tap water and then subjected to surface sterilization successively with sterile distilled water for one minute, 70% ethanol for one minute, 3% NaOCl (Sodium hypochlorite) for three minutes, 70% ethanol for 30 s (Souza et al. 2004). Leaf samples were finally washed thrice with sterile distilled water. As a surface-sterilization process control, aliquots of water from the last wash were plated on 2% MA (Malt Agar) (Acumedia) and PDA (Potato Dextrose Agar) (Acumedia), then incubated at 28 ℃ for process efficiency evaluation (Araújo et al. 2010; Schulz and Boyle 2005). After sterilization, 0.5 cm-diameter fragments were cut from each leaf and inoculated in PDA, 2% MA and OA (Oatmeal Agar) media, containing streptomycin sulphate (20 µg/mL) for avoiding possible bacterial growth. The samples were plated in quintuplicate, totalizing 25 fragments through cultivation at 28 ℃ for 20 days. The plates were daily observed and, once the mycelia had grown, they were transferred to plates containing PDA and incubated under the same conditions, aiming the purification of the isolates. The plates showing reproductive growth and the presence of soluble pigments and exudates were selected for the further experiments. The promising fungi were deposited in the collection "Coleção Brasileira de Micro-organismos de Ambiente e Indústria" under the identification numbers LMA1705, LMA1793, LMA1719, LMA1721 and LMA1684.

Preparation of fungal extract and chemical screening

On the basis of phenotypic traits exhibited during the purification, the endophytic fungi were treated for the solvent extraction process. The isolates were cultured for seven days in PDA medium plates and after this period, four to five 0.5 cm agar discs containing mycelium were inoculated in 250 mL Erlenmeyer flasks for fermentation with 100 mL of potato dextrose broth (Souza et al. 2004; Khiralla et al. 2015). The flasks were incubated at 28 ℃, under shaking condition (120 rpm). After 15 days, the fermented broth was filtered by Whatman No. 1 filter paper. The filtrate was partitioned twice with EtOAc (Ethyl acetate) (1:1), followed by BuOH (Butanol) (1:1). The EtOAc and BuOH fractions were then evaporated to dryness using rotary vacuum evaporator, weighed and stored at 4 ℃ for further experiments. The main classes of chemical compounds (alkaloids, phenolics, flavonoids, tannins and saponins) was determined according to the procedure indicated by Matos (2009) with modifications.

Molecular identification of endophytic fungi

The fungal isolates with outstanding features in the chemical screening were identified by their morphological characteristics and by sequencing the ITS (Internal Transcribed Spacer) region. Genomic DNA of the isolates was extracted with physical lysis of the mycelium using glass microspheres (425–600 µm in diameter, Sigma) according to Aamir et al. (2015), modified. The ITS1-5.8S-ITS2 region was amplified using ITS1 (5′-CCGTAGGTGAACCTGCGG-3′) and ITS4 primers (5′-TCCTCCGCTTAT TGATATGC-3′) (White et al. 1990). DNA fragments sequencing was carried out with the amplification primers using the Big Dye Kit (Life Technologies) in the ABI 3500 Genetic Analyzer XL system. The forward and reverse sequences of each isolate were assembled in contigs using editor BioEdit software (Hall 1999). The sequences obtained were compared with reference sequences from Genbank, NCBI (National Center for Biotechnology Information) database and the Westerdijk Fungal Biodiversity Institute–(The Netherlands) database. Based on identity score, closest reference sequences of each endophytic fungus were obtained and used for further phylogeny analysis. The sequences were aligned with the CLUSTALW function (Thompson et al. 1994) and phylogenetic tree was constructed using the MEGA (Molecular Evolutionary Genetics Analysis) X software (Kumar 2018). The evolutionary relationship was established using Neighbor-Joining algorithm and distances were calculated with the Kimura 2-parameter model (Kimura 1980). The sequence of a *Donadinia lusitanica* strain was used as the outgroup. The statistical support of nodes was estimated by bootstrap analysis with 1000 replications (Felsenstein 1985). The visualization of phylogenetic affiliations was carried out in the Interactive Tree of Life web-based tool (https://itol.embl.de) (Letunic and Bork 2016).

The ITS sequences are deposited in Genbank database under accession numbers MN179336-MN179340.

