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Anti-*Escherichia coli* activity of the crude extract produced by the endophyte *Diaporthe* sp. GQ461588isolated from *Trichilia elegans*

Atividade anti-Escherichia coli do extrato bruto produzido pelo endófito Diaporthe sp. GQ46158 isolado de Trichilia elegans

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ABSTRACT

Endophytic microorganisms, usually fungi and bacteria, live in the interior of healthy plants without causing apparent damage to them. Endophytic fungi from *Diaporthe* (=*Phomopsis*) genus represent an important group with biotechnology potential and can be isolated of several medicinal plants, such as *Trichilia elegans* (Meliaceae family) which is popularly known in Brazil as "pau-de-ervilha" and has anti-inflammatory, antiviral and anti-rheumatic activities. Considering that endophytic fungi constitute an alternative for the control of pathogens because they can synthesize bioactive natural products, this study aimed to evaluate the antibacterial activity of the crude ethyl acetate extract from endophytic fungus *Diaporthe* sp. GQ461588, isolated from *T. elegans*, against *E. coli* ATCC 25922. The results showed that the fungus tested is promising for the production of compounds capable of inhibiting the *E. coli* growth, since inhibition halos with 13.98±0.39 mm of diameter were observed. This work suggests that the crude extract tested herein can be investigated in further studies aiming to expand the knowledge about its possible pharmaceutical use.
Keywords: antibacterial action; cup plate assay; pathogenic bacterium.

RESUMO

Microrganismos endofíticos, geralmente fungos e bactérias, vivem no interior de plantas sadias sem causar-lhes danos aparentes. Fungos endofíticos do gênero *Diaporthe* (=*Phomopsis*) representam um importante grupo com potencial biotecnológico e podem ser isolados de diversas plantas medicinais, como a *Trichilia elegans* (família Meliaceae), popularmente conhecida como pau-de-ervilha e com atividade anti-inflammatória, antiviral e antirreumática. Considerando que fungos endofíticos representam uma alternativa para o controle de patógenos devido à síntese de produtos naturais bioativos, esse estudo teve como objetivo avaliar a ação antibacteriana do extrato bruto (obtido por fracionamento com acetato de etila) do fungo endofítico *Diaporthe* sp. GQ461588 (isolado de *T. elegans*) contra *E. coli* ATCC 25922. Os resultados mostraram que o fungo testado é promissor para a produção de compostos capazes de inibir o crescimento de *E. coli*, já que foram observados halos de inibição com 13,98±0,39 mm de diâmetro. Este trabalho sugere que o extrato bruto testado poderá ser investigado em trabalhos futuros visando ampliar o conhecimento sobre uma possível aplicação farmacêutica.
Palavras-chave: ação antibacteriana; ensaio *cup plate*; bactéria patogênica.

INTRODUCTION

Endophytic fungi or endophytes are those which live in the interior of plants generally colonizing their aerial parts, such as stems and leaves, without causing apparent damage to their hosts. They are distinguished from phytopathogens that cause diseases in plants and epiphytes that live on the surface of plants. Bary, in 1866, differentiated at first time the endophytic and the phytopathogenic fungi. However, only in the end of 1970's, these fungi have gained importance and it was verified that they have symbiotic interactions with the host plant, protecting it from the insect attack, diseases and herbivores (Azevedo, 1999). Since then, different biotechnological properties of endophytes have been researched as: biological control of plant pathogens and production of industrial enzymes such as: production of enzymes of industrial interest (Choi et al., 2005; Orlandelli et al., 2015; Sunitha et al., 2013) and biological control of plant pathogens (Rocha et al., 2011; Rubini et al., 2005). Nowadays, it is know that endophytic fungi represent new possibilities in the investigation for metabolically active substances (Kalyanasundaram et al., 2015) with pharmaceutical application, as recently reviewed by Specian et al. (2014).

