

Full Length Research Paper

Antibacterial activity of endophytic fungi from the medicinal plant *Uncaria tomentosa* (Willd.) DC

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This study was designed to determine the diversity and antibacterial activity of endophytic fungi isolated from *Uncaria tomentosa*. Leaf and stem were disinfected superficially and inoculated in PDA and SDA medium, with and without plant extract and incubated at 18 and 28°C for isolation of endophytic fungi. Endophytic fungi were inoculated in BD medium and the metabolites extracted with ethyl acetate. Endophytic fungi extracts were tested for antibacterial activity by the disk diffusion test. One hundred and seventy endophytic fungi were isolated and identified as *Aspergillus*, *Asterosporium*, *Aureobasidium*, *Botrytis*, *Colletotrichum*, *Curvularia*, *Didymostilbe*, *Fusarium*, *Guignardia*, *Nigrospora*, *Penicillium*, *Pestalotiopsis*, *Phomopsis*, and sterile mycelium. *Staphylococcus aureus* was the most resistant bacterium, with only two fungal extracts inhibiting its growth, while the most sensitive was *Escherichia coli*, with 23 extracts inhibiting its growth. Five extracts inhibited *Enterococcus faecalis* and four *Klebsiella pneumoniae*. No fungal extract was able to inhibit the four tested bacteria. Extracts from endophytic fungi isolated from *U. tomentosa* showed *in vitro* antibacterial activity against gram-positive and gram-negative bacteria.

Key words: Cat's claw, microbial ecology, antibiotics.

INTRODUCTION

Endophytes are microorganisms that colonize internal tissues of plants for at least part of their life cycle without causing disease symptoms in their hosts (Petrini, 1991). Fungal endophytes can inhabit host tissues in different organs, including leaves, stems, barks, roots, fruits, flowers, and seeds (Stone et al., 2004). In this symbiotic relationship, fungal endophytes receive protection and nutrients from the host, while the host plant receives protection against natural enemies, such as pathogens

and herbivores (Azevedo et al., 2000), promoting plant growth (Hamayun et al., 2010) and increasing its resistance to abiotic stress factors (Khan et al., 2014).

Many medicinal plants are known to harbor endophytic fungi, which are producers of important bioactive secondary metabolites for the industry. Therefore, efforts have been made to characterize and identify endophytic fungi isolated from medicinal plants (Strobel et al., 2004). *Uncaria tomentosa* (Willd.) DC belongs to the

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Rubiaceae family, being a medicinal plant widely used by Amazon peoples. This species is used to treat infections, rheumatism, gastritis, cancer, asthma, cirrhosis, fever, and has a wide range of other medicinal applications (Keplinger et al., 1998; Dreifuss et al., 2013). Several chemical compounds, such as oxindole alkaloids and quinolinic acids, have anti-inflammatory (Akhtar et al., 2011), anticancer (Dietrich et al., 2014), and antimicrobial activity (Sá et al., 2014). However, there are no studies on the endophytic community of this plant. Thus, this study was designed to determine the diversity and antibacterial activity of endophytic fungi isolated from *U. tomentosa*.

MATERIALS AND METHODS

Plant samples

Healthy and mature plant tissues were collected from three *U. tomentosa* trees in Rio Branco, Acre, Brazil (10°01' S and 67°42' W) in September 2015. Voucher specimens were deposited in the Herbarium of the Universidade Federal do Acre under the identification number 22.002. Leaves and stems were collected from each plant and brought directly to the laboratory, being processed within 24 h after collection (Azevedo et al., 2010).

Isolation of endophytic fungi

Each sample of plant material was washed with running water and surface sterilized with 70% ethanol for 1 min, followed by treatment with 2.5% active chlorine solution for 3 min, 70% ethanol for 30 s, and final rinsing in sterile water (Pereira et al., 1993). Prior to surface decontamination, the ends of stem fragments were sealed with paraffin to prevent the entry of germicidal agents into the plant tissue and thus inhibit the death of endophytic fungi. To assess whether the disinfection method was effective in the removal of fungi from the surface, 200 µL wash water were inoculated in the same culture media used for the isolation of endophytic fungi, and these plates were observed for emergent fungi (Azevedo et al., 2010).