Evaluation of the antioxidant activity of endophytic extracts

One of the most commonly used methods to determine antioxidant activity is to evaluate the scavenging activity of the purple-colored-free radical DPPH, which absorbs it at 517 nm. Through an antioxidant or a radical species, DPPH is reduced, forming diphenyl picryl hydrazine, yellow in color, with consequent absorbance disappearance, potentially being monitored through decreased absorbance (Brand-Williams et al. 1995). The dried fractions were dissolved in methanol. A total of 100 µL of methanolic fraction (in various concentrations) were mixed with 2900 µL of methanol solution of DPPH (100 µM). The mixture was incubated in the dark for 30 min at room temperature. The decrease in absorbance was measured at 517 nm. A mixture of 100 µL of methanol and 2900 mL of DPPH served as negative control. BHT reference substances, ascorbic acid and Rutin were evaluated in the same concentrations (0.25–5 mg mL^{-1}). Considering 100% absorbance of negative control, the radical scavenging activity was calculated using the following formula:

Antioxidant activity (AA) (%) = $[(A_0 - A_i)/A_0] \times 100$

where A_0 is the absorbance of control and A_i = absorbance of samples or reference substances.

Estimation of total phenolic content

The content of total phenolic substances was determined by Folin–Ciocalteu's spectrophotometric method, using gallic acid as a reference standard. Folin–Ciocalteau's phenol reagent is a solution of complex polymeric ions formed from heteropoly phosphomolybdic and phosphotungstic acids. This reagent oxidizes phenolates, turning the acids into a blue W/Mo complex (Singleton et al. 1999). An aliquot of 0.5 mL (5 mg g^{-1}) of each sample was transferred to a tube with screw cap, inside of which 2.5 mL of Folin–Ciocalteau reagent diluted in distilled water 1:10 was added. The mixtures remained at rest for eight minutes and received the addition of 2 mL of sodium carbonate at 4%. The tubes were left to stand for 2 h in the dark at room temperature and then absorbance reading was carried out at 765 nm. Methanol was used as blank and an analytic curve containing 100, 80, 60, 40, 20 and 10 µg mL^{-1} of gallic acid was constructed. Total phenolic contents were expressed in mg equivalent in gallic acid (Singleton et al. 1965; Minussi et al. 2003) and

obtained from regression equation: $y = 0.012x + 0.0436$ with $R^2 = 0.997$. All analyses were performed in triplicate.

Estimation of total flavonoid content

Initially, a fermented extraction using the liquid–liquid partition method was performed. An equal volume of organic solvent was added to the filtrate (1:1). At first, the filtered was extracted with ethyl acetate, resulting in the acetate fraction and the aqueous fraction (medium), then the aqueous fraction (filtered) was extracted with n-Butanol twice, providing butanolic and aqueous fraction. The different extracts (EtOAc and BuOH) were transferred to round-bottomed flasks and vacuum dried in a rota-evaporator device, providing dry fractions that were dissolved in 5 mL methanol and stored at 4 ℃ (Liu et al. 2007). A total of 1 mL (5 mg g^{-1}) of each fraction obtained was mixed with 1 mL of a methanolic solution of aluminum chloride at 2%. After incubation for 30 min, the absorbance of the reaction was measured at 430 nm. An analytical curve was built containing 5, 10, 20, 40, 60 and 80 µg mL^{-1} of rutin (Sigma® standard). Total flavonoid contents were expressed in mg Rutin (mg g^{-1}) using the regression equation based on the calibration curve: $y = 0.003x - 0.009$ with $R^2 = 0.997$. All analyses were performed in triplicate.

Gas chromatography–mass spectrometry analysis of antioxidant potential

In order to characterize antioxidant compounds, the fractions were subjected for GC–MS (Gas Chromatography–Mass Spectrometry) analyses. This was carried out using an Agilent 6890 N gas chromatograph and in an Agilent 5975 mass-selective detector, HP-5MS column (J & W Scientific, 30 m × 0.25 mm × 0.25 µm). Chromatographic conditions employed were: Temperatures: 280 ℃ injector; 300 ℃ detector; column 150 ℃/2 min, 5 ℃·min^{-1} up to 240 ℃, 10 ℃·min^{-1} up to 300 ℃ for 34 min. Injection volume was 1.0 µL with flow of carrier gas Helium of 1.0 mL min^{-1}. Bioactive compounds were identified by comparison of mass spectra available with data from the NIST (National Institute of Standard and Technology, US) 05 library.

