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Article

Antimicrobial activity of leaf and root extracts of tough lovegrass

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Abstract

This study aimed to evaluate tough lovegrass leaf and root extracts antimicrobial activity. The extracts (plant material: solvent, 1:10) were prepared by maceration with methanol:water (1:1) during ten days followed by a concentration in a rotary evaporator under reduced pressure. The extracts were resuspended in water containing 1% of dimethylsulfoxide (DMSO) to obtain a final concentration of 100 mg/mL and then filtered through a sterilizing membrane with 0.22µm. The antibacterial activity of the leaf and root extracts were evaluated against pathogenic and phytopathogenic bacteria by agar well diffusion and microdilution broth methods for the minimum inhibitory concentrations (MIC) determination. The antifungal activity of tough lovegrass leaf and root extracts were evaluated by micelial growth inhibition and conidial germination inhibition. The extracts presented low antibacterial activity against *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Xanthomonas translucens*, but the leaf extracts presented significant antifungal activity against the phytopathogenic fungus *Drechslera tritici-repentis*. The results support the continuity of the study in improving the effectiveness of the active extract for a possible use in pharmacology and agronomy and in attempting to determine the probable active antimicrobial compound.

Keywords: antibacterial activity, antifungal properties, *Eragrostis plana*, *phenolic compounds*

Atividade antimicrobiana de extratos foliares e radiculares de capim annoni

Resumo

Este estudo teve como objetivo avaliar a atividade antimicrobiana dos extratos foliares e radiculares radiculares do capim-annoni. Os extratos (material vegetal: solvente 1:10) foram preparados por maceração com metanol: água (1: 1) durante dez dias seguido de concentração em evaporador rotatório sob pressão reduzida. Os extratos foram ressuspendidos em água contendo 1% de dimetil sulfóxido (DMSO) para obter uma concentração final de 100 mg / mL e depois filtrados através de uma membrana esterilizante de 0,22 um. A atividade antibacteriana dos extratos foliares e radiculares foi avaliada contra bactérias patogênicas e fitopatogênicas por meio de técnicas de difusão em ágar pelo método dos poços e microdiluição em caldo para a determinação das concentrações inibitórias mínimas (MIC). Avaliou - se a atividade antifúngica de extratos de folhas e raízes de capim-annoni resistentes por inibição do crescimento micelial e inibição da germinação conidial. Os extratos apresentaram baixa atividade antibacteriana contra *Staphylococcus aureus*, *Staphylococcus epidermidis* e *Xanthomonas translucens*, porém os extratos foliares apresentaram atividade antifúngica significativa contra o fungo fitopatogênico *Drechslera tritici-repentis*. Os resultados apoiam a continuidade do estudo na melhoria da eficácia do extrato ativo para um possível uso em farmacologia e agronomia e na tentativa de determinar o provável composto antimicrobiano ativo

Palavras-chave: atividade antibacteriana, propriedades antifúngicas, *Eragrostis plana*, compostos fenólicos

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Introduction

Tough lovegrass (*Eragrostis plana* Nees), native from South Africa, was introduced in Rio Grande do Sul (RS), Brazil, in the 1950s as an impurity in seed lots (Reis, 1993). It began to be sold as forage due to its tolerance to climatic fluctuations, vigorous size, and plentiful yield of green mass (Reis & Oliveira, 1978; Medeiros & Focht, 2007). However, in 1979, the Brazilian Department of Agriculture prohibited the transportation, importation and exportation because the species was recognized as low quality forage, having a large capacity to spread, characteristics of dominance over other species and as being difficult to eradicate (Reis & Oliveira, 1978; Medeiros & Focht, 2007). Since tough lovegrass was introduced, it has become quickly established on the natural fields of RS, leading to a reduction in native species (Medeiros et al., 2004) and to a decrease in cattle ranching productivity (Reis, 1993). Tough lovegrass became the most invasive grass in the fields of southern Brazil, and it was estimated that over to two million hectares were invaded by this specie (Medeiros & Focht, 2007).

The aggressive characteristic of tough lovegrass is observed in its high persistence in cutting, trampling, and frosts; high capacity for regrowth; low acceptability by animals (Reis, 1993); large seeds production (Kissmann, 1991); allelopathy (Coelho, 1986); and the absence of natural enemies (Reis, 1993). This grass is not infected by phytopathogens, which has prompted the investigation its antimicrobial property. The only pathogen reported to cause damage to tough lovegrass was the fungus *Uromyces* sp. (Nachtigal et al., 2009).

