

Full Length Research Paper

Antimicrobial activity of crude extracts of endophytic fungi from *Oryctanthus alveolatus* (Kunth) Kuijt (Mistletoe)

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The use of antibiotics has unleashed high bacterial resistance. This outcome triggered the urgent need for effective new antibacterial agents to treat infectious diseases. A promising source for the production of antibiotics and several other bioactive substances are endophytic fungi. These microorganisms inhabit in and bring benefits to living plant tissues. Thus, the aim of this paper was to know the endophytic fungi associated with *Oryctanthus alveolatus* (mistletoe) and assess their potential to inhibit pathogenic bacteria. A total of 86 endophytic fungi were isolated from the stems and leaves of *O. alveolatus*. Of these fungi, 29 were selected for the assessment of antimicrobial activity. The antimicrobial activity of the obtained extracts was evaluated using the agar diffusion method towards two Gram-positive pathogenic bacteria (*Staphylococcus aureus* and *Staphylococcus epidermidis*) and two Gram-negative bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*). The extracts were tested at concentrations of 200, 500, 700, 800, 900 and 1000 µg.mL⁻¹. The antimicrobial test showed that two (COA 009 and 014) of the 29 extracts inhibited bacterial growth of at least one of the strains each, from both Gram-positive and Gram-negative bacteria. The extracts with the inhibitory activity were derived from the fungi *Curvularia* sp. (COA 009) and *Diaporthe* sp. (COA 014).

Key words: Gram-positive and Gram-negative bacteria, microorganisms, Loranthaceae.

INTRODUCTION

There are an estimated 2.2 to 3.8 million fungal species worldwide, and more than 120,000 of these have been formally described (Hawksworth and Lücking, 2017). These microorganisms include endophytic fungi, which, according to the definition of Bacon and White, inhabit

internal plant tissue without causing any immediate harm (Chapla et al., 2013; Kharwar et al., 2011; Kaul et al., 2012).

Endophytes are an important component of plant microbiota. They reside in the living tissue of almost all plants in a range of relationships, from symbiosis to

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balanced antagonism (Qadri et al., 2014). This group of microorganisms has multiple functions within plant communities, including nutritional effects, protection against pathogens that harm roots, modifications of environmental tolerances, and involvement in the dynamics of plant communities (Bonfim et al., 2016).

For this study, we assessed the production of antimicrobial secondary metabolites produced by endophytic fungi obtained from the species *Oryctanthus alveolatus*. This plant, commonly known as mistletoe, like other species of the family Loranthaceae, is a parasite found on the branches and trunks of trees (Vinod et al., 2005). The species occurs in temperate to tropical climates of Central and South America, Europe, Africa, the Middle East, and throughout Asia and Australia, and it currently consists of 76 genera and 1076 species (Grimsson et al., 2017). The species *O. alveolatus* is used in folk medicine to treat several diseases, such as stomach cancer (Scudeller et al., 2009), malaria, and the treatment of fractures (Salgado, 2007). It has antioxidant properties and chemical studies have shown the presence of flavonoids, tannins, and polyphenols in its leaves and stems (Coba et al., 2010).

Antibiotics or antimicrobial substances are a special group of therapeutic agents, usually produced and obtained from living organisms (Cowan, 1999). Endophytic fungi are known to produce important bioactive agents that inhibit or eliminate a wide variety of microorganisms, including plant pathogens, bacteria, fungi and protozoa, considered harmful to humans and animals (Cai et al., 2017).