Statistical analysis

Differences of phenolic and flavonoid content and antioxidant activity between the isolates were analyzed by one-way analysis of variance. ANOVA´s assumptions were revised by equal variance (Levene Median) and normality test (Shapiro–Wilk). Post hoc analysis included Tukey HSD test when $p < 0.01$. Statistical analyses were carried out in SigmaPlot 12.0.

Results

Isolation and selection of endophytic fungi

The disinfection process of leaf surface was clearly effective, as there was no microbial growth on inoculated plates with water from the last wash. In total, 90 leaves (five per individual) were evaluated, from which was obtained 315 endophytic fungi. A total of 60 isolates was selected for analysis of main chemical constituents. After the chemical analyses, all isolates tested exhibited at least one class of metabolite (Table S1). Five endophytic isolates (LMA 1705, LMA 1793, LMA 1719, LMA1721 and LMA 1684) showed outstanding traits (Table 1) and were chosen for tests of antioxidant activity.

Identification of promising fungi

Morphological characterization from the five promising endophytes revealed the prevalence of high sporulating ascomycetes in the subphylo Pezizomycotina, being three well-known anamorphic forms (*Aspergillus, Fusarium* and *Trichoderma*) and one characteristic sexual phase (*Chaetomium*). Regarding to molecular identification, the ITS sequences of fungi selected showed > 99.8% similarity with reference sequences. From the alignment matrix, 398 positions were used for construction of the phylogenetic tree. The taxonomic affiliations of five isolates were determined, which were supported by > 60% bootstrap values. The phylogenetic tree showed similar results to similarity search by BLAST. The ITS1-5.8S-ITS2 sequence of isolate LMA 1705 formed a clade with the sequence of *Emericella dentata* (MK108395.1). However, recently all species of the genus *Emericella* were transferred to *Aspergillus* (Samson et al. 2014) within the *Nidulantes* section, thus *E. dentata* is also known as *Aspergillus nidulans* var. *dentatus.* The isolate LMA 1793 clustered with two sequences of *Chaetomium globosum* (MK281559.1 and MN069626.1). Although the isolates LMA 1719 and LMA 1721 formed a monophyletic group with the ITS1-5.8S-ITS2 sequences of *Fusarium*

Table 1 Results of the crude fermentation broth from endophytic fungi isolated from *P. incarnata* and their secondary metabolites

Endophytic fungi	Secondary metabolism of endophytic fungi			
	Flavonoids	Phenols	Tannins	Saponins
LMA 1705	+	+	+	+
LMA 1793	+	+	−	+
LMA 1719	+	+	−	+
LMA 1721	+	+	−	+
LMA 1684	+	+	−	+

oxysporum (MG975622.1, KY678301.1 and MG407705.1), supported by a 97% bootstrap value. Based on phylogenetic tree the isolate LMA 1684 were classified within the order Hypocreales (marked in red in Fig. 1) and affiliated with the species *Trichoderma longibrachiatum* (MH153613.1).

Quantification of total flavonoid and phenolic content

As can be seen in Table 2, the both ethyl acetate and butanolic fractions showed levels of total flavonoids expressed as rutin and total phenols expressed as gallic acid. Fungi LMA 1705, LMA 1793, LMA 1719, LMA 1721 and LMA 1684 presented larger values for total flavonoids in the butanolic fraction, indicating the solvent extractor n-Butanol was better suited for these metabolites. In the phenolic compounds case, all samples (except the LMA 1684 isolate) showed higher values in the ethyl acetate fraction. Strain LMA 1719 showed the greatest levels of total phenols and flavonoids, 208.43 mg g^{-1} (EtOAc fraction) and 204.59 mg g^{-1} (BuOH fraction), respectively.