Despite the limited phytochemical characterization of tough lovegrass, the presence of bioactive compounds in its extracts has been suggested. Phenolic compounds – catechin and epicatechin (Cowan, 1999) – observed in tough lovegrass leaves (Favaretto et al., 2015) have antimicrobial properties (González-Lamothe et al., 2009; Lin et al., 2013). In the aerial portion of a plant with the same genus, *Eragrostis viscosa* (Retz.) Trin., labdane were isolated (Sebastião et al., 2010; Sebastião et al., 2012), class of compounds with already stated with antimicrobial properties (Demetzos & Dimas, 2001).

Obtaining new bioactive compounds, with fewer side effects safe use and with greater effectiveness is a constant need in health, agronomy, and industry. This investigation is important for antimicrobial compounds due to the ability of resistance selection of antimicrobials by microorganisms, including phytopathogens. Based on the intrinsic resistance of tough lovegrass to phytopathogens with the production of secondary metabolites with antimicrobial activity by the genus and the need for new alternatives for the treatment of fungal and bacterial infections, the present study aimed to evaluate the antimicrobial activity of tough lovegrass leaf and root extracts.

Material and Methods

Plant material

The leaves and roots of tough lovegrass were collected in April, 2013 at Passo Fundo University (UPF) (Passo Fundo, RS, Brazil) experimental field. The specimen was identified and deposited in the UPF Herbarium with the registration number 11832.

Extract preparation

The vegetal material was washed and air-dried at 40°C for 48 hours until constant weight. The extracts (plant material: solvent, 1:10) were prepared by maceration with methanol:water (1:1) for ten days followed by a concentration in a rotary evaporator under reduced pressure. The extracts were resuspended in water containing 1% dimethylsulfoxide (DMSO) to obtain a final concentration of 100 mg/mL and then filtered through a sterilizing membrane with 0.22µm.

Antibacterial test

In this study, the antibacterial activity of the leaf and root extracts of tough lovegrass were evaluated in controlling the following pathogenic bacteria: *Staphylococcus aureus* (ATCC 27213), *Staphylococcus epidermidis* (ATCC 12228), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhi* (ATCC 19196). The extracts were also evaluated against the following phytopathogenic bacteria: *Agrobacterium*

tumefaciens (ATCC 4720), *Pectobacteria* sp., *Xanthomonas translucens* (ATCC 35935) and *Acidovorax avenae* (ATCC 19860).

Inoculum preparation

Surfaces of Mueller Hinton agar containing the grown colonies were washed with sterile saline and diluted to 25% of transmittance at a wavelength of 580 nm in a spectrophotometer to obtain approximately 10^6 CFU/mL. The antibacterial activity was evaluated by agar diffusion and microdilution broth methods for the minimum inhibitory concentrations (MIC) determination (Michelin et al., 2005; Ostrosky et al., 2008).

Agar well diffusion

Over the surface of the Petri dishes containing 20 mL of Mueller-Hinton Agar, 100 µL of the inoculum (10^6 CFU/mL) were dispersed with a sterile swab. Wells of 6 mm were made in agar and filled with 60 µL of tough lovegrass leaf or root extracts (100 mg/mL) or with a control. Chloramphenicol (30 µg/disk) was used as a positive control for *S. epidermidis, S. aureus, and S. typhi*; polymyxin B was used for *P. aerug*inosa; cefotaxime (30 µg/disk) was used for *A. tumefaciens* and *X. translucens*; gentamicin (50 µg/disk) was used for *Pectobacteria* sp.; and tetracycline (30 µg/disk) was used for *A. avenae*. DMSO (1 %) was the negative control for all organisms. After the incubation of the plates for 24 h at 37 °C, the zones of inhibition were measured. The assay was performed in six replicates.

Microdilution broth

MIC were determined using the microdilution broth method. The tough lovegrass extracts were diluted in a nutrient broth at the concentrations of: 1:1, 1:2, 1:4, 1:8, and 1:16. The microplates of 96 wells were used in which 90 µL of nutrient broth, 10 µL of standardized inoculum (10^6 CFU/mL), and 100 µL of extracts dilutions were added in each well. After 24 hours at 37 °C, the turbidity of the solution was observed. Turbid solutions indicated the development of the organism, and, hence, the absence of antibacterial activity. The assay controls were: 1) positive (inoculum, nutrient broth, and the respective antibiotic, the same used for agar well diffusion assay), 2) negative (inoculum and nutrient broth), 3) negative (inoculum, extracts diluent, and nutrient broth), and 4) medium sterility control.

Antifungal activity

In this study, the antifungal activity of tough lovegrass leaf and root extracts against the following fungi was evaluated: *Fusarium* sp., *Alternaria* sp., *Bipolaris sorokiniana*, *Drechslera tritici-repentis, Phomopsis sojae, Sclerotinia sclerotiorum*, and *Colletotrichum truncata*.