These microorganisms produce a broad spectrum of antimicrobial substances belonging to various structural classes, including peptides, steroids, alkaloids, terpenoids, phenols, phenylpropanoids, aliphatic compounds, polyketides, quinones, and flavonoids (Malhadas et al., 2017). In the genus *Phoma* for example, the antimicrobial activity of dominant species isolated from the plant *Salvia involucreta* in China, together with *Cladosporium*, can fight human pathogenic fungi and bacteria (Bonfim et al., 2016). As in the study of Zhao et al. (2015), one of the isolated substances of the endophytic fungus *Neopestalotiopsis* sp. showed antimicrobial activity against three bacteria. The fungi *Colletotrichum pisi*, *Fusarium oxysporum*, *Fusarium solani*, *Phoma terrestris*, and another two unknown fungi showed antimicrobial activity against some of the pathogens of ginseng (Park et al., 2015). These data indicate endophytic fungi help synthesise bioactive agents used by plants as protection against other pathogens (Ayob and Simarani, 2016).

The bacterial species known ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter*) cause most hospital-acquired infections and effectively "escape" from the effects of antibacterial drugs. The resistance of

these bacteria to antibiotics has become a global concern and calls for the search for new antibacterial agents (Radić and Strukelj, 2012).

Consequently, the aim of this paper was to assess the crude extracts of 29 endophytic fungi isolated from leaves and stems of the plant *O. alveolatus* against Gram-positive and Gram-negative bacteria.

METHODOLOGY

Plant materials

Samples of healthy-looking leaves and stems of the species *O. alveolatus* were collected from the Institute of Exact Sciences and Technology/UFAM, Campus in Itacoatiara – AM, in March and May 2013. A voucher herbarium specimen (n° 244064) was deposited at the herbarium of the National Research Institute of the Amazon (INPA).

Isolation of endophytic fungi

Following the method of Maier (1997), the stems were washed, immersed in a sequence of 70% ethanol (3 min), 1% NaClO (5 min), again in 70% ethanol (3 min), and double washed in sterile water for 5 min per wash. The leaves were soaked in 70% ethanol (1 min), 1% NaClO (3 min) and again in 70% ethanol (1 min), followed by double washing in sterile water for 5 min per wash. A small portion of the second water collected during from the second double washing of the stems and leaves were plated as negative control.

After disinfection, parts of the stems and leaves were cut with a scalpel into fragments with approximate 0.5 cm². The fragments were placed in Petri dishes containing potato dextrose agar medium supplemented with gentamicin (100 µg.mL⁻¹) to prevent bacterial growth. The plates were incubated for 24 to 72 h in incubator at 25°C and microbial growth was monitored with the naked eye. After fungal growth, samples were purified in a solid medium, characterised and enumerated with codes according to part of the plant that was removed.

The isolates were coded as follows: FOA corresponds to the fungi removed from the leaves and COA corresponds to the fungi removed from the stems of the plant. Once the colonies were isolated, the endophytic fungi were preserved in agar slants and sterile water.

Preparation of extracts

The extracts were prepared according to the method of Oliveira et al. (2011). Each of the 29 fungi were picked and placed into two Petri dishes containing a potato dextrose agar medium and incubated at 26°C in incubator for up to ten days, depending on the growth period of each fungus. After this time, each isolate was picked and transferred into four Erlenmeyer flasks containing 200 mL of the potato dextrose broth medium, totalling 800 mL of fungus culture. The cultures were grown statically at room temperature (28°C) for 20 days. After incubation, the broth was separated from the mycelium by filtration and extracted with EtOAc three times (50% of the broth volume each). The fraction containing organic solvent was placed in a rotational-evaporator under reduced pressure to obtain the crude extracts.

Antimicrobial activity test

The test to determine antimicrobial activity was conducted using the

Table 1. Antibacterial activity of extracts obtained from endophytic fungi isolated from *Oryctanthus alveolatus* against human pathogenic bacteria.