After superficial disinfection, two plates of potato dextrose agar (PDA), Sabouraud dextrose agar (SDA), PDA+plant extract, and SDA+plant extract, supplemented with chloramphenicol (100 µg mL⁻¹), each of them containing 10 fragments of plant material, were prepared for each of the two types of samples (leaf and stem) and maintained in the dark at 18 and 28°C. For producing the plant tissue extract, 100 g of fresh tissue were ground in 500 mL distilled water, filtered on filter paper, and 500 mL of an infusion of 200 g of potato were added to prepare PDA+extract medium or 500 mL distilled water for SDA+extract (Lima et al., 2011).

Fragments of mycelium emerging from plant fragments were transferred to new PDA plates without chloramphenicol to obtain pure cultures for identification (Azevedo et al., 2010).

Identification of endophytic fungi

Fungal cultures were maintained at ambient temperature (22 to 25°C) under natural photoperiod for 14 days and then visually examined regarding macroscopic (morphology, size, mycelial and agar color) and microscopic (presence of spores or other reproductive structures) characteristics (Barnett and Hunter, 1998). Isolates with similar morphological characteristics were grouped

into morphospecies. Each morphospecies is represented by several isolates, being an isolate representative of each selected for microscopic identification and antibacterial activity (Azevedo et al., 2010).

Antibacterial test

A fungus from each morphospecies was inoculated in PDA medium and incubated at 28°C for 14 days, and ten 5 × 5 mm plugs were inoculated into 20 mL potato dextrose broth (PD) incubated at 28°C, without agitation, for 14 days. Moreover, 2 mL of medium containing fungal metabolites were extracted by a liquid-liquid partition with ethyl acetate and solubilized in 300 µL dimethylsulfoxide 99.9%(DMSO) (Azevedo et al., 2010).

Antibacterial activity of fungal extracts was performed by the disc diffusion method against the pathogenic bacteria *Staphylococcus aureus* (ATCC 12598), *Streptococcus pneumoniae* (ATCC 11733), *Enterococcus faecalis* (ATCC 4083), *Escherichia coli* (ATCC 10536), and *Klebsiella pneumoniae* (ATCC 700603) (CLSI 2003).

Pathogenic bacteria were cultured at 3°C for 4 to 6 h and their turbidity adjusted to 0.5 McFarland scale. Bacteria were inoculated into Petri dishes containing Muller-Hinton (MH) medium, deposited on these paper discs, and then 20 µL of endophytic fungal extracts and incubated at 37°C for 24 h. The endophytic extract that did not allow bacterial growth around the disc was considered as having antibacterial activity and the inhibition halos produced were measured in millimeters (CLSI, 2003). Antibacterial tests were done in triplicate.

Statistical analysis of data

The infection index (FI) was calculated from the relationship between the number of fragments from which the endophytic fungi emerged and the total number of fragments used in the experiment (Azevedo et al., 2010).

The relative frequency of isolation (RF) was calculated as the number of isolates of a species divided by the total number of isolates, being expressed as a percentage.

For the diversity analysis of the endophytic community of *U. tomentosa*, the number of dominant species was calculated by using the Simpson and Shannon indices. The formula for calculating the Simpson diversity index is $1 - \sum (pi)^2$. Shannon-Wiener diversity (H') = $-\sum pi \ln pi$, where pi is the proportion of species colonization frequency in a sample. Equivalence of Evenness (E) was calculated by using the following formula: $E = H' / \ln S$, where S is the number of species in the sample (Bezerra et al., 2015).

RESULTS AND DISCUSSION

Isolation and identification of endophytic fungi

A total of 170 isolates belonging to 101 morphospecies, including isolates from sterile mycelium, were obtained from leaves and stems of *U. tomentosa* (Table 1). Isolation frequency of endophytic fungi was 89.6%, being higher in leaves (93.7%) than in stem (85.6%).

More endophytes were recovered from leaves (54.12%) than stems (45.88%) (Table 1). This difference may be related to the anatomical characteristics of *U. tomentosa*, which is a liana vine with more stems than leaves, facilitating the entry of microorganisms by

Table 1. Number and relative frequency percentages of endophytic fungi isolated from *Uncaria tomentosa* according to plant tissue, culture medium, and temperature.