The Gram-negative bacterium *Escherichia coli* normally inhabits the intestinal tract of animals (including humans) and has a beneficial effect on organism, reducing the multiplication of harmful bacteria and synthesizing a considerable amount of vitamins. However, not all the *E. coli* strains are harmless, and some of them can cause debilitating diseases in human (Jafari et al., 2012). The self-medication habit and antibiotics overuse can cause the selection of antibiotic-resistant strains (Orlandelli et al., 2012a) as a natural result of the bacterial adaptability to the increased drug exposure. Multidrug-resistant *E. coli* are widely spread in hospitals and are increasingly being isolated in communities, were they are responsible for urinary tract infections with difficulty treated, with few oral treatments remaining effective (Ibrahim et al., 2012; Theuretzbacher, 2012). Therefore, the discovery of new drugs effective against antibiotic-resistant bacteria is an extreme necessity. One alternative for the use of chemical drugs is the use of natural compounds obtained from biological sources, such as endophytic fungi, which can synthesize bioactive products identical or similar to those from some medicinal plants (Strobel, 2003). The variety of secondary metabolites produced by a single endophyte has not been estimated, but it is probably high due to the ease and versatility for adaptation presented by these microorganisms (Azevedo et al.,2002).

Since January 2013, with the deletion of the Art. 59 from the International Code of Nomenclature for Algae, Fungi and Plants, the asexual and sexual names of fungi were substituted by a unique name based on the priority. Because the name *Diaporthe* (sexual state) predates *Phomopsis* (asexual state), the name *Diaporthe* is currently being adopted to represent the fungal group originally identified as *Diaporthe* or *Phomopsis* species, as shown by Gomes et al. (2013). Several species of endophytes from this genus have been isolated from several medicinal plants (Anitha et al., 2013; Bernardi-Wenzel et al., 2010; Garcia et al., 2012a; Huang et al., 2009; Orlandelli et al., 2012b; Qadri et al., 2013). Some of them represent rich source of biologically active secondary metabolites with antimicrobial activity against different human pathogens (Garcia et al., 2012b; Jayanthi et al., 2011; Specian et al., 2012).

The medicinal plant *Trichilia elegans* A. Juss. belongs to Meliaceae family and is abundant in southern Brazil. Preparations using leaves, seeds, bark and roots of this plant have been employed in traditional medicine, including the treatment of rheumatism and malaria, the induction of vomit and the use as purgative (Garcez et al., 1996). This plant harbors many endophytic fungi and among those molecularly identified (13 fungal isolates), 11 endophytes belong to *Diaporthe* (= *Phomopsis*) genus, as

shown by Rhoden et al. (2012a). However, few *T. elegans* endophytes were already investigated about their activity against human pathogenic bacteria: *Cordyceps memorabilis* GQ461583, *Diaporthe longicolla* GQ461584 and *Dothideomycete* sp. GQ461591 (Flores et al., 2013; Rhoden et al., 2012b).

OBJECTIVE

In virtue of the urgent necessity of finding new antimicrobial drugs and considering the shortage of information about antibacterial activity of *T. elegans* endophytes, the objective of this study was to evaluate the anti-*E. coli* activity of metabolic extracts produced by the endophytic fungus *Diaporthe* sp.GQ461588.

MATERIAL AND METHODS

Microorganisms and culture media

The endophytic fungus *Diaporthe* sp. (GenBank accession number GQ461588) belongs to the fungal culture collection of Laboratório de Biotecnologia Microbiana, Universidade Estadual de Maringá, and was isolated from the leaves of *T. elegans* located in the "Horto Florestal Dr. Luiz Teixeira Mendes", Maringá, Paraná, Brazil. Its molecular identification was based on sequencing of the ITS1-5.8S-ITS2 region of ribosomal DNA (rDNA) (Rhoden et al.,2012a).

The bacterium *Escherichia coli* ATCC 25922 was provided by the Laboratório de Microbiologia from the Universidade Estadual de Maringá, Brazil. Potato Dextrose Agar (PDA) and Potato Dextrose Broth (PDB) were prepared according to Smith & Onions (1983) modified by Pamphile et al. (2004). These media were respectively used for the fungal growth and obtainment of bioactive extract. Luria Bertani Agar (LBA) and Luria Bertani Broth (LBB) were prepared according to Sambrook & Russel (2001) and employed in the cup plate assay.

