Journal of Applied Pharmaceutical Science Vol. 7 (04), pp. 038-043, April, 2017
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2017.70405
ISSN 2231-3354

Endophytes isolated from passion fruit plants: molecular identification, chemical characterization and antibacterial activity of secondary metabolites

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ARTICLE INFO

Article history:
Received on: 14/07/2016
Accepted on: 24/11/2016
Available online: 30/04/2017

Key words:
Endophytic fungi, antimicrobial action, *Passiflora* genus, rDNA sequencing, thin layer chromatography.

ABSTRACT

Endophytic fungi inhabit the interior of healthy plants without any apparent harm and may synthesize bioactive compounds, which constitute an alternative for the control of human pathogens. Current study reports the molecular identification of two endophytes from passion fruit plants: isolate PE1-13 (from *Passiflora edulis* f. *flavicarpa*) has been identified as *Phyllosticta* sp. and isolate PA8-2 (from *Passiflora alata*) is a *Cercospora beticola* strain. Thin layer chromatography showed that steroids and triterpenoids were present in the secondary metabolites produced by the two endophytes. The antibacterial assay against *Enterococcus faecalis*, *Salmonella typhi* and *Streptococcus pyogenes* indicated that the fungal metabolites reduced up to 98.1±0.5% of bacterial growth. Results suggest that these endophytes are capable of producing antibacterial compounds.

INTRODUCTION

Although enterococci are natural colonizers of the gastrointestinal tract in most humans and animals, the bacterial group may cause nosocomial infections (Arias and Murray, 2012) since *Enterococcus faecalis*is a highly relevant pathogenic species (Gregersen *et al.,* 2010). Glycopeptide antibiotics, including vancomycin and teicoplanin, are often used against *Enterococcus* infections because the bacterial group is multi-resistant to several antibiotic classes (Arias and Murray, 2008). However, glycopeptide resistance in enterococci has been reported (Breidenstein *et al.,* 2015). Approximately 200,000 people die annually worldwide as a result of typhoid fever, a systemic disease caused by *Salmonella typhi* (Crump and Mintz, 2010; Butler, 2011). Typhus is an important public health problem and there is an increasing need for antimicrobial treatment since its resistance to ciprofloxacin, ceftriaxone and azithromycin has been proved (Wong *et al.,* 2014). *Streptococcus pyogenes*is transmitted primarily through direct contact with skin lesions or throat secretions of infected patients and colonizes the human skin and throat by causing pharyngitis, impetigo, necrotizing fasciitis and streptococcal toxic-shock syndrome (Wasserzug *et al.,* 2009; Siljander *et al.,* 2010). Some *S. pyogenes* isolates have been reported to be resistant to erythromycin and tetracycline (Rubio-López *et al.,* 2012; Silva-Costa *et al.,* 2012).

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Considering the increasing resistance of pathogenic bacteria to current antibiotics, in-depth research to discover new antibacterial substances is of the utmost importance.

Endophytic fungi colonize intra- and inter-cellular plant tissues during all or part of their life cycle without causing apparent damages (Fouda *et al.,* 2015). These microorganisms establish a symbiotic relationship with the host plant, in which the fungus obtains nutrients and shelter, while the plant receives protection against pathogens and insect-pests (Giménez *et al.,* 2007; Alvin *et al.,* 2014). Endophytes constitute a novel source of bioactive substances that may be employed in different biotechnological industries (Orlandelli *et al.,* 2015), including aliphatic compounds, alkaloids, flavonoids, peptides, phenols, quinones, steroids and terpenoids with antimicrobial properties, reviewed by Yu *et al.* (2010). So that the biotechnological properties of these fungi could be expanded, current study establishes the chemical characteristics of the secondary metabolites produced by two endophytes isolated and molecularly identified from passion fruit plants and reports their antibacterial activity against *Enterococcus faecalis*, *Salmonella typhi* and *Streptococcus pyogenes*.

