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Chromatography and bioautography of endophytic fungi extracts of *Uncaria tomentosa* (Willd.) DC with antibacterial activity

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Endophytic fungi of different species have already proved their efficacy against a range of pathogenic bacteria. However, the separation of the compounds contained in these extracts that actually have an effective activity is not a constant, but it is essential for the scientific knowledge to formulate a new medication. Thus, this study aimed to separate compounds from two endophytic fungi extracts of *Uncaria tomentosa* with positive results against Gram-positive and Gram-negative bacteria. The separation was carried out by thin-layer chromatography and bioautography. For this, it was necessary to produce fungal extracts from the endophytes Colletotrichum (23916) and Fusarium (23952), which were separated from the liquid state by filtration. The remaining filtrate was divided into ethyl acetate and then concentrated in a rotary evaporator at 40°C. Antimicrobial activity was evaluated and the Minimum Inhibitory Concentration (MIC) was determined with the following test microorganisms: *Escherichia coli, Enterecoccus faecalis, Klebsiella pneumoniae* and *Staphylococcus aureus*. Subsequently, it was carried out the thin layer chromatography (TLC) of the extracts using hexane, ethyl acetate/hexane, ethyl acetate and acetate/methanol. The reading was performed in 312 nm UV light, and right after the chromatography, the bioautography was carried out. The following Rf values were obtained: 0.30 for K. pneumoniae and 0.35 for *E. coli*, in ethyl acetate, for the extract 23952, and 0.81 for *K. pneumoniae* and 0.41 for *S. aureus*, in styrene acetate, for the extract 23916, confirming different compounds by bioautography. Therefore, it was concluded that several compounds present in the extracts tested have antibacterial activity.

Key words: Chromatography, bioautography, endophytic.

INTRODUCTION

Thin Layer Chromatography (TLC) is a technique used to separate compounds of a sample of a given substance using two phases, a stationary phase and a mobile one, on silica gel plates (Niessem, 2017).

The scope of its applicability is the identification of molecular compounds by comparing them with previously existing standards, separating the components of a mixture through the purification technique (Bonnin et al.,

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2018). Bioautography is a technique that combines thin-layer chromatography with agar diffusion (Choma and Jesionek, 2015). Both techniques are tools for the separation of molecules at laboratory level, capable of biologically identifying which specific compounds of an extract act actively in that compound, such as the endophytic fungi extracts from several plant species that act as potent antimicrobials, since some known drugs and microorganism derivatives (Fanali et al., 2016) are already on the market as antibiotics (Luo et al., 2016), being used in the treatment of various diseases such as Alzheimer's, from bioactive (Wang et al., 2016), anticancer (Petersen et al., 2014; Zaiyou et al., 2015) and anti-allergic compounds, among others (Sakurai et al., 2003).

Among these microorganisms that produce active metabolites, the endophytes that are fungi or bacteria that reside within plant tissues without causing apparent damage (Li et al., 2018) stand out. These are sources of secondary metabolites with many biological activities (Yan et al., 2018). The use of these compounds is already a promising reality in the field of biotechnology, especially in pest control in agriculture, due to their low cost and benefits to human health, especially when compared to the use of agrochemicals (Silva et al., 2008), and are also applied in the medical and pharmacological fields in the inhibition of pathogenic bacteria that are resistant to the main antibiotics currently available in the market (Arora and Kaur, 2018). An example of this is the significant number of compounds produced from fungal endophytes with proven inhibitory activity against several Gram-positive and Gram-negative bacteria (Banhos et al., 2014).

The Brazilian Amazon is a wide source of new compounds from its rich biodiversity for the development of new drugs, mainly from its endophytic fungi (Banhos et al., 2014). Also, the analysis of their biological activity is an appropriate option in the fight against resistant microorganisms; however, there are few studies on this subject in the literature, which becomes a limiting factor in the contribution to the formulation of new drugs (Cheng et al., 2018).