Concentration ($\mu\text{g.mL}^{-1}$)	Bacteria	Halo diameter (mm \pm SD)
COA 014 (1000)	<i>E. coli</i>	9.00 \pm 0.00 ^a
COA 014 (1000)	<i>P. aeruginosa</i>	8.50 \pm 0.71 ^{ab}
COA 009 (1000)	<i>S. aureus</i>	7.67 \pm 1.15 ^{abc}
COA 014 (900)	<i>E. coli</i>	7.02 \pm 0.50 ^{bc}
COA 009 (1000)	<i>E. coli</i>	7.00 \pm 0.00 ^{bc}
COA 014 (1000)	<i>S. aureus</i>	6.00 \pm 0.00 ^c

The data presented are means \pm the standard deviation of three independent experiments. Different letters indicate significant differences ($P < 0.05$) by the Tukey's test.

agar diffusion method originally described by Bauer et al. (1966) and updates from the National Committee for Clinical Laboratory Standards (NCCLS, 2009). The bacteria used were Gram-positive bacteria *S. aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228) and Gram-negative *P. aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922).

Initially, bacterial strains were inoculated on a sterile Muller Hinton agar plate and incubated at 37°C for 24 h. Bacterial strains were then sub-cultured in tryptic soy broth medium for 24 h before testing. Antibiotic discs were placed on the surface of the plate containing the bacteria using sterile tweezers. The crude extracts were diluted in dimethyl sulfoxide at concentrations of 200-1000 $\mu\text{g.mL}^{-1}$. For each concentration, 10 μL of liquid extract was placed on filter paper discs of approximately 6 mm in diameter. All plates were incubated at 37°C for 24 h. The term active is attributed to paper discs containing crude extracts displaying any halo zone of inhibition of bacterial growth (CLSI, 2009). The diameter of inhibition zones were recorded (in mm) using a calliper. All the trials were conducted in triplicate, considering the average diameter of the respective inhibition halos (Bauer et al., 1966). As the positive control, 5 μg ciprofloxacin was used for the bacteria *S. aureus* and *S. epidermidis* and discs of amikacin at 30 μg were used for the bacteria *P. aeruginosa* and *E. coli*. As the negative control 10 μL dimethyl sulfoxide was used.

Fungal identification

The fungal isolates that produced extracts with inhibitory activity were identified by morphology and DNA sequencing. Since the fungi exhibited asexual spores, we carried out axenic cultures by single-spore isolation. Subsequently, the fungi were grown on malt agar 2% (MA2%) at 25°C for seven days, in darkness. The macroscopic characteristics of the colonies and the microscopic reproductive structures (conidiophores and conidia) were examined. For the latter assessment, semi-permanent slides were prepared using water as the mounting fluid. The structures were examined under a compound microscope (Leica DM500).

Genomic DNA was extracted from mycelia of seven days-old cultures grown in MA2% (conditions same as above). DNA extraction followed the protocol used in Montoya et al. (2016). The DNA barcoding region ITS (Internal Transcribed Spacer) was amplified using primers ITS4/ITS5 and bidirectionally sequenced by the Sanger method in an ABI sequencer. PCR and sequencing protocols were the same as in Montoya et al. (2016).

The generated sequences were assembled in consensus sequences in Bioedit v. 7.0.5 which were later compared with those deposited in NCBI-GenBank and MycoBank. Sequences were deposited in the NCBI-GenBank under accessions #: MG847099 (for isolate COA 009) and MG847100 (for isolate COA 014).

Homologous sequences as well as others from previous studies (Udayanga et al., 2012; Jeon et al., 2015) were retrieved from the NCBI-GenBank. Alignments were performed separately for each isolate using MAFFT v.7. The phylogenetic trees were inferred in MEGA7 using the neighbor joining method with 1000 pseudoreplicates to calculate the bootstrap value.

RESULTS AND DISCUSSION

A total of 86 fungi were isolated, of which 29 were active for posterior use in the study. Of the 29 crude extracts, only extracts COA 009 and COA 014, showed activity against two or more bacteria. The present finding is in agreement with Nascimento et al. (2015) and Sebastianes et al. (2013), that the stem is the most vascularized organ of the plant, allows greater nutrient acquisition and favours the development of other microorganisms such as bacteria, leading the fungus to produce antimicrobial substances for its protection.