MATERIALS AND METHODS

Endophytic fungi

The endophytes tagged as PE1-13 and PA8-2, belonging to the fungal culture collection of the Laboratório de Biotecnologia Microbiana, Universidade Estadual de Maringá, Maringá PR Brazil, were isolated from *Passiflora edulis f. flavicarpa* and *Passiflora alata*, respectively. For fungal isolation, healthy *Passiflora* leaves were collected from the University´s Experimental Didactic Garden, washed in running tap water and in an aqueous solution 0.01% Tween 80 to remove epiphyllous debris. The leaves were then rinsed with 70% ethanol for 1 min, surface-disinfected with sodium hypochlorite solution (4% available Cl^-) for 3 min, and rinsed in 70% ethanol (30 s) and twice in sterile distilled water. Leaves were cut into 5-mm^2fragments and equally placed on Potato Dextrose Agar (PDA) dishes to which tetracycline was added. All dishes were incubated at 28°C in biochemical oxygen demand (BOD) for seven days and periodically checked (Silva *et al.,* 2013). Fungi were maintained on PDA medium (HiMedia, Mumbai, MH, India) at 4ºC.

Molecular identification

Genomic DNA was extracted according to Felber *et al.* (2015). DNA concentration and integrity were analyzed by electrophoresis in 1% agar gel with Lambda DNA digested by HindIII (Gibco-Invitrogen, Carlsbad, CA, USA) as molecular weight standard. PCR amplification of ITS1-5.8S-ITS2 of rDNA region was performed according to Magnani *et al.* (2005) with primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCCCGCTTATTGATATGC-3′) (White *et al.,* 1990) with an initial denaturation at 92ºC for 4 min, followed by 35 denaturation cycles at 92 ºC for 40 s, annealing at 52ºCfor 1 min and 30 s, extension at 72ºC for 2 min and a final extension at 72ºC for 5min. PCR products were purified with GFX PCR DNA kit and Gel Band Purification (Amersham Biosciences, Piscataway, NJ, USA), following manufacturer's instructions.

Sequencing was performed in a MegaBACE TM 1000 sequencer (Amersham Biosciences), with injection and electrophoresis conditions of 2 kV/60 s and 6 kV/230 min, respectively. Nucleotide sequences were analyzed and edited, and compared to those deposited in GenBank (http://www.ncbi.nlm.nih.gov). The BLASTN program was used for research into genera or species. Determination was based on the best result obtained for identity. In the case of phylogenetic analysis, a dendrogram was constructed with the sequences obtained coupled to those available in the GenBank. Sequences were aligned by Clustal X (Thompson *et al.,* 1997) and the dendrogram was constructed by MEGA program version 6 (Tamura *et al.*, 2013) with grouping by the neighbor-joining method (Saitou and Nei, 1987), using p-distance for nucleotides with the option of pair-wise gap deletion and bootstrap with 10,000 replications.

Extraction of secondary metabolites

A slightly modified version of the protocol described by Rukachaisirikul *et al.* (2008) was used to obtain secondary metabolites. Endophytes were grown on PDA at 28 ºC for seven days. Further, three 5-mm^2mycelium fragments of each endophyte were inoculated into 500-mL Erlenmeyer flasks containing 250 mL of Potato Dextrose Broth (PDB) (HiMedia, Mumbai, MH, India) and incubated at 28ºC for 25 days under stationary condition. Broth cultures were then filtered with sterile gauze to separate the fungal mycelia, which were discarded. The cell-free media were centrifuged at 1300 $\times g$ for 20 min to separate cellular debris.

The supernatants were transferred to a separating funnel in which ethyl acetate (EtOAc) P.A. (Fmaia, Cotia, SP, Brazil) was added at a ratio of 1:5. After strong agitation, the separation of phases occurred by polarity difference and the EtOAc phase was collected. After extracting the fermented medium three more times with EtOAc, the solvent was removed by 98% concentration in a MA-120 rotary evaporator (Marconi, Piracicaba SP Brazil) at 40ºC. The metabolites were tagged SM_{PE1-13} and SM_{PA8-2}.