Uncaria tomentosa (Willd.) DC belongs to the Rubiaceae family, it is a plant species of the Amazon region, popularly known as cat's claw (Alvarenga et al., 2018). This species has various medicinal properties such as anti-inflammatory (Yatoo et al., 2018), anticancer (Zhang et al., 2015), contraceptive (Nogueira et al., 2011), antiasthmatic (Azevedo et al., 2018), antiarthritic (Rajamanikyam et al., 2017), and immunostimulant (Baraya et al., 2017) activities, mainly antibacterial activity of endophytic fungi extracts, especially against *Enterecoccus faecalis* (Rodrigues et al., 2018), which is a Gram-positive bacterium, ubiquitous, highly resistant and commonly found in the root canal system (Qian-Wang et al., 2012). In the contemporary world, the indiscriminate use of antimicrobials is frequent, so they no longer have the same efficacy against a specific group of bacteria, known as superbugs or resistant bacteria (Yelin and Kishony, 2018). Based on the instability of the immune system, several opportunistic bacteria can cause infections and establish acute pathological conditions that lead the host organism to unpleasant consequences (Sedghizadeh et al., 2017), such as *Escherichia coli*, leading to food poisoning and diarrhea (Gomez-Duarte, 2014). *Klebsiella pneumoniae* is another pathogenic bacterium capable of causing pneumonia by different types of transmission, mostly in the hospital environment (Xiong et al., 2016). *Staphylococcus aureus* can cause various infections, such as acne, boils, pneumonia, meningitis, endocarditis, sepsis and others (Koch et al., 2014). Strains resistant to various drugs have become increasingly prevalent (Otto, 2012).

In this context, the antimicrobial activity of endophytic fungi of *U. tomentosa* makes this species a promising source for the development of a new drug. However, it is not entirely known, what compounds or molecules are contained in the endophytic fungi extracts of this plant that actually inhibit bacterial growth. For this reason, the separation of these substances is as important as the extract activity. Thus, this study aimed to separate the compounds of two endophytic fungal extracts of *U. tomentosa*: *Colletotrichum* and *Fusarium* which had positive results against Gram-positive and Gram-negative bacteria from the collection of the microbiology laboratory of the Federal University of Acre (UFAC).

MATERIALS AND METHODS

Obtainment of extracts and purification of endophytic fungi of *U. tomentosa*

The fungi *Fusarium* with laboratory record (23952) and *Colletotrichum* (23916), isolated from *U. tomentosa* of the collection of the microbiology laboratory of the Federal University of Acre (UFAC), with positive results of antibacterial activity were cultivated on a large scale, in a broth using a fermentor to obtain fungal extracts to be used in *in vitro* biological assays. For this purpose, they were cultivated in Petri dishes using potato dextrose agar (PDA) medium for 14 days at 28°C. The content from 5 dishes (80 × 15 mm) with the grown fungus was inoculated into a 1000 ml Erlenmeyer flask containing 500 ml of PD broth and then conducted for 14 days at 28°C without shaking. For each fungus, 3000 ml of PD broth was used and distributed into six Erlenmeyer flasks of 1000 ml each. After incubation, the fungal mycelium was separated from the broth by filtration using filter paper. The filtrate was subjected to the liquid-liquid partition with 200 ml of ethyl acetate five times, and then the extraction product was concentrated on a rotary evaporator under reduced pressure in a water bath at 40°C (Cechinel and Yunes, 1998) until the dry yield was calculated dividing the weight value of two dry extracts by the weight of empty flasks, and solubilizing them with Dimethylsulfoxide (DMSO) for the antibacterial activity bioassay and determination of the Minimum Inhibitory Concentration (MIC).

Bioassays of antibacterial activity

The antibacterial activity of the endophytic fungi *Fusarium*

Table 1. Mean and standard deviation of the halos of the antibacterial activity of endophytic fungi extracts isolated from *U. tomentosa*: Colletotrichum 2.3916 and Fusarium 2.3952 against Gram-positive and Gram-negative bacteria.

Extract (EtOAc)	Antibacterial activity (mm)			
	E. coli	***S. aureus***	***K. pneumonia***	***E. faecalis***
Colletotrichum 2.3916	10.3 ±0.57	13.0±2.6	15 mm±1.0	13.6±0.5
Fusarium 23952	12.3±0.57	11.6±1.5	14.3±2.0	13.3±1.5

Table 2. MIC of endophytic fungi extracts *of U. tomentosa against Escherichia coli, S. aureus, K. pneumoniae,* and *E. faecalis.*

Extract	Dry matter yield (mg)	Susceptibility of bacteria mg/mL			
		E. coli	***S. aureus***	***K. pneumoniae***	***E faecalis***
(EtOAc)		**MIC**	**MIC**	**MIC**	**MIC**
Colletotrichum 2.3916	49.83	31.25	7.81	7.81	7.81
Fusarium 23952	32.12	31.25	7.81	7.81	15.62