Obtainment of the crude extract from *Diaporthe* sp. GQ461588

The obtainment of the crude extract was performed according to the protocol described by Li et al. (2005) and outlined by Rhoden et al. (2012b). The endophytic fungus was cultivated on Petri dishes with PDA at 28ºC for seven days for obtaining young colonies. Then, tree mycelia fragments (5 mm^2) were inoculated into 500-mL Erlenmeyer flasks containing 250 mL of PDB and incubated at 28ºC for 15 days under stationary condition. After, the contents of the flasks were filtered with sterile gauze to separate the fungal mycelium from the fermented liquid medium and the mycelium was discarded. The liquid medium was filtered and centrifuged at 1300 x *g* for 20 min to separate the cellular debris. The supernatant (cell-free fermented medium) was transferred to a separatory funnel in which was added ethyl acetate (EtOAc) at a ratio of 1:1.After strong agitation, the separation of phases occurred by polarity difference and the EtOAc phase was collected. The fermented medium was extracted two more times with EtOAc (1:1). After, all the EtOAc phase collected was concentrated in a rotary evaporator (Marconi MA 120) at 50ºC for the solvent evaporation. Then the extract residue was dissolved in 1 mL of absolute methanol P.A. (99.8%) and stored at 4ºC until its use in cup plate assay.

Evaluation of *in vitro* anti-*E. coli* activity

For the cup plate assay, the bacterium *E. coli* ATCC 25922 was grown sequentially in LBA and LBB, for 24 h in each medium, adjusted at a concentration of 10^6 cells/mL and spread (100 µL) on Petri dishes with LBA. Each dish received four sterile Whatman No. 4 filter paper disks (6 mm) placed equidistant and inoculated with 10 µL of the crude extract. Paper disks were also inoculated with autoclaved distilled water and methanol (negative controls) and Tetracycline (Sigma) (50 µg/mL in absolute ethanol) as positive control. The tests were performed in triplicate. Dishes were incubated at 37ºC for 24 h and then the antibacterial activity was evaluated by the formation of inhibition halos, measured using the program Image j. 1.45S. The means

of inhibition halos (in mm) were considered for results.

RESULTS AND DISCUSSION

The crude extract from *Diaporthe* sp.GQ461588 presented anti-*E. coli* activity, since inhibition halos with means of 13.98±0.39mm diameter were measured (Figure 1a). The positive control with antibiotic Tetracycline produced inhibition halos with diameters of 27.77±0.09 mm (Figure 1b), while the negative controls showed the absence of inhibition halos (Figures 1c and 1d). Regarding the inhibition halos observed in this cup plate assay, Rocha et al. (2009) state that they can be considered an indication of antibiosis caused by antagonistic substances possibly produced in the culture medium. This result can be considered satisfactory even if the action of the fungal extract was inferior to the antibiotic, because it is a crude material that can be purified and/or produced in optimal conditions (that can be optimized in future studies), increasing its antibacterial action.