Genus	Plant tissue		Culture medium						Temperature		T	RF(%)
	Leaf	Stem	PDA	PDA+Leaf	PDA+Stem	SDA	SDA+Leaf	SDA+Stem	18°C	28°C		
<i>Penicillium</i>	7	8	5	2	-	5	-	3	4	11	15	8.82
<i>Nigrospora</i>	12	1	1	-	-	10	2	-	5	8	13	7.65
<i>Colletotrichum</i>	10	2	-	3	-	5	4	-	4	8	12	7.06
<i>Pestalotiopsis</i>	1	10	4	1	1	3	-	2	4	7	11	6.47
<i>Curvularia</i>	2	8	5	-	2	1	1	1	2	8	10	5.88
<i>Phomopsis</i>	9	-	5	-	-	2	2	-	3	6	9	5.29
<i>Fusarium</i>	1	4	5	-	-	-	-	-	4	1	5	2.94
<i>Guignardia</i>	3	-	-	-	-	3	-	-	-	3	3	1.76
<i>Aspergillus</i>	-	2	1	-	-	-	-	1	-	2	2	1.18
<i>Asterosporium</i>	-	1	-	-	-	1	-	-	1	-	1	0.59
<i>Aureobasidium</i>	-	1	1	-	-	-	-	-	1	-	1	0.59
<i>Botrytis</i>	-	1	-	-	1	-	-	-	-	1	1	0.59
<i>Didymostilbe</i>	1	-	-	1	-	-	-	-	-	1	1	0.59
Unknown	46	40	27	7	11	15	13	13	50	36	86	50.59
Total	92	78	54	14	15	45	22	20	78	92	170	-
RF (%)	54.12	45.88	31.76	8.24	8.82	26.47	12.94	11.76	45.88	54.12	-	-

T: Total identified in the sample; RF: relative frequency of endophytic fungi (%).

stomata and leaf grooves, as well as some fungi with hyphal growth on the leaf surface (Wagner and Lewis, 2000).

Among the total isolated species, 28.23% were Hyphomycetes, 21.18% were Coelomycetes, and 50.59% were sterile mycelium. Among the endophytic species, *Penicillium* (8.82%), *Nigrospora* (7.65%), *Colletotrichum* (7.06%), and sterile mycelium (50.59%) predominated. As specialists and isolated only once, *Asterosporium*, *Aureobasidium*, *Botrytis*, and *Didymostilbe* were observed. These fungi indicated an intimate relationship with this plant, which suggests a genotypic interaction between fungus and plant, which may depend exclusively on the plant for its survival (Malcolm et al., 2013).

The highest recovery rate of endophytic fungi of *U. tomentosa* may also be related to the variation in the used nutritional and environmental conditions (Huang et al., 2007; Putra et al., 2015).

A different genus of endophytic fungi was isolated. Some of them are common in tropical regions and are often isolated in this type of study, being called generalists. However, other endophytic fungi are not very frequent and are known as specialists. Those with a preference for a particular culture medium, tissue, and/or temperature were classified as specialists of this isolation condition (Toju et al., 2013).

Among the culture media used, the highest fungal recovery occurred in PDA regardless of the type of tissue used, with 54 isolates (31.76%), followed by SDA, with 45 isolates (26.47%) (Figure 1). Some fungal genera showed

to be specialists in relation to the culture medium. *Fusarium* was isolated only in PDA medium, *Botrytis* and *Didymostilbe* in PDA+extract, and *Guignardia*, *Asterosporium*, and *Aureobasidium* in SDA, showing the need to use different nutritional sources to increase the recovery rate and richness of endophytic fungi. Studies on endophytic fungal isolation usually use only PDA medium (Hilarino et al., 2011; Katoch et al., 2014; Bezerra et al., 2015).

Another factor of relevance in this study was the isolation temperature. Lower temperatures, such as 18°C, allow the development of fastidious fungi (Souza Leite et al., 2013). However, for *U. tomentosa*, a temperature of 28°C provided the highest number of endophytes with 90 isolates (52.94%).

Some fungal genera also presented specificity to the isolation temperature, being isolated only at 18 or 28°C. *Asterosporium* and *Aureobasidium* were isolated only at 18°C, while *Guignardia*, *Aspergillus*, *Botrytis*, and *Didymostilbe* only at 28°C. Studies on endophytic fungi generally use temperatures between 25 and 28°C (Premalatha and Kalra, 2013; Campos et al., 2015; Ferreira et al., 2015).

Some fungi were not identified due to the absence of reproductive structures, called sterile mycelium. In *U. tomentosa*, 86 isolates could not be identified, representing 50.59%.