(23952) and *Colletotrichum* (23916) was evaluated using the well method for the allocation of extract, according to the National Committee for Clinical Laboratory Standards, using the following test-microorganisms: *E. coli* (ATCC 10536)*, S. aureus* (ATCC 12598)*, K. pneumoniae* (ATCC 700603) and *E. faecalis* (ATCC 4083). For this, Petri dishes containing Müeller-Hinton agar medium were prepared and incubated at 37°C for 24 h to verify the sterility of the medium, where the bacterial purification process was carried out, using the streak plate technique. Afterward, they were incubated at 37°C for 24 h. Five isolated bacterial colonies were spread in Luria Bertani broth (LB) and then incubated for up to 3 h. The turbidity degree was then checked and adjusted to the McFarland 0.5 scale (1.5×10^8 cells/ml) with a sterile saline solution. After adjustment, the bacteria were spread onto Petri dishes containing Müeller-Hinton agar medium using a swab in three distinct directions and around the dish uniformly. Prior to the inoculation of the bacteria, wells of approximately 5 mm in diameter were made in pipette tips to place the extract. After spreading the test-bacteria on the wells, 20 µl of the fungal extracts were added. Subsequently, the dishes were incubated at 37°C for 24 h and then the formation of the inhibition halo was observed. Samples considered to have positive antibacterial activity were those that did not allow the microbial development around the well, and these halos were measured in millimeters. A millimetre (mm) graduated ruler was used to measuring the size of the halos obtained from the well diffusion bioactivity test.

Determination of minimum inhibitory concentration

After obtaining the dried fungal extracts, they were solubilized in dimethyl sulfoxide (DMSO) at an initial concentration of 1, 250, 125, 62.5, 31.25, 15.62, 7.81 and 3.90 mg mL^{-1}. After the solubilization of the extracts in the respective concentrations, the *in vitro* antibacterial bioactivity test against *E.coli, S. aureus, K. pneumoniae,* and *E. faecalis* was performed to determine MIC, by the well method in Müeller-Hinton medium; then the extracts were applied at their respective concentrations (Montero, 2017).

Chromatography and bioautography of the tested fungal extracts

After obtaining the results of the bioactivity test against *E. faecalis*, the extracts were subjected to the chromatography process to separate the compounds with biological activity. For this, 10 µl of each extract were placed on the plates of TLC (Silica gel 60), eluted in the mobile phase using different low, medium and high polarity systems: hexane, ethyl acetate-hexane, ethyl acetate (EtOAc) (1:1), ethyl acetate/methanol (EtOAc/MeOH) (1:1), and analyzed in ultraviolet (UV), 312 nm. Soon afterward, to perform the bioautography, each TLC plate was placed on dishes containing Müeller-Hinton medium, properly identified according to each solvent used, and stored for 24 hours in a refrigerator. After this period, the test-bacteria were spread and incubated in a bacteriological oven at 37°C for 24 h. Then, the inhibition zone of the bioactive compounds was analyzed, as described above, and the retention factors (Rf) (Di Ciaccio et al., 2018) were calculated by measuring the distance traveled by the compound (DC) divided by the distance traveled by the solvent (DS) (Kagan and Flythe, 2014).

Data analysis

The data were analyzed from the mean and standard deviation of the halos obtained from the antibacterial activity test, which were performed in triplicate, in addition to the calculation to measure the retention factors (Rf) of the compounds with the equation: (Rf = DC / DS).

RESULTS

The antimicrobial activity was positive for the two fungal extracts tested, *Fusarium* (23952) and *Colletotrichum* (23916), for all bacteria tested: *E. coli, S. aureus, K. pneumoniae* and *E. faecalis* with formation of inhibition halo measured in mm (Table 1) and MIC of 7.81 for the majority of the bacteria tested for the two extracts, except for *E. coli* with 31.25, for both extracts, and for *E. faecalis* with 15.62, for extract 23952 (Table 2).

From the reading of the UV chromatographic plates, several components were found in the extracts used in this study and, according to the bioautography, these substances have high antibacterial potential (Figure 1). The bioautography results confirm antimicrobial activity

for *Fusarium* fungal extract with values of Rf (0.30) for *K. pneumoniae* in ethyl acetate eluent and 0.85 for acetate/methanol; Rf (0.74) for *S. aureus* in hexane/ethyl acetate eluent and 0.35 for ethyl acetate; Rf (0.67) for *E. coli* in ethyl acetate/methanol and Rf (0.64) for *E. faecalis* in ethyl acetate/methanol (Figure 1). For the *Colletotrichum* extract, the following Rf values were obtained: 0.81 against *K. pneumoniae*, 0.41, 0.36 and 0.45, all in ethyl acetate, for *S. aureus*, *E. coli* and *E. faecalis*, respectively, and 0.63 *E. coli*, in ethyl acetate/methanol.