Growth micelial inhibition

Aliquots (1mL) of leaf or root extracts of tough lovegrass (100 mg/mL) or a positive (fungicide)/negative (DMSO 1%) control were uniformly distributed on Petri plates with potato dextrose agar (PDA) with the aid of a Drigalsky handle. A mycelial disc (8 mm diameter) taken from the edge of a growing colony was positioned in the center of each plate (Li et al., 2008). The plates were incubated at 28 °C for 5 days for Fusarium sp., 8 days for *Alternaria sp*., 7 days for *Bipolaris sorokiniana* and *Colletotrichum truncata*, 6 days for *Drechslera tritici-repentis*, 3 days for *Phomopsis sojae*, and 2 days for *Sclerotinia sclerotiorum*. The positive controls were glyphosate (10 mg/mL) for *Alternaria* sp.; carbendazim (20 ppm) for *Fusarium* sp., *P. sojae*, *S. sclerotiorum*, and *C. truncate*; and iprodione (30 ppm) for *B. sorokiniana* and *D. tritici-repentis*. The negative control was the DMSO (1%). The diameters of growth were measured daily in four directions until the negative control colony reached the edges of the Petri plate. The test was conducted in five replicates. The percentage of inhibition of the diameter growth (PIDG) of the extracts on the fungal growth was determined according to the formula below:

$$PIDG = \{[(Ed / Cd) \times 100] - 100\}$$

in which Ed means the average diameter (mm) of the mycelia with the extract or fungicide, and Cd means the average diameter (mm) of the mycelia with the negative control (Yang et al., 2012, with brief modifications).

The statistical analysis was performed using a one-way analysis of variance (ANOVA), and the means were compared using the Tukey´s test at a 5% of significance. The experiment was carried out in a completely randomized design.

Conidial germination inhibition

To evaluate the inhibition of conidial germination, the fungi were grown on PDA until the formation of conidia, which was collected in a vial containing sterile distilled water and a drop of Tween (20 %). For the inoculum preparation, the concentration of conidia in the solution was determined by counting in a Neubauer chamber and adjusted to 105 conidia/mL. Then, in Petri plates containing 20 mL of PDA medium, 200 µl of inoculum (10^5 conidia/mL) were homogeneously dispersed with the aid of a Drigalski handle. The wells (6 mm) were made in the agar to subsequent addition of 60 ml of each treatment (extract or control). The plates were incubated at 28 °C until the complete development of the fungus. In order to evaluate the effect of the treatment on the conidia germination, the formation of the inhibition zone was observed (Li et al., 2008; Magnusson & Schnurer, 2001; Schreiber et al., 2011). The positive and negative controls were the same controls used for the growth micelial inhibition method. The test was conducted in five replicates.

Results

Antibacterial test

Using the agar well diffusion method, the leaf and root extracts of tough lovegrass did not presented antibacterial activity; however, in the microdilution broth assay, the leaf extract of tough lovegrass inhibited the growth of *S. epidermidis*, *S. aureus*, and *X. translucens* at the highest concentration used (1:1). The root extract of tough lovegrass was only active against *S. epidermidis* at the MIC of 1:2 (Table 1). For the other bacteria, no inhibition by the extracts was observed.

Table 1. Minimum inhibitory concentration of leaf and root extracts of tough lovegrass by broth microdilution method

Bacteria	Extract origin	
	leaves	roots
S. epidermidis	1:1	1:2
S. aureus	1:1	X
X. translucens	1:1	X
S. typhi	X	X
P. aeruginosa	X	X
A.tumefaciens	X	X
A. avenae	X	X
Pectobacterium	X	X

X indicates no inhibition

Antifungal activity

The leaf extract of tough lovegrass significantly inhibited the mycelial growth of the phytopathogen *Dreschlera tritici-repentis* after the second day of culture (Figure 1, Table 2). For the other fungi analyzed, no significant inhibition of mycelial growth was observed (Figure 1). For *Phomopsis sojae*, a tendency of a reduction of mycelia growth was observed, but it was not significant. The root extract did not show antifungal activity (Figure 1). None of the evaluated tough lovegrass extract was able to inhibit the conidial germination of the confronted fungus.

Discussion

In this study, we observed that the leaf extract from tough lovegrass has a greater antimicrobial activity when compared to the root extract. These results are similar to those described by Favaretto et al. (2015), who detected phenolic compounds only or in higher concentration on leaf extracts, chemical class associated with antimicrobial activity.