The COA 014 extract displayed antimicrobial potential against the bacteria *P. aeruginosa* and *S. aureus* at a concentration of 1000 $\mu\text{g.mL}^{-1}$. Similar data were obtained in the studies of Atiphasaworn et al. (2017) for the crude extract obtained from the endophytic fungus *Aspergillus* sp. MFLUCC16-0613, active in this same concentration. However, when compared to activities against *E. coli*, the COA 014 extract had an average inhibition halo of 9 mm with no variations in the three samples. This value is higher than the value obtained for the fungus *Aspergillus* sp. MFLUCC16-0613 of (7.1 \pm 1.2) at a concentration of 1000 $\mu\text{g.mL}^{-1}$. None of the extracts exhibited inhibitory activity against the bacterium *S. epidermidis*. The higher susceptibility of extracts in the Gram-negative bacteria can be related to the morphological differences between them and the Gram-positive bacteria. The Gram-negative bacteria exhibited greater sensitivity, indicating the raw extracts have selective action against the chemical composition of the bacterial cell wall. This positive action may be caused by the polarity of the extract and its chemical constituents. The concentrations of 900 $\mu\text{g.mL}^{-1}$ and 1000 $\mu\text{g.mL}^{-1}$ were active in the tests.

In the statistical analyses (Table 1), the average

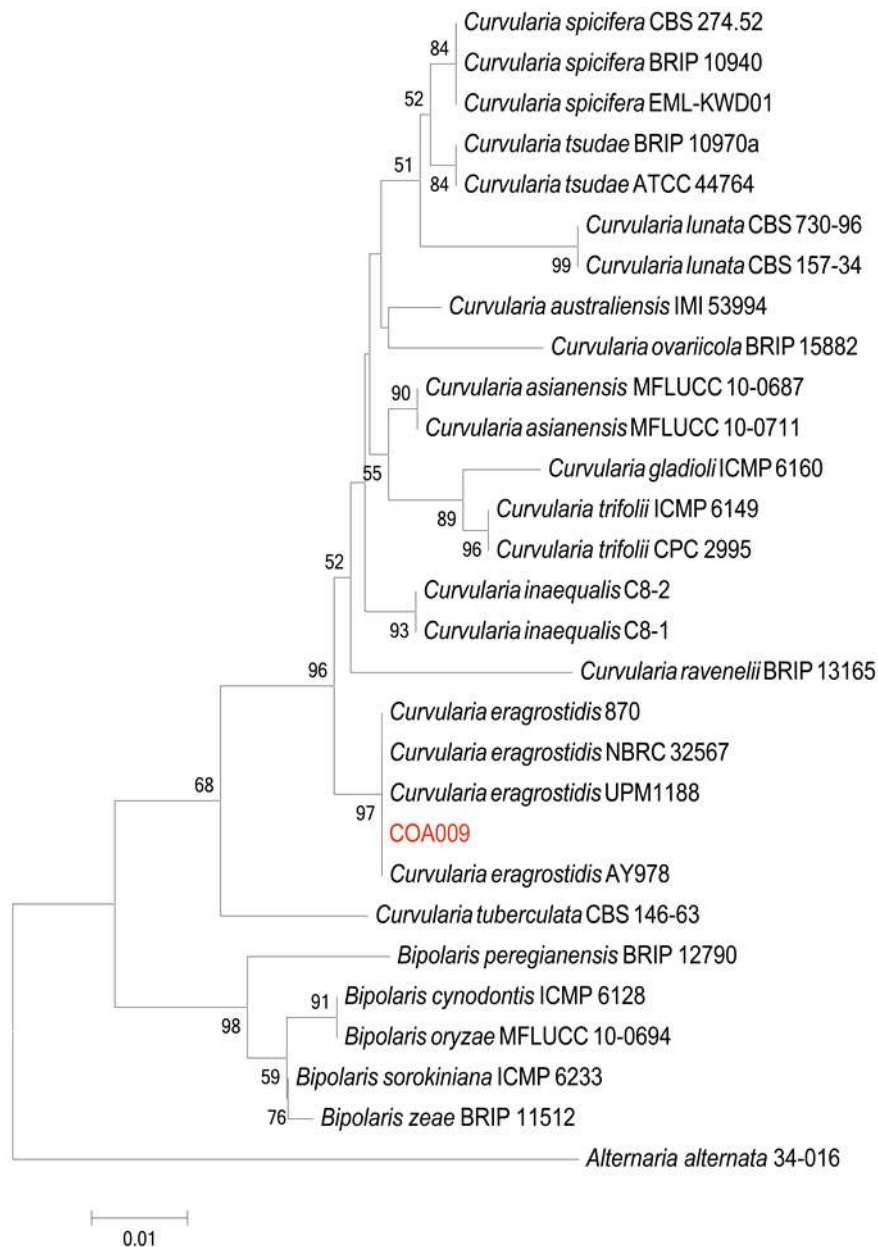