DISCUSSION

The antibacterial activity of endophytic fungi of several plant species is already described in the literature from many works in the field of biotechnology (Sudha et al., 2016) as it can be observed in studies on extracts of endophytic fungi of *Ipomea biloba*, which showed positive result against pathogenic bacteria (Prakash et al., 2016) as well as on extracts of endophytic fungi from *Azadirachta indica* A. Juss, which have proved to be potent antibacterial agents (Marcinkevicius et al., 2019). In this context, the antibacterial activity of extracts of endophytic fungi (*Colletotrichum* 23916 and *Fusarium* 23952) based on ethyl acetate from *U. tomentosa* presented in this study was confirmed, proving to be effective against bacteria, both for Gram-positive and Gram-negative bacteria.

The endophytic fungi *Colletotrichum* and *Fusarium* are considered sources of defense metabolites with several biological activities, mainly with antibacterial activity (Juang et al., 2019), corroborating the results found in this study. The MIC is the lowest concentration of the extract capable of stopping the growth of a microorganism (Tripathi, 2013). In this study, the MIC was 7.81 for both fungal extracts for most of the bacteria tested, except for *E. coli* (31.25) and *E. faecalis* (15.62) for the *Fusarium* 23952 extract, similarly to the results of other studies on MIC determination in antimicrobial activity (Nisa, 2015; Ochoa-Velasco et al., 2018; Naime-Shamel et al., 2019).

Thin-layer chromatography reveals that from a stationary and a mobile phase it is possible to separate compounds that travel the entire surface of the plate (TLC), according to the degree of polarity of the compound and solvent used (Agostini-Costa et al., 2012). In addition to TLC, there are other types of chromatography, such as gas chromatography and liquid diffusion (Marathe et al., 2019). The advantage of TLC is the adsorption (Kalindere et al., 2018), for this reason, the TLC was chosen in this research.

In this study, it was demonstrated that the fungal extracts used for both *Colletotrichum* 23916 and *Fusarium* 23952 have different UV-visible compounds, which are mostly of medium polarity with better separation in ethyl acetate. Similar results are presented in other studies using chromatography (Li et al., 2019; Marcinkevicius et al., 2019), demonstrating the appearance of different compounds in ethyl acetate. Ethyl acetate is a solvent that has medium polarity and has an affinity with adsorptive silica (polar) molecules, and this facilitates the separation of the compounds from an extract (Sui et al., 2018). Recalling that nonpolar compounds have greater difficulty in silica plates with acidic and basic solvents (Churms, 2002). On the other hand, some authors question the fidelity of the chromatography information due to the spreading of the fractions and colonization of the TLC plates (Mccalley, 2010). The solvents used in the mobile phase may have an effect on bacterial growth and may interfere with the results (Arshad et al., 2018). However, correct drying of TLC plates to eliminate solvents is necessary for the effectiveness of bioautography (Choma and Jesionek, 2015), even though the technique of plate bioautography by evaluating the mark left by diffusion is considered a reliable method of confirmation (Choma and Grzelak, 2011).

The bioautography technique associates thin layer chromatography with the confirmation of the antibacterial activity of the compounds separated by TLC from antimicrobial analysis on plates containing Müller Hilton agar (Matanna et al., 2010). The bioautography can be performed using different techniques, diffusion and dilution (Choma and Grzelak, 2011), but the use of the mark in solid state was chosen because it is a practical technique, validated for microbiological control in the laboratory, that is, to evaluate the antimicrobial substance of a mixture, simultaneously with its quantification regarding the activity of a standard (Colorado et al., 2007).

The results of the bioautography obtained in this study confirmed that the compounds revealed in TLC have antibacterial activity against the bacteria tested. These results are in agreement with other studies on chromatography and bioautography reported in the literature (Chen and Schwack, 2014), in which bioactive compounds were found in fungal extracts of *Terminalia arjuna* (Gill and Vasundhara, 2019). Similarly, more compounds with antibacterial activity were also isolated in endophytic fungi extracts of *Helianthus annuus* (Farhat et al., 2019).