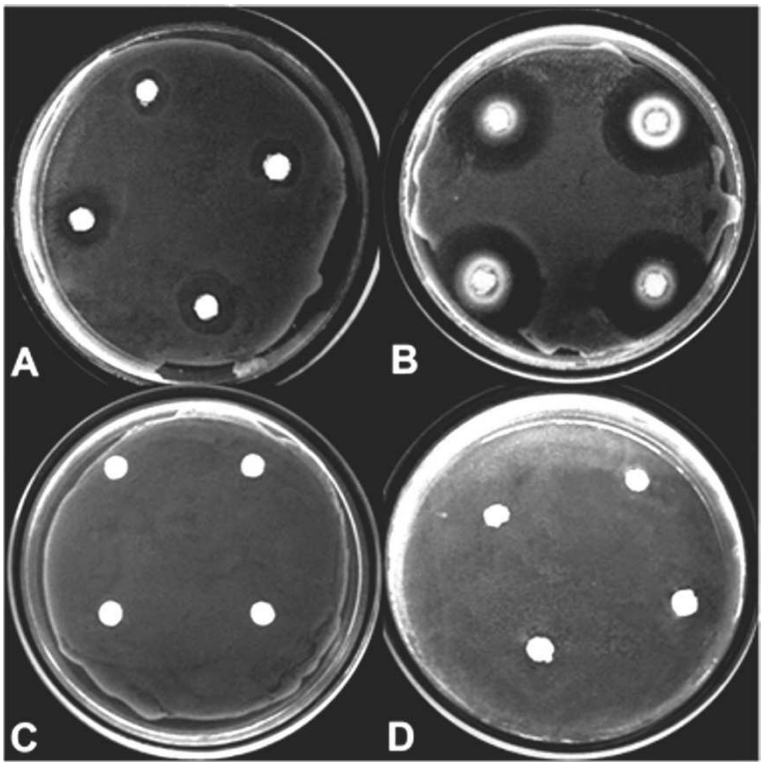

Figure 1. Cup plate assay: a) 13.98±0.39mm diameter halos (means of triplicates) produced by the fungal extract; b) 27.77±0.09mm diameter halos produced by antibiotic Tetracycline (positive control); c) negative control with distilled water and d) negative control with methanol, showing the absence of inhibition halos.

Figura 1. Ensaio *cup plate*: a) halos de 13,98±0,39 mm de diâmetro (média das triplicatas) produzidos pelo extrato fúngico; b) Halos de 27,77±0,09 mm de diâmetro produzidos pelo antibiótico Tetraciclina (controle positivo); c) controle negativo com água destilada e d) controle negativo com metanol, mostrando a ausência de halos de inibição.

Previously, Rhoden et al. (2012b) used the same protocols (culture conditions and cup plate assay) to evaluate the antimicrobial activity of crude extracts produced by some endophytes from *T. elegans*. The results of cup plate assay showed that *Diaporthe longicolla* GQ46158 inhibited *Salmonella typhi* ATCC 19430 and *Enterococcus hirae* ATCC 1227, without inhibiting *E. coli* ATCC 25922. A subsequent study (Flores et al., 2013) revealed that this fungal strain produced a secondary metabolite that was separated by column chromatography in five fractions including one identified as 3-nitropropionic acid; but over again, no anti-*E. coli* activity was detected,

although it was effective against other bacteria (*S. typhi* ATCC 19430, *Micrococcus luteus* ATCC 9341, and *Xanthomonas axonopodis* pv. *phaseoli* I227). Consistent with these previous investigations, the positive results verified for *Diaporthe* sp. GQ461588 can be highlighted: this fungal strain presented a biological activity not described for another endophyte from the same genus isolated from the same host plant. According to Orlandelli et al. (2015), different strains even members of the same fungal species, can exhibit specific metabolic productions, a fact that could explain the differences in the anti-*E.coli* activity of metabolic extracts produced by two closely related endophytes from same genus when cultivated under same conditions.

Similarly, Specian et al. (2012) investigated the bioactive metabolite from the endophyte *Diaporthe helianthi*, isolated from *Luehea divaricata*, against six different bacteria. Expressive anti-*E. coli* action was observed in cup plate test when the crude extract was used (inhibition halos of 10.7±0.5 mm). After separation of this extract into 10 fractions, the halos of each fraction varied between 7.2±0.2 and10.0±0.9 mm. The fraction identified as tyrosol generated halos of 7.8±0.0 mm against *E. coli* ATCC 25922. The tyrosol identified by Specian and co-authors and 3-nitropropionic acid (Flores et al., 2013) are only two of the antimicrobial compounds secreted by *Diaporthe* species. As reviewed by Flores et al. (2013), other bioactive secondary metabolites already reported for *Diaporthe* endophytes include: phomol, dicerandrol A and B, phomopsicalasine and isocumarin. Therefore, a diversity of substances could be involved in the anti-*E. coli* activity of the extract produced by *Diaporthe* sp. GQ461588. Only a chemical characterization (commonly given by nuclear magnetic resonance) will correctly determine the correlation between the structure and biological activity of this metabolic extract.