Figure 1. Phylogenetic tree of *Curvularia* species including isolate COA009 (in red). The phylogenetic analysis was based in 29 sequences of the ITS region. The final alignment has 476 bases pair. The tree was built using the neighbour joining algorithm with 1000 of pseudoreplicates. The numbers on branches indicate bootstrap values. *Alternaria alternata* was used as outgroup.

inhibition halo measurements of the endophytic fungi (COA 009 and COA 014) against the bacteria (*E. coli*, *S. aureus* and *P. aeruginosa*) were compared using the Tukey test ($p < 0.05$). Extracts COA 009 ($1000 \mu\text{g.mL}^{-1}$) and COA 014 ($900 \mu\text{g.mL}^{-1}$) against the bacterium *E. coli* only exhibited a significant difference with extract COA 014 ($1000 \mu\text{g.mL}^{-1}$) against the same bacterium. The COA 014 extract ($1000 \mu\text{g.mL}^{-1}$) showed no significant difference against the bacteria *E. coli* and *P. aeruginosa* but showed a significant difference when compared with

S. aureus. The COA 009 extract ($1000 \mu\text{g.mL}^{-1}$) against the bacterium *S. aureus* showed no significant difference with any other case.

The morphological characteristics of each fungi coupled with the phylogenetic analyses made it possible the identification of isolates COA 009 and COA 014 to the genus level (Figures 1 and 2). Isolate COA 009 showed 99% similarity with sequences belonging to *Curvularia* species. The ITS sequence of isolate COA 014 showed 97% similarity with sequences of two *Diaporthe* species:

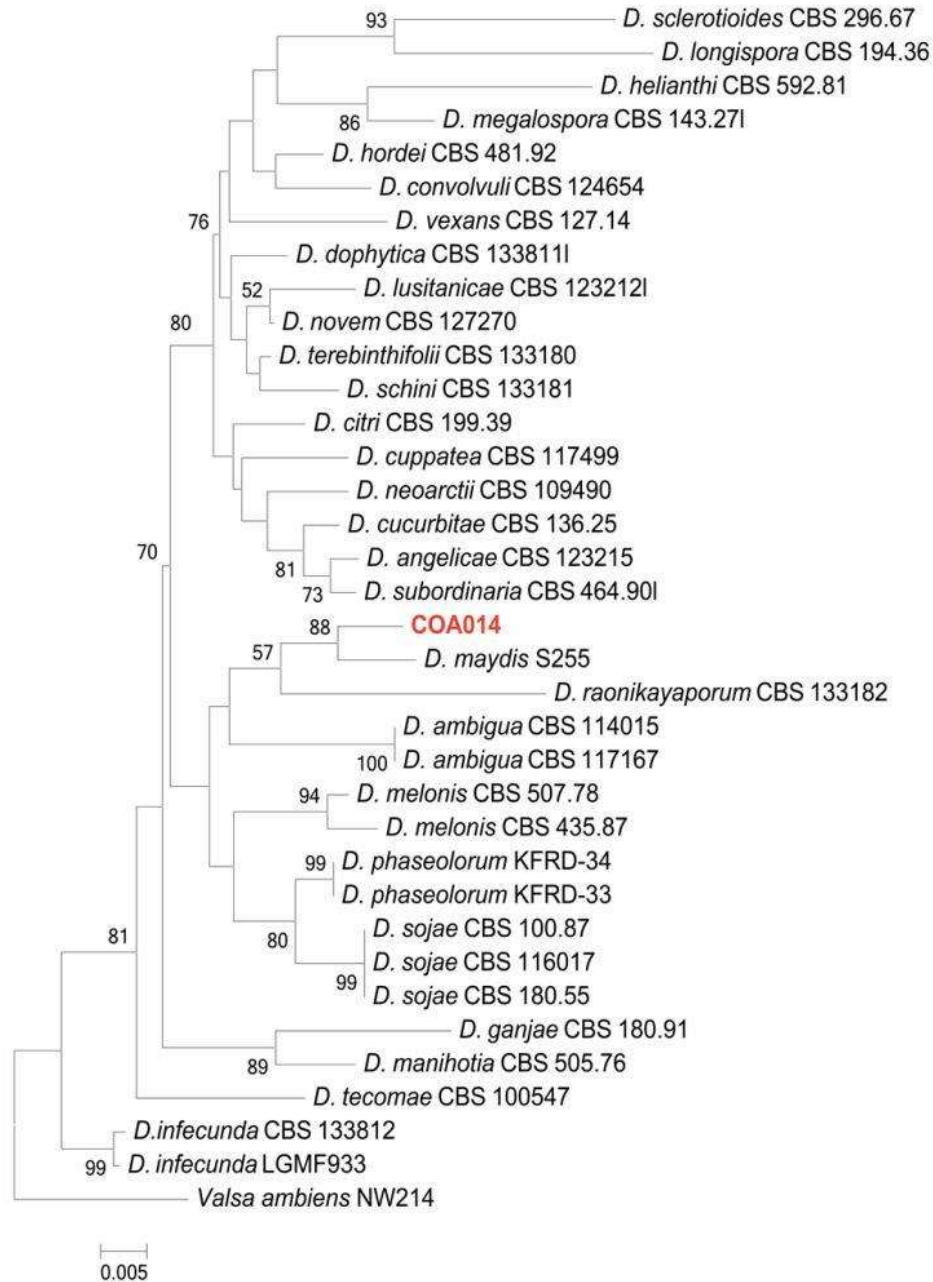


Figure 2. Phylogenetic tree of *Diaporthe* species including the strain COA014 (in red). The phylogenetic analysis was based in 36 sequences of the ITS region. The final alignment has 587 bases pair. The tree was built using the neighbour joining algorithm with 1000 of pseudoreplicates. The numbers on branches indicate bootstrap values. *Valsa ambiens* was used as outgroup.

D. miriciae BRIP 54736j and *D. ambigua* CBS 114015 deposited in NCBI-GenBank. Contig COA 014 also showed 97% similarity with an unidentified *Diaporthe* species deposited in Mycobank. Due to the low similarity with sequences found in the databases, isolate COA 014 may represent an undescribed species. To further identify both isolates to the species level, further taxonomic

studies including analysis of additional DNA regions and an in-depth morphological analysis is required.

Conclusion

The results of this work indicate the potential of

endophytic fungi *Diaporthe* sp. and *Curvularia* sp. isolated from the species *O. alveolatus* as producers of compounds with antimicrobial activity. Further studies to isolate and identify the biologically active compounds of these endophytic fungi will be conducted to explore their potential. It should be stressed, however, that these were the first isolated microorganisms to be obtained from the plant species *O. alveolatus*.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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