The diversity of the endophytic community isolated from different *U. tomentosa* tissues was compared by using α -diversity indices. Simpson diversity of endophytic

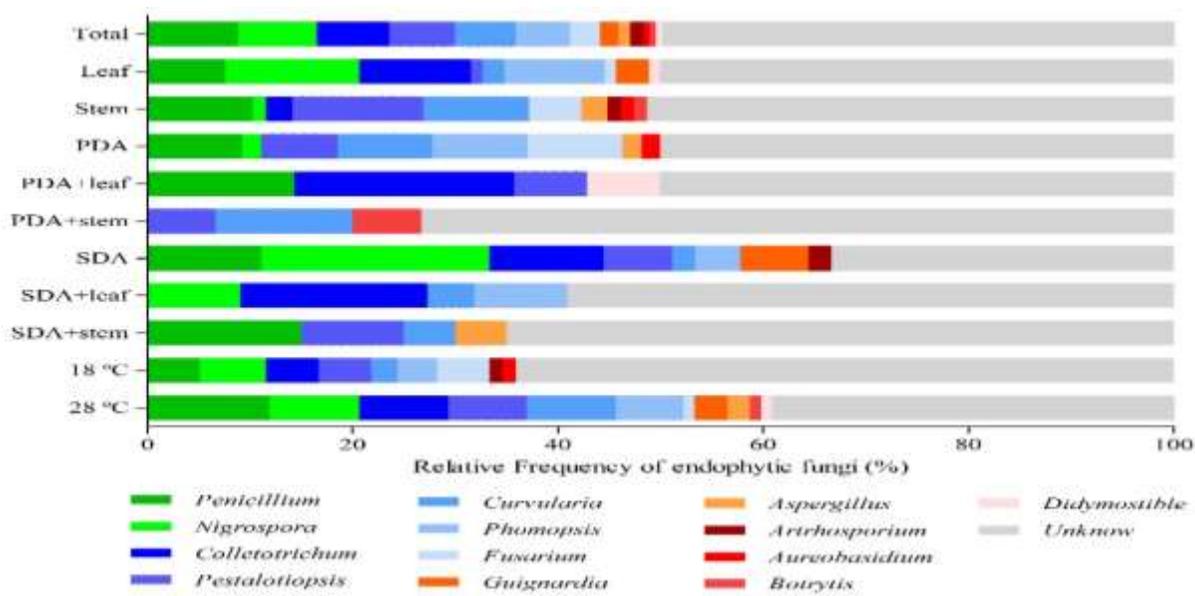


Figure 1. Endophytic fungal communities isolated from *Uncaria tomentosa* according to plant tissue, culture medium, and temperature.

Table 2. Diversity indices of endophytic fungi from *Uncaria tomentosa* according to plant tissue, culture medium, and temperature.

Diversity index	Abundance	Species richness	Shannon-Wiener diversity	Simpson diversity	Species evenness
Tissue					
Leaf	92	49	3.79	0.98	0.84
Stem	78	52	3.85	0.98	0.88
Culture medium					
PDA	54	36	3.47	0.97	0.87
PDA+leaf	14	08	1.97	0.85	0.75
PDA+stem	15	10	2.21	0.88	0.82
SDA	45	24	3.07	0.95	0.81
SDA+leaf	22	11	2.33	0.90	0.75
SDA+stem	20	12	2.39	0.90	0.80
Temperature					
18°C	78	46	3.72	0.98	0.85
28°C	90	54	3.88	0.98	0.86
Total sample	170	101	4.51	0.99	0.88

fungi was the same for both tissues. Both the Shannon-Wiener diversity and Evenness indices were higher in the stem. Species richness was also higher in the stem (Table 2).

Antibacterial activity

Among the 98 endophytic fungal extracts selected for

testing against pathogenic strains, 23 were positive against at least one of the tested pathogenic bacteria. Five extracts were active against *E. faecalis*, two against *S. aureus*, four against *K. pneumoniae*, and 23 against *E. coli* (Table 3).

Extracts from *Penicillium* spp. 2 (2.378), *Penicillium* spp. 4 (2.4055), and *Fusarium* spp. 1 (2.3952) showed antibacterial activity against gram-positive and gram-negative bacteria (*S. aureus*, *E. coli*, and *K. pneumoniae*)

Table 3. Antibiosis results of extract from endophytic fungi isolated from of *U. tomentosa*, which presented some activity against pathogenic strains.