Antioxidant activity of endophytic fungi from P. incarnata

DPPH is a relatively stable radical and widely used to evaluate the antioxidant activity of several biological samples. The ethyl acetate and butanolic fraction of all five promising fungi were evaluated for their antioxidant activity in different concentrations (0.25, 0.5, 1, 2 and 5 mg mL^{-1}). Three reference substances (Ascorbic acid, Rutin and BHT) were include in this assay (Fig. 2) (Table S2). The butanolic

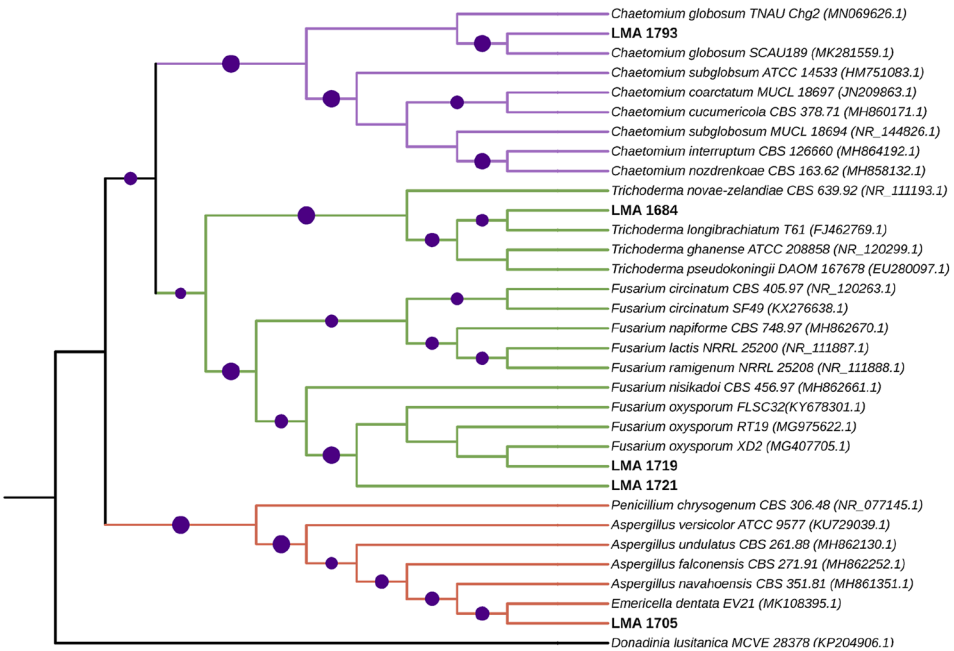

Fig. 1 Neighbor-Joining phylogenetic tree of endophytic fungi selected based on their ITS1-5.8S-ITS2 rDNA sequences. The branch colors indicate different fungal order: Sordariales (purple), Hypocreales (green) and Eurotiales (red). Only bootstrap values equal and greater than 50% are displayed as circles with increasing size up to 100%

Table 2 Total phenolic and flavonoid contents in EtOAc fraction and n-Butanol fraction of endophytic fungi from *P. incarnata*

Endophytic fungi	Total phenolic*		Total flavonoids**	
	EtOAc fraction	n-Butanol fraction	EtOAc fraction	n-Butanol fraction
Aspergillus nidulans var. *dentatus* LMA 1705	161.29 ± 0.134**a**	141.72 ± 0.341**a**	71.05 ± 0.456**a**	76.44 ± 0.314**a**
Chaetomium globosum LMA 1793	164.52 ± 0.556**b**	155.77 ± 0.346**b**	128.57 ± 1.165**b**	161.94 ± 0.452**b**
Fusarium oxysporum LMA 1719	208.43 ± 0.876**c**	140.46 ± 0.942**a**	139.84 ± 0.986**c**	204.59 ± 0.331**c**
Fusarium oxysporum LMA 1721	146.98 ± 0.976**d**	143.22 ± 0.854**ac**	124.77 ± 1.004**b**	122.80 ± 1.352**d**
T. longibrachiatum LMA 1684	103.62 ± 0.324**e**	140.07 ± 0.652**ad**	105.07 ± 0.984**d**	162.81 ± 2.213**b**

Same letters mean no statistical differences among values according to the Tukey test ($p < 0.01$)

Data are expressed as mean ($n = 3$) ± SD (Standard deviation)

Values with different letters within the columns are significantly different according to the Tukey test ($p < 0.01$)

*Total flavonoids equivalent to Rutin (mg g^{-1} extract)