Chemical characterization of secondary metabolites

Thin layer chromatography (TLC) was carried out on 20 $\times$ 20 cm glass plates with silica gel 60 G and 60 GF254 (Merck, Darmstadt, HE, Germany), suspended in distilled water at a ratio of 1:2, and distributed at an approximate thickness of 0.25 mm. Plates were dried at 120ºC for 30 min before use. They were developed in organic solvents hexane, dichloromethane, methanol and ethyl acetate, at a polarity-increasing order (0, 3.1, 5.1 and 6.2, respectively), used in pure form or as binary mixtures. Chromatograms were developed by the ascending technique in which each plate was immersed in the developing solvent to a depth of ~0.5 cm. Plates were then washed and dried at room

temperature and spots on TLC plates were visualized by spraying with Liebermann-Burchard reagent (to detect steroids/ triterpenoids) and with Dragendorff reagent (to detect alkaloids).

Antibacterial activity assay

Enterococcus faecalis ATCC 29212, *Salmonella typhi* ATCC 19430 and *Streptococcus pyogenes* ATCC 19615 were selected for the antibacterial activity assay. They were grown in Luria Bertani Broth (LBB) (Sambrook and Russel, 2001) for 24 h at 37ºC, and the diluted bacterial suspension (1.5×10^8 cfu mL^{-1}) was ready for detection. A modified version of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-dephenyl tetrazolium bromide] assay (Zhao et al, 2010) was used to detect the antibacterial activity of SM_{PE1-13} and SM_{PA8-2}. Briefly, the metabolites were dissolved in 2.5% DMSO solution (in LBB) at an initial concentration of 1000 µg.mL-1, and then diluted with LBB to obtain the following concentrations: 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 µg mL^{-1}. The tetracycline (Sigma-Aldrich, St. Louis, MO, USA) solution, used as positive control, was prepared under the same conditions.

Aliquots of each bacterial solution (5 µL) and LB(100 µL) were applied in each well of 96-well plates (except for blank wells). The bacteria were treated (three replicates) with 100 µL of secondary metabolite solution (100-1000 µg mL^{-1}), Tetracycline solution (100-1000 µg mL-1) or 2.5% DMSO solution (negative control).

After incubation for 24 h at 37ºC, the treatments were discarded and 10 µL of MTT solution (5 mg mL^{-1} in LBB) was added to each well. Plates were centrifuged at 1300 ×g for 20 min, the supernatants were discarded and150 µL of DMSO were added. Plates were kept at room temperature for 30 min and absorbance was measured at 550 nm in a FlexStation microplate reader (Molecular Devices, Sunnyvale, CA, USA). Absorbance means of negative control was taken as 100% of bacterial growth. The inhibition ratio (%) was calculated as follows (1):

$$\text{Inhibition ratio } (\%) = \frac{A_C - A_T}{A_C} \times 100 \qquad (1)$$

where: A_T and A_C were the absorbance means measured for treated groups and negative control group, respectively. Data were analyzed by analysis of variance (ANOVA) and averages were compared by Tukey test ($p<0.05$) with statistical program SISVAR 5.3.

RESULTS AND DISCUSSION

Molecular identification of endophytes

The sensitivity and specificity of molecular biology techniques employed for the genetic differentiation of species forwarded important advances in the identification of fungal species. The amplification of the internal transcribed spacer region (ITS) of ribosomal DNA (rDNA) by the polymerase chain reaction (PCR) is frequently used. Further, rDNA-ITS sequences obtained may be compared to those available at GenBank database (Magnani *et al.,* 2005). Herein, molecular techniques were employed to identify two fungal endophytes isolated from passion fruit plants. Sequencing of ITS1-5.8S-ITS2 regions identified the two endophytes isolated from passion fruit plants (Table 1 and Figure 1).