Useful antimicrobial phytochemicals can be divided into several categories: simple phenols and phenolic acids; quinones; flavones, flavonoids and flavonols; tannins; terpenoids and essential oils; alkaloids; lectins, and polypeptides (Cowan, 1999). A complete description of the

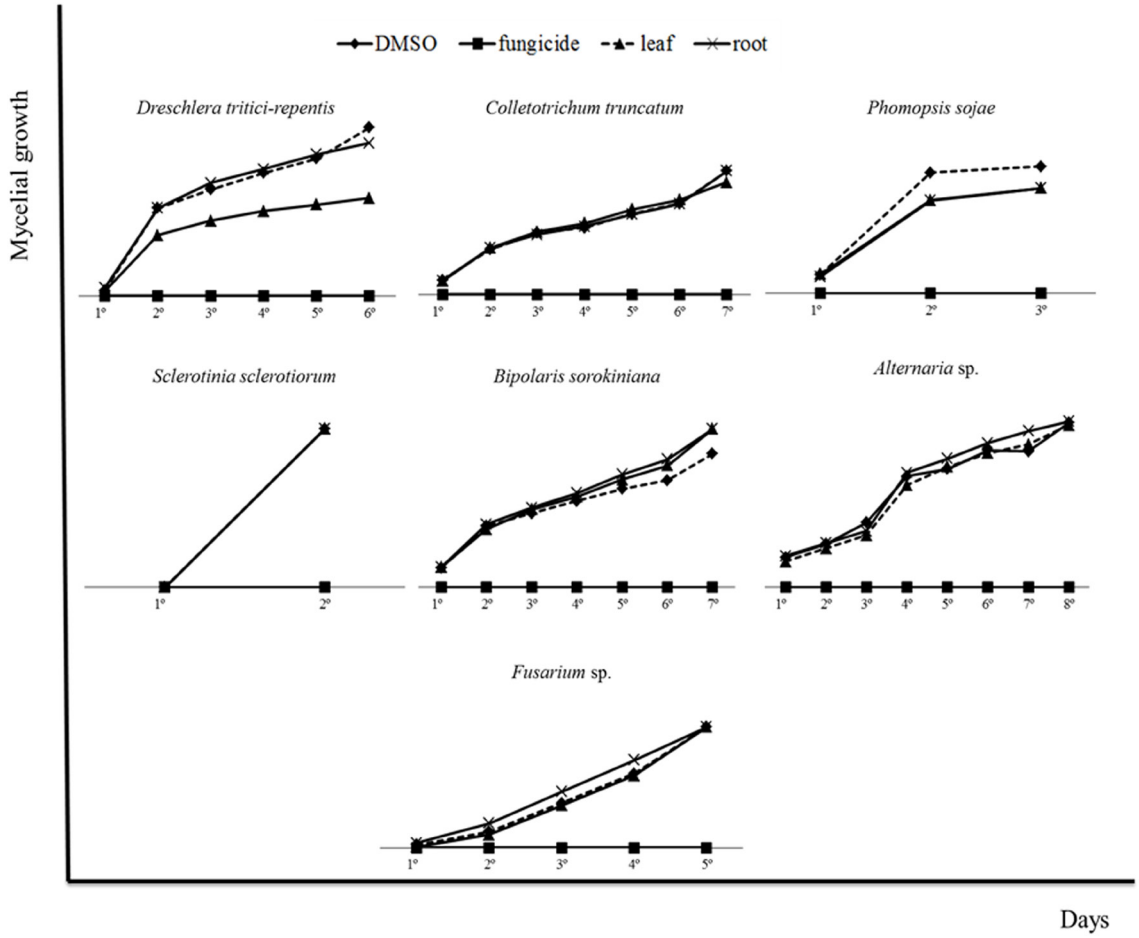

Figure 1. Daily analyzes of the action of the tough lovegrass leaf or root extracts on the mycelial growth.

Table 2. Daily and average measures of *Dreschlera tritici-repentis* mycelial growth (mm) under action of tough lovegrass leaf or root extracts and significance* in relation to positive (fungicide) or negative (DMSO 1%) control

Incubation day	Treatments			
	DMSO 1%	Fungicide	Leaves	Roots
1°	2,60 A	0,00 A	2,40 A	3,80 A
2°	40,75 A	0,00 C	28,15 B	40,75 A
3°	49,40 A	0,00 C	34,80 B	52,40 A
4°	57,05 A	0,00 C	39,20 B	58,75 A
5°	63,75 A	0,00 C	42,30 B	65,65 A
6°	78,00 A	0,00 C	45,35 B	70,90 A
Average	48,59 