Endophytic fungus	Isolate	Antagonistic activity against*			
		Efa	Sau	Eco	Kpn
<i>Colletotrichum</i> spp. 1	2.4078	-	-	20.3±0.5	-
<i>Colletotrichum</i> spp. 2	2.3916	13.7±0.4	-	-	-
<i>Colletotrichum</i> spp. 3	23916	-	-	13.7±0.4	-
<i>Colletotrichum</i> spp. 4	2.3837	-	-	9.7±0.4	-
<i>Colletotrichum</i> spp. 5	2.4042	-	-	10.0±0.0	-
<i>Colletotrichum</i> spp. 6	2.3895	-	-	9.0±0.0	-
<i>Nigrospora</i> spp. 1	2.3831	-	-	18.0±0.0	-
<i>Nigrospora</i> spp. 2	2.3972	-	14.0±0.0	-	-
<i>Nigrospora</i> spp. 3	2.3907	-	-	9.7±0.4	-
<i>Nigrospora</i> spp. 4	2.3909	-	-	8.0±0.0	-
<i>Nigrospora</i> spp. 5	2.3799	-	-	7.7±0.4	-
<i>Nigrospora</i> spp. 6	2.4088	-	-	6.0±0.0	-
<i>Penicillium</i> spp. 1	2.3964	19.3±0.5	-	-	-
Concl.					
Endophytic fungus	Isolate	Antagonistic activity against*			
		Efa	Sau	Eco	Kpn
<i>Penicillium</i> spp. 2	2.3788	17.7±0.4	-	-	10.0±0.0
<i>Penicillium</i> spp. 3	2.3964	-	16.0±0.0	-	-
<i>Penicillium</i> spp. 4	2.4055	-	-	17.7±0.4	11.0±0.0
<i>Penicillium</i> spp. 5	2.3828	-	-	8.3±0.4	-
<i>Penicillium</i> spp. 6	2.3758	-	-	7.0±0.0	-
<i>Pestalotiopsis</i> spp. 1	2.4084	-	-	18.0±0.0	-
<i>Pestalotiopsis</i> spp. 2	2.3794	-	-	10.7±0.4	-
<i>Pestalotiopsis</i> spp. 3	2.3800	-	-	-	10.0±0.0
<i>Curvularia</i> spp. 1	2.4034	-	-	21.0±0.0	-
<i>Curvularia</i> spp. 2	2.3761	-	-	10.0±0.0	-
<i>Fusarium</i> spp. 1	2.3952	16.7±0.4	-	25.0±0.0	-
<i>Fusarium</i> spp. 2	2.3949	-	-	6.0±0.0	-
<i>Phomopsis</i> spp. 1	2.3934	-	-	-	10.0±0.0
<i>Aspergillus</i> spp. 1	2.3959	-	-	11.0±0.0	-
Unknown spp. 1	2.4090	-	-	20.7±0.4	-
Unknown spp. 2	2.3903	20.0±0.0	-	-	-
Unknown spp. 3	2.3773	-	-	19.7±0.4	-
Unknown spp. 4	2.4061	-	-	19.0±0.0	-
Unknown spp. 5	2.4014	-	-	-	-
Unknown spp. 6	2.3815	-	-	-	-
Unknown spp. 7	2.3903	-	-	-	-
Unknown spp. 8	2.3951	-	-	-	-
Chloramphenicol	-	13.3±0.4	19.3±0.5	26.0±0.0	13.7±0.5
Total		5	2	23	4

*Efa: *Enterococcus faecalis*; Spn: *Streptococcus pneumoniae*; Sau: *Staphylococcus aureus*; Eco: *Escherichia coli*; Kpn: *Klebsiella pneumoniae*.

(Figure 2). The extracts with the best antibacterial activity against *S. aureus* and *K. pneumoniae* were *Penicillium* spp. 3 (2.3964) and *Penicillium* spp. 4 (2.4055),

respectively. The extract from *Fusarium* spp. 1 (2.3952) was the best for *E. coli*, while for *E. faecalis*, the fungus Unknown species 2 (2.3903), which did not produce