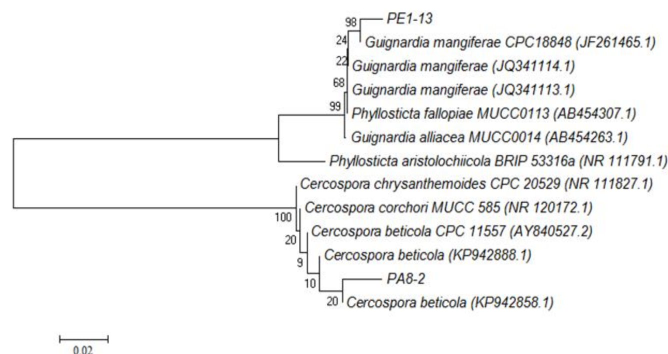

Fig. 1: The evolutionary history was inferred by the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10,000 replicates) is shown next to the branches. The evolutionary distances were computed by p-distance method and are in the units of the number of base differences per site. Evolutionary analyses were conducted in MEGA6.

Isolate PE1-13 (98% identity with *Phyllosticta fallopiae* MUCC0113 at BLAST) was grouped with *Phyllosticta* (= *Guignardia*) genus with 99% BP, confirming classification at genus level. Sequence data were submitted to GenBank under accession number KU641754. After the deletion of art. 59 from the International Code of Nomenclature for Algae, Fungi, and Plants, valid since January 2013, the asexual and sexual names of fungi received equal status and binomial nomenclature was replaced by a single name based on priority. Since the genus *Phyllosticta* was introduced by Persoon in 1818 (a much older name than *Guignardia* introduced by Viala and Ravaz in 1892), *Phyllosticta* has been currently used as the legitimate genus name. Species belonging to this genus have been reported as plant pathogens (Stringari *et al.,* 2009; Escanferla *et al.,* 2009) and several strains have already been isolated as endophytes as, for instance, from the mangrove plants *Laguncularia racemosa* and *Rhizophora mangle* (Costa *et al.,* 2012), and from the medicinal plants *Piper hispidum* (Orlandelli *et al.,* 2012) and *Luehea* divaricata (Bernardi-Wenzel *et al.,* 2010).

Isolate PA8-2 (99% identity with *Cercospora beticola* CPC 11557 at BLAST) was grouped with *Cercospora* genus with 100% BP and grouped with *C. beticola* with 20% BP, confirming the species' classification. Sequence data were submitted to GenBank under accession number KU641755. The fungus genus has been commonly associated with leaf spots on a wide range of host plants such as maize (*Zea mays*), faba bean (*Vicia faba*)and sugar beet (*Beta vulgaris*) (Meisel *et al.,* 2009; Kimber and Paull, 2011; de Coninck *et al.,* 2012). Recently, the genus was reported as an endophyte from the macrophytes *Eichhornia azurea* and *Eichhornia crassipes* (Almeida *et al.,* 2015).

Table 1: Molecular identification of endophytic strains based on the alignment of ITS region sequences with those deposited at GenBank and phylogenetic analyses.

Endophytes from *Passiflora* plants				Closely related GenBank sequences		
Code	**Host**	**Identification**	**Genbank code**	**Fungal species**	**GenBank code**	**Similarity**
PE1-13	P. *edulis* f. *flavicarpa*	*Phyllosticta* sp.	KU641754	*Phyllosticta fallopiae* MUCC0113	AB454307.1	98%
PA8-2	*P. alata*	*Cercospora beticola*	KU641755	*Cercospora beticola* CPC 11557	AY840527.2	99%

Table 2: Antibacterial activity of secondary metabolites obtained from endophytic fungi against *Enterococcus faecalis* ATCC 29212, *Salmonella typhi* ATCC 19430 and *Streptococcus pyogenes* ATCC 19615.