**Total phenols equivalent to Gallic Acid (mg g^{-1} extract)

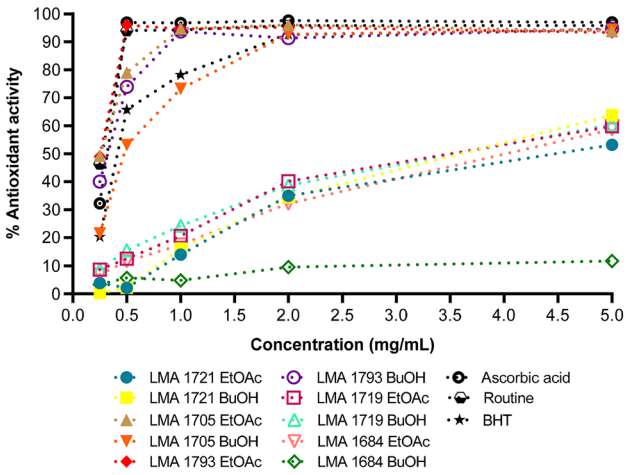

Fig. 2 DPPH radical scavenging activity of EtOAc and BuOH fractions of the promising endophytic fungi

fraction from LAM 1793 strain showed higher antioxidant capacity, even, than BHT when tested 5 mg mL^{-1} from each one. Similar values were determined in the ethyl acetate and butanolic fraction from LMA 1705 strain.

It is noteworthy that the IC_{50} values, in relation to DPPH radical eliminating abilities of fungi *Aspergillus nidulans* var. *dentatus* LMA 1705 and *Chaetomium globosum* LMA 1793, were not only comparable to those of the ascorbic acid and rutin patterns, but also significantly higher than those of the BHT standard, as shown in Table 3.

Chromatographic analysis

Extracts obtained in liquid–liquid extraction process of the five selected fungi through ethyl acetate and butanol were subjected to analysis by gas chromatography coupled to mass spectrometry. The volatile metabolites detected in fungal extracts, which were identified comparatively with available compounds in the NIST-05 library, were: orcinol, present in the ethyl acetate fraction of the fungus *Aspergillus nidulans* var. *dentatus* LMA 1705, 4-methoxymethylphenol, sorbicillin and ergosterol, those present in the ethyl acetate fraction of the fungus *T. longibrachiatum* LMA 1684. In the case of the fungus *F. oxysporum* LMA 1719, the presence of the 7-Isopropenyl-1,4-dimethyl-1,2,3,4,5,6,7,8-octahydroazulene compound was determined, as well as in the ethyl acetate fraction (Table 4).

Discussion

Recently, endophytic fungi have been drawing attention of researchers because they have shown major potential for medicinal compounds; but the most interesting is that in several studies these compounds were initially described in their host plants (Stierle et al. 1993; Krings et al. 2007). Between 2002 and 2012, approximately half of the newly discovered fungal metabolites were resulted from studies involving endophytic microorganisms (Pan et al. 2017). This study represents an evidence on ability of endophytes to synthesis compounds originally characterized in its host plant, since it determined antioxidant activity in fungi isolated from a plant (*P. incarnata*) rich in antioxidant compounds (Miroddi et al. 2013).

In determining the phytochemical profile of five selected isolates, we observed the presence of saponins, flavonoids and polyphenols and tannin production by *Aspergillus nidulans* var. *dentatus* LMA 1705. Tannins (commonly referred to in the form of tannic acid) are water-soluble polyphenols present in many foods of plant origin and in endophytic fungi. Due to its characteristics (complexation of metal ions, antioxidant activity and complexation of macromolecules), tannins have multiple pharmacological applications, such as astringent action, healing, hemostatic, antiseptic and antioxidant activity (Chung et al. 1998).