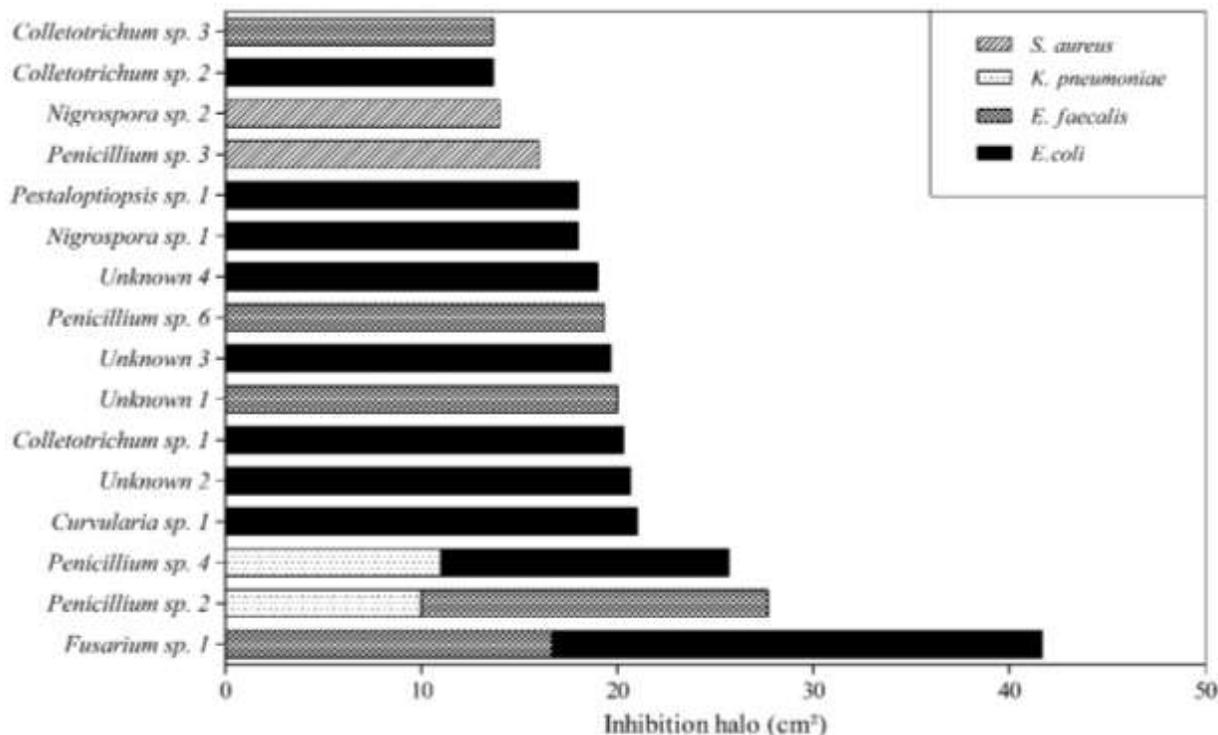


Figure 2. Antimicrobial activity (inhibition halo, in cm^2) against gram-positive and gram-negative bacteria presented by endophytic fungi isolated from *Uncaria tomentosa*. Each value is expressed as the average of three independent.

reproductive structures. Any fungal extracts presented antibacterial activity against the four bacteria tested.

Penicillium spp. is the most studied bioprospecting fungus since penicillin was discovered and produces several defense metabolites with several biological activities such as antibacterial and antifungal agents (Supaphon et al., 2013). Endophytic *Penicillium* was observed with antibacterial activity in other studies (Jouda et al., 2004; Padhi and Tayung, 2015).

Colletotrichum isolated as endophytic fungus also showed antibacterial activity against gram-positive, gram-negative, and *Candida albicans* bacteria (Katoch et al., 2014; Ferreira et al., 2015).

Nigrospora has not been a fungus commonly reported as a producer of antibiotics. However, in this study, six morphospecies presented this activity and *Nigrospora* spp. 1 (2.3831) presented strong activity against *E. coli*.

The endophytic fungus *Pestalotiopsis* spp. proved to be an important producer of antibacterial substances (Banhos et al., 2014; Pinheiro et al., 2017).

Antimicrobial activity is frequently detected among species of the genera *Fusarium* and *Phomopsis* (Radić and Štrukelj, 2012), as confirmed in this study.

Conclusion

This study demonstrated the diversity of endophytic fungi

in the medicinal species *U. tomentosa* as the first report of endophytic studies for this plant. Crude extracts prepared from endophytic fungi isolated from leaves and stems demonstrated *in vitro* antibacterial activity against gram-positive and gram-negative bacteria.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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