Treatment ($\mu g\ mL^{-1}$)	*Enterococcus faecalis**			*Salmonella typhi**			*Streptococcus pyogenes**		
	SM_{PA8-2}	SM_{PE1-13}	PC	SM_{PA8-2}	SM_{PE1-13}	PC	SM_{PA8-2}	SM_{PE1-13}	PC
100	80.7 ± 3.8^{c}	93.9 ± 1.4^{a}	79.8 ± 5.9^{c}	-	80.3 ± 1.0^{c}	102.2 ± 4.7^{a}	94.2 ± 0.8^{a}	97.1 ± 0.5^{a}	91.9 ± 3.1^{a}
200	83.0 ± 2.3^{c}	96.2 ± 3.7^{a}	74.2 ± 4.9^{c}	-	78.9 ± 2.8^{c}	103.6 ± 5.2^{a}	92.9 ± 2.4^{a}	96.9 ± 1.1^{a}	90.6 ± 2.7^{a}
300	81.0 ± 0.2^{c}	93.8 ± 8.9^{a}	71.6 ± 0.9^{c}	-	84.3 ± 8.6^{c}	104.1 ± 4.2^{a}	92.7 ± 1.9^{a}	94.2 ± 0.9^{a}	88.3 ± 2.0^{b}
400	80.1 ± 3.0^{c}	90.4 ± 1.0^{a}	70.8 ± 3.5^{c}	-	89.5 ± 9.1^{b}	103.3 ± 2.1^{a}	93.4 ± 0.4^{a}	93.9 ± 3.1^{a}	87.2 ± 3.2^{b}
500	81.3 ± 3.4^{c}	96.1 ± 3.3^{a}	67.6 ± 4.4^{c}	3.2 ± 0.5^{g}	88.0 ± 4.3^{b}	101.6 ± 3.1^{a}	93.3 ± 1.0^{a}	93.8 ± 1.7^{a}	86.0 ± 2.3^{b}
600	78.4 ± 1.6^{c}	94.8 ± 3.3^{a}	64.8 ± 4.2^{d}	18.4 ± 2.4^{f}	90.2 ± 0.3^{b}	99.8 ± 4.9^{a}	92.1 ± 1.3^{a}	92.4 ± 1.3^{a}	84.5 ± 3.5^{c}
700	80.4 ± 1.2^{c}	93.5 ± 4.6^{a}	62.4 ± 3.0^{d}	37.9 ± 2.4^{e}	94.2 ± 2.7^{b}	90.4 ± 2.2^{b}	91.7 ± 0.1^{c}	93.8 ± 1.7^{a}	81.7 ± 1.1^{c}
800	80.5 ± 1.3^{c}	92.2 ± 4.3^{a}	61.7 ± 3.7^{d}	59.9 ± 0.3^{d}	98.1 ± 0.5^{a}	90.7 ± 8.8^{b}	91.9 ± 1.8^{a}	93.4 ± 3.1^{a}	83.4 ± 4.0^{c}
900	79.2 ± 1.0^{c}	95.7 ± 2.1^{a}	62.6 ± 3.0^{d}	62.9 ± 4.1^{d}	97.6 ± 0.5^{a}	87.8 ± 3.1^{b}	91.1 ± 1.3^{a}	93.6 ± 4.6^{a}	82.3 ± 4.7^{c}
1000	79.7 ± 4.7^{c}	93.8 ± 5.7^{a}	61.1 ± 1.9^{d}	79.6 ± 2.1^{c}	93.9 ± 0.0^{b}	79.3 ± 2.3^{c}	91.1 ± 0.2^{a}	93.0 ± 4.3^{a}	81.5 ± 5.4^{c}

Note: *For each bacterium, means followed by different letters are significantly different according to Scott-Knott test ($p<0.05$).
Abbreviations: SMPA8-2 = secondary metabolite from *Cercospora beticola* KU641755; SMPE1-13 = secondary metabolite from *Phyllosticta* sp. KU641754; PC = positive control, that is, Tetracycline (Sigma-Aldrich).

TLC chromatography of secondary metabolites

Dragendorff test for alkaloids was negative for metabolic extracts from *Phyllosticta* sp. KU641754 (SM_{PE1-13}) and *Cercospora beticola*KU641755 (SM_{PA8-2}), whereas both extracts were positive by Liebermann-Burchard test for steroids and triterpenoids. Manufactured steroid compounds may be used for therapeutic purposes, as anti-inflammatory, immunosuppressive, progestational, diuretic, anabolic and contraceptive drugs (Bracco *et al.,* 2013). Terpenoids and triterpenoids are described as potent antimycobacterial agents (Cantrell *et al.,* 2001; Jadulco *et al.,* 2011).