Table 3 IC_{50} of DPPH free radical capture of the ethyl acetate and butanolic fractions of endophytic fungi

Endophytic fungi	IC_{50} value (mg/mL ± SD)	
	EtOAc fraction	n-Butanol fraction
Aspergillus nidulans var. *dentatus* LMA 1705	0.23 ± 0.235**a**	0.49 ± 0.214**a**
Chaetomium globosum LMA 1793	0.21 ± 0.324**a**	0.28 ± 0.567**a**
Fusarium oxysporum LMA 1719	3.28 ± 0.487**b**	3.22 ± 0.986**a**
Fusarium oxysporum LMA 1721	4.10 ± 0.134**b**	3.28 ± 1.314**a**
T. longibrachiatum LMA 1684	3.77 ± 0.023**b**	304.18 ± 0.313**b**
Ascorbic acid	0.23 ± 0.095	
BHT	0.42 ± 0.101	
Rutin	0.25 ± 0.301	

Same letters mean no statistical differences among values according to the Tukey test ($p < 0.01$)

Data are expressed as mean ($n = 3$) ± SD (Standard Deviation)

Values with different letters within the columns are significantly different ($p < 0.01$)

Table 4 Metabolites identified by GC–MS in selected ethyl acetate extracts

Chemical name	Chemical formula	RT (min)	Abundance of compounds identified in each extract (%)*				
			LMA 1705	LMA 1793	LMA 1719	LMA 1721	LMA 1684
4-(Methoxymethyl) phenol	$C_8H_{10}O_2$	4.18	–	–	–	–	0.05
Sorbicillin	$C_{14}H_{16}O_3$	17.31	–	–	–	–	0.34
Ergosterol	$C_{28}H_{44}O$	28.59	–	–	–	–	0.23
7-Isopropenyl-1,4-dimethyl-1,2,3,4,5,6,7,8-octahydroazulene	$C_{15}H_{24}$	9.18	–	–	0.96	–	–
Orcinol	$C_7H_8O_2$	3.54	0.03	–	–	–	–

*Relative abundance of extract components was generated from electronic integration of individual pics of the chromatogram relative to the total pics area

In this study, phenolic compounds and flavonoids showed affinity both with ethyl acetate solvent and *n*-Butanol. Tung et al. (2007) isolated eleven phenolic compounds from the EtOAc fraction from the bark of *Acacia confusa* Kasote et al. (2011). reported the presence of polyphenols with antioxidant activity in the BuOH fraction of linseed flour (*Linum usitatissimum* L.). Phenolic compounds and flavonoids play an important role in stabilizing lipid oxidation and are associated with antioxidant activity, as emphasized in some studies (Yin et al. 2007). Studying an endophytic fungus isolated from the pigean peas roots (*Cajanus cajan* L.) Zhao et al. (2014) used three solvents (n-Hexane, n-Butanol and ethyl acetate) of varying polarities to determine the content of total phenols and flavonoids in the three fractions. The best results were gathered from the ethyl acetate fractions 578.12 mg g^{-1} and 356.89 mg g^{-1}, followed by the butanolic fractions 322.65 mg g^{-1} and 201.27 mg g^{-1}, respectively. In summary, the content of phenols from the different fractions had the same order of flavonoids: ethyl acetate fraction > n-Butanol fraction > n-Hexane fraction > water fraction, these data corroborate with the data obtained in our study, seeing that the presence of polyphenols and flavonoids was observed in both solvents used.

We highlight that this is the first report on the antioxidant activity of endophytes from the leaves of passion flower. The butanolic extract of the LMA 1705 strain showed antioxidant activity greater than Rutin (91.98%) and BHT (90.27%) standards, being able to eliminate 94% of the DPPH-free radical, whereas *C. globosum* LMA 1793 showed values of antioxidant activity in the ethyl acetate and butanolic fractions equal to 93.6% and 94.7%, respectively. Corroborating with our results, Yadav et al. (2014), studying 21 endophytic fungi isolated from *Eugenia jambolana* Lam. (Myrtaceae), found the highest percentage of antioxidant activity in members of the genera *Aspergillus* and *Chaetomium* with values higher than 80%. In addition, Selim et al. (2014), in his study with *C. globosum* isolated from the medicinal plant *Adiantum capillus-veneris,* concluded that the fungus presents a high antioxidant activity, aside from having a wide spectrum of bioactivity (antimicrobial, antiviral and antineoplastic) in vitro and rich in secondary metabolites. This species as endophyte can be considered a promising source for drug discovery through the study of their active principles.