Conclusion

According to the *in vitro* assays and the chromatographic and bioautographic analysis performed to separate the chemical compounds from the extract, with subsequent verification of the retention factors (Rf), there are different compounds in the fungal extracts (*Colletotrichum* 23916 and *Fusarium* 323952) with an inhibition reaction of bacterial growth in all samples tested, especially with

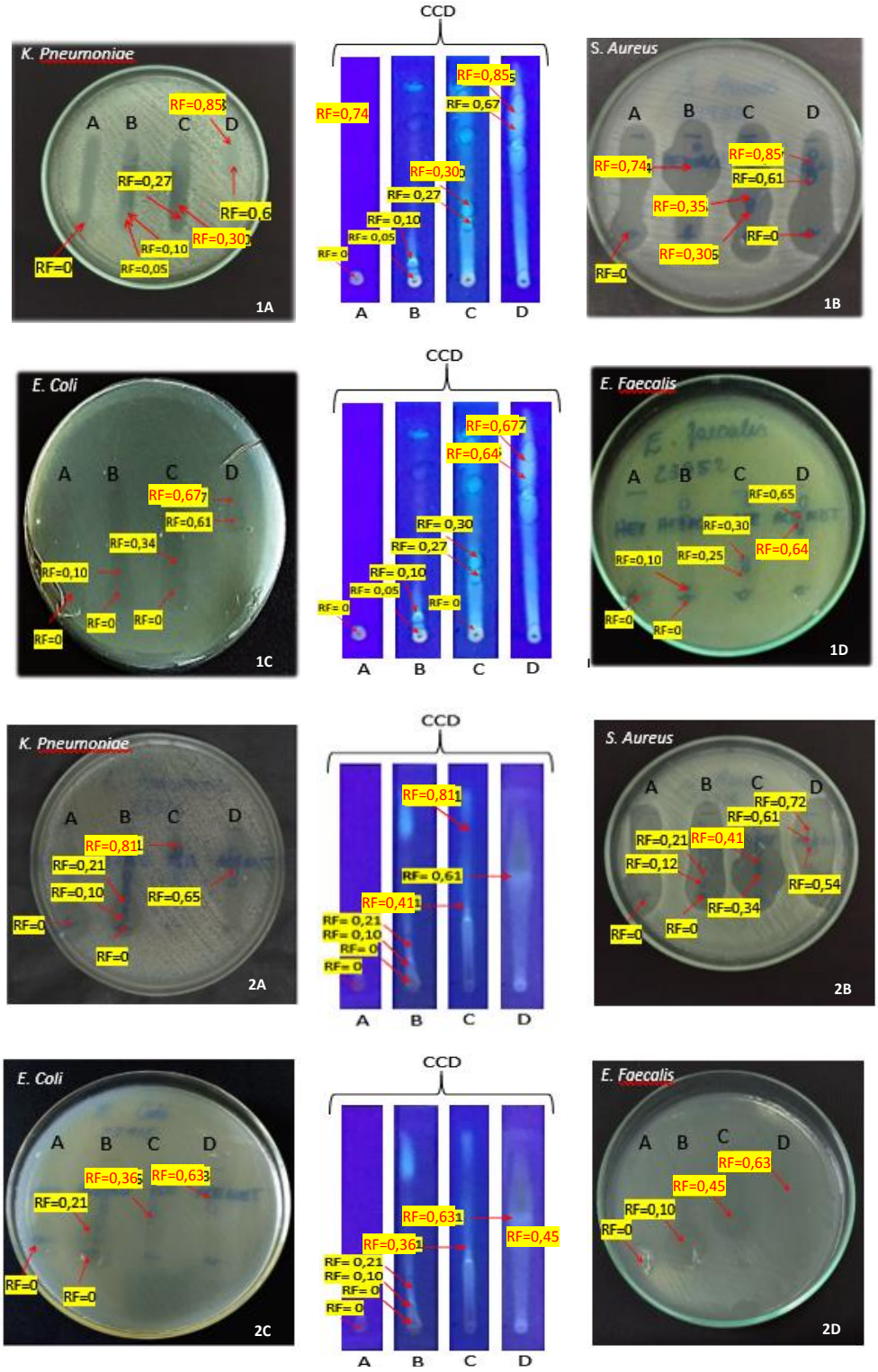

Figure 1. Bioautography of fungi metabolites (*Fusarium* 23952 and *Colletotrichum* 23916) with antibacterial activity. Figure 1A-1D (*Fusarium* 23952), respectively, in the following order of bacteria test: *K. pneumoniae, S. aureus, E. coli* and *E. faecalis*. Figure 2A-2D (*Colletotrichum* 23916), respectively, in the following order of bacteria test*: E. coli, S. aureus, K. pneumoniae* and *E. faecalis,* TLC (thin layer chromatography) of A-D in Figures 1 and 2, respectively, represent (A) eluent system in hexane, (B) eluent system ethyl acetate/hexane (1:1), (C) eluent system in ethyl acetate and (D) eluent system in ethyl acetate/methanol (1:1). Rf = retention factor.

ethyl acetate solvent.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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