When the chromatoplates were analyzed with pure solvents, the metabolic extracts showed a better resolution and distribution of substances with the dichloromethane solvent, which has intermediate polarity. Therefore, analyses were performed with different proportions of a binary mixture of dichloromethane and ethyl acetate. The proportion of 50% dichloromethane and 50% ethyl acetate provided the best separation of the compounds contained in SM_{PE1-13} when compared to the use of 100% dichloromethane as a mobile phase. Since acetate increased the phase´s polarity, the compounds left the point of origin and they were carried by the column, being visually more separated. On the contrary, the best separation of SM_{PA8-2} occurred when 100% dichloromethane was used.

Herein, the production of steroids and triterpenoids by *Phyllosticta* and *Cercospora* endophytes was reported. The genus *Phyllosticta* is often reported due to its capacity of producing phytotoxins, including phyllostoxin and phyllostin for the production of herbicides (Evidente et al, 2008). Moreover, Kumaran *et al.* (2009) reported the isolation and identification of an anticancer drug taxol from *P. tabernaemontanae*. *Cercospora beticola* is the economically most important foliar pathogen of sugar beet (*Beta vulgaris*). The latter and other *Cercospora* species are mainly described due to the production of cercosporin, a photo-activated toxin that generates reactive oxygen species when exposed to light (Staerkel *et al.,* 2013).

Antibacterial activity of secondary metabolites

As seen in Table 2, all concentrations of SM_{PE1-13} (from *Phyllosticta* sp. KU641754) were effective against *E. faecalis* ATCC 29212, with highest inhibition ratio at 96.2±3.7%. This result was statistically more effective than that obtained for SM_{PA8-2} (from *C. beticola* KU641755) and positive control. Similarly, the inhibition ratio for SM_{PE1-13} (up to 98.1±0.5%) was higher than that described for SM_{PA8-2} (up to 79.6±2.1%) when the bacterium *S. typhi* ATCC 19430 was tested. In the case of the antibacterial activity against *S. pyogenes* ATCC 19615, the two metabolites showed positive results with an inhibition ratio of 94.2±0.8% (SM_{PA8-2}) and 97.1±0.5% (SM_{PE1-13}).

Current study is the first report on antibacterial activity of *C. beticola* metabolites. In the case of *Phyllosticta* species, the metabolic extracts were already investigated against *Escherichia coli, Bacillus cereus, Bacillus subtilis, Micrococcus luteus* and *Pseudomonas aeruginosa* (Wikee *et al.,* 2013; Chukeatirote *et al.,* 2015). There are no reports in the literature on the effects of *Phyllosticta metabolites* against *E. faecalis, S. typhi* and *S. pyogenes*. Therefore, further studies should be undertaken to provide in-depth knowledge on antibacterial properties of these fungal metabolites.

CONCLUSIONS

Research on microbial sources of bioactive compounds with commercial interest is of great importance to replace those of animal and plant origin, which are subject to predation, extractive activity and consequent extinction. Moreover, the obtainment of

natural compounds involves lower costs and environmental impacts when compared to those obtained by synthetic routes. In fact, current investigation revealed that *C. beticola* KU641755 and *Phyllosticta* sp. KU641754 are potential sources of steroid and triterpenoid metabolites with antibacterial action against *E. faecalis*, *S. typhi* and *S. pyogenes*.

ACKNOWLEDGEMENTS

The authors are grateful to CNPq (311534/2014-7 and 447265/2014-8) and Fundação Araucária (276/2014) for financial support. M.S. Santos was recipient of an undergraduate scholarship from CNPq/PIBIC.

Conflict of Interests: There are no conflicts of interest.

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How to cite this article:

Santos MS, Orlandelli RC, Polonio JC, Ribeiro MAS, Sarragiotto MH, Azevedo JL, Pamphile JA. Endophytes isolated from passion fruit plants: molecular identification, chemical characterization and antibacterial activity of secondary metabolites. J App Pharm Sci, 2017; 7 (04): 038-043.