Aspergillus and *Chaetomium* are admittedly important genera in the food sector and, especially in the pharmaceutical industry, which focuses on producing enzymes, organic acids and fermentation. Arora and Chandra (2011) studied the antioxidant activity of the fungus *A. fumigatus* using different organic solvents and observed that the ethyl acetate was a better solvent extractor of the components responsible for the antioxidant potential, followed by chloroform and butanol. The results obtained in our study are remarkable when compared with those achieved by Arora and Chandra (2011), because the selected strain of *Aspergillus* presented great percentage of antioxidant activity in both ethyl acetate and butanolic fractions, 96.4% and 94.8%, respectively, while the results of the EtOAc fraction were better compared with the ascorbic acid (95.8%), used as positive control. The isolates LMA 1705 and LMA 1793 presented significantly higher IC_{50} than fungi *F. oxysporum* LMA 1719 and LMA 1721 and *T. longibrachiatum* LMA 1684 in the two solvent extractors used in this study. Kanagasabapathy et al. (2011) found that the butanolic fraction of the fungus *Pleurotus sajor-caju* showed better results compared with the ethyl acetate fraction. These data showed that the antioxidant compounds can be found in both fractions.

Between samples, the BuOH fraction of the isolate LMA 1684 showed the lowest eliminating activity of DDPH-free radical (value of IC_{50} at 304.18 mg mL^{-1}), while the EtOAc fraction of the fungus *C. globosum* LMA 1793, with a value of IC_{50} at 0.21 mg mL^{-1}, revealed a higher antioxidant activity, compared with BHT positive controls (0.42 mg mL^{-1}), rutin (0.25 mg mL^{-1}) and ascorbic acid (0.23 mg mL^{-1}) (Table 3). Zhang et al. (2008) isolated the fungus *Cephalosporium* sp. from the roots of the *Trachelospermum jasminoides,* producer of graphislactone A, a substance of strong

antioxidant activity. Pestacine and isopestacine antioxidants are produced by *Pestalotiopsis microspora*, a fungal species that inhabits the interior of the plant *Terminalia morobensis,* which is native to Papua New Guinea (Harper et al. 2003). Phenylpropanoid amide, another antioxidant compound, was isolated from the endophytic fungus *Penicillium brasilianum* living in *Melia azedarach* (Fill et al. 2010).

Other compounds with antioxidant activity were isolated from different endophytic fungi (Oliveira et al. 2009). Antioxidants protect the cells from damage caused by free radicals. Reactions mediated by free radicals are associated with various disorders, including Alzheimer's disease, diabetes, cardiovascular disorders and cancer (Dorko 1994; Market Research Future 2019). Antioxidant action of fungal extracts is yet not well understood, but studies show that many species of endophytic fungi can scavenge-free radicals (Jaszek et al. 2013; Sugiharto 2016). The antioxidant activity measured by the stable radical DPPH shows the capacity of molecules belonging to the fungal extracts to scavenge these radicals in the existing medium. The methodology is simple, quick and efficient in the evaluation of extracts obtained from solvents with different polarities.

Orcinol, a phenolic compound present in the ethyl acetate fraction of the endophytic strain *Aspergillus nidulans* var. *dentatus* is considered a polyketide (Staunton and Weissman 2001; Jorgensen et al. 2014), a class of secondary metabolites known as one of the most important among the fungi (O'Hagan 1991; Cox 2007). Fungal polyketides range from simpler monocyclic aromatic compounds, such as orsellinic acid and orcinol, to the polycyclic aromatic, such as citrinin (Staunton and Weissman 2001; Cox and Simpson 2009; Pan et al. 2017). In addition to showing an impressive range of functional and structural diversity, they possess a richness of pharmacologically important activities, such as antibacterial, anticancer, antifungal, antiparasitic and immunosuppressive (Staunton and Weissman 2001). Wu et al. (2016) uncovered orcinol in *A. versicolor* and determined this biomolecule has antioxidant activity. It is assumed that the antioxidant activity presented in ethyl acetate fraction of the endophytic strain LMA 1705 can be attributed to the orcinol.