According to Schulz et al. (2002), approximately 80% of endophytic fungi studied produce biologically active compounds. Therefore, besides *Diaporthe* species, the *in vitro* antibacterial activity of different fungal genera has been investigated. Recently, Kalyanasundaram et al. (2015) observed varying degrees of growth inhibition of *E. coli* by the extracts produced by *Aspergillus niger* (4.0±0.10 mm), *Fusarium* sp. (4.3±0.57 mm), *Alternaria alternata* (5.6±0.57 mm), *Cladosporium* sp. (6.6±0.57 mm), *Aspergillus terreus* (9.3±1.52 mm), *Penicillium* sp. (9.6±0.57 mm) and *Meyerozyma* sp. (11.3±0.57 mm), endophytes isolated from the salt marsh plants (*Suaeda maritima* and S. *monoica*). As protocol difference, these authors inoculated 50 µL of fungal compound in each 6-mm cup plate. Therefore, we can highlight that a lower aliquot (10 µL) of the *Diaporthe* sp. GQ461588 extract presented an anti-*E. coli* activity(13.98±0.39 mm) more satisfactory than those observed for the ascomycetous fungi researched by Kalyanasundaram and co-authors.

In this current study the disk diffusion (cup plate) method was used for evaluating the antibacterial activity of the fungal extract against *E. coli* ATCC 25922. According to the American Type Culture Collection (ATCC), it is a strain originally isolated from a human clinical sample collected in Seattle, Washington, USA, in 1946 (Minogue et al., 2014). Lorian (2005) state that the ATCC strains are widely accepted as quality control strains for testing rapidly growing aerobic bacteria. Furthermore, this strain has identity (GenBank accession number CP009072) and physiological characteristics already defined, assuring the maintenance of the same culture conditions in any research study. An effective result against *E. coli* ATCC 25922 indicates that the antimicrobial agent can be further evaluated against other pathogenic *E. coli* strains.

Regarding the disk diffusion method, it consists in diffuse the drug or substance of interest through a solid medium commonly using substance-impregnated paper disks. The microbial sensitivity can be determined by measuring the zones (halos) of growth inhibition around the disks (Ghatage et al.,

2014; Rippere, 1978). As advantages, it is more flexible and cheaper than other methods, being easily performed (Edelmann et al., 2007; Gould, 2000). Although this method is often used, it is disadvantages includes the inability in determining an exact minimal inhibitory concentration (MIC) value and the difficulty in evaluating the susceptibility of slow-growing or fastidious microorganisms (Lee, 2009), being also little reproducible and standardized (Gould, 2000). According to Andrews (2001), MIC tests are considered the "gold standard" for determining the susceptibility of organisms to antimicrobial drugs, being used to judge the performance of all other methods. Future MIC tests could confirm and expand the result showed herein, defining the lowest concentration of crude extract necessary to inhibit the *E. coli* growth.

One of the most important fungal properties is the metabolic capacity of producing a wide range of bioactive small molecules. However, these microorganisms are also responsible for the production of substances highly toxic to mammals (Pinto et al., 2002). To guarantee the safety of a new drug its possible toxic action needs to be tested. The toxicity tests can be defined as procedures in which the responses of test-organisms are used to detect and quantify the presence of adverse effects of one substance (Laitano & Matias, 2006). According to Rogero et al. (2003), the International Standards Organization (ISO) 10993 considers the *in vitro* cytotoxicity assay as the first test for evaluating the biocompatibility of any substance for use in biomedical devices. These reproducible, sensitive and rapid tests use mammalian cells to evaluate cell damages, formation of cell colonies and cell viability. Once confirmed as no citotoxic, the product can be applied in the *in vivo* toxicity assays, as those animals-based tests (Bednarczuk et al., 2010; Rogero et al., 2003). Consistent with it, this current study with *Diaporthe* sp. GQ461588 can be considered an important initial result for the obtainment of a new antibacterial compound. In future investigations, it is expected to use toxicity tests to evaluate the safety of this fungal extract.

CONCLUSION

The crude extract produced by the endophytic fungus *Diaporthe* sp. GQ461588 isolated from *T. elegans* was effective for the inhibition of *E. coli*. The use of this endophyte and its metabolic extract can be investigated in subsequent studies aiming to expand the knowledge about its possible pharmaceutical use.

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