Volatile compounds may appear as intermediate and final products of various metabolic pathways (Siddiquee et al. 2012; Hung et al. 2013), as it may have happened in the case of the production of 4-methoxymethylphenol by the isolate *T. longibrachiatum,* LMA 1684. Another compound identified in the extract of this isolate was sorbicillin. According to Salo et al. (2016), the sorbicillinoids, which are derivatives of sorbicillin, constitute a class with various biological activities, such as: antioxidant, antiviral, antiinflammatory and antimicrobial. Studying a strain of *Trichoderma* sp. isolated from saline lands on the coast of Bohai Bay, China, Ma et al. (2011) isolated two compounds analogous to sorbicillin. The cytotoxic effects of the isolated compounds were preliminarily assessed against leukemic cells (P388 and HL-60). The results showed that these compounds were slightly active against both cellular strains. In addition, the compounds have shown some antioxidant activity against the DPPH-free radical. It is possible that we could assign to the sorbicillin the antioxidant activity of the fungus LMA 1684 isolated in our study. It is noteworthy that this is the first report of this compound as a product of the endophyte *T. longibrachiatum* associated with *P. incarnata*.

In their research of endophytic fungi associated with *Paeonia delavayi* Wu et al. (2011), isolated a *Trichoderma* endophyte from where metabolites, such as trichodermic acid, 2β-hydroxy-trichoacorenol, cyclonerodiol, cyclonerodiol oxide and sorbicillin were extracted. All these biomolecules showed inhibitory activity when facing an *Escherichia coli* and a *Staphylococcus albus*, as well as in varying levels of activity against *Shigella sonnei* and phytopathogenic fungi, such as *Botrytis cinerea, Fusarium avenaceum*, *Fusarium* from *fujikuroi* complex and *Pyricularia oryzae*. In addition, these compounds showed inhibitory activity against the human opportunistic *Exophiala dermatitidis*. Data obtained by Wu et al. (2011) corroborated with our results, since the presence of sorbicillin in isolates of *Trichoderma* was observed. Future studies might validate its biological activity.

Our results corroborate with the earlier reports claiming that phytochemical compounds present in endophytic fungi can contribute to the future development of the synthesis of new drugs (Castillo et al. 2007). Several classes of compounds earlier considered to be exclusively of plant origin have already been seen in endophytic fungi, such as alkaloids (Liu et al. 2010), polyphenols (Yadav et al. 2014), tannins (Selvi and Balagengatharathilagam 2014; Ladoh-Yemeda et al. 2015), flavonoids (Huang et al. 2007; Cheng et al. 2013), saponins (Khanna and Kannabiran 2008), sterols (Carvalho et al. 2016) and terpenes (Saxena et al. 2015). The compounds produced by endophytic fungi are a diverse group as to structures and functions, being either low-molecular-weight compounds (pyrones, aromatic compounds and their derivatives, terpenes, amino acids, lactones, bicyclic and tricyclic compounds, among others), or high-molecular-weight compounds (proteins, glycoproteins and polysaccharides) (Liebermann et al. 2000; Larsen et al. 2003). Our results provide a basis for subsequent studies on bioactive compounds produced by endophytes of *P. incarnata*, a medicinal plant widely used in practically all regions around the world. Flavonoids, phenols and alkaloids are the compounds that contribute the most to its medicinal properties (Speroni and Minghetti 1988; Marchart et al. 2003; Dhawan et al. 2004).

Conclusion

The present study has evidenced the potential of *Passiflora incarnata* endophytic fungi as antioxidant compound source. The results show that the microbial extracts obtained from ethyl acetate and n-butanol are rich in phenolic compounds and flavonoids. By the phytochemical screening performed, all isolates analyzed are able to produce at least a class of secondary metabolite (phenols, flavonoids, tannins and/or saponins) of pharmaceutical importance.

Endophytes from *P. incarnata* produce phenolic compounds, methoxymethylphenol, orcinol and sorbicillin and the strains *Chaetomium globosum* LMA 1793 and *Aspergillus nidulans* var. *dentatus* LMA 1705, specifically, present notable antioxidant activities in vitro, which may be regarded as promising sources of new drugs. There is a vast potential of passion flower endophytes for application in biotechnology, what also justifies the initiatives to organize collections of endophytic strains as provisional resources for innovation and discoveries.

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Author contributions MHRS and DAA designed the work. MHRS and SBJ conducted the experiments. MHRS and DAA analyzed and interpreted the results. MHRS, LGCY and DAA drafted the manuscript. AS, VLG and SBJ supervised the antioxidant activity and GC–MS analysis. DAA, DFA and LGCY supervised the isolation and identification of endophytic fungi. All authors read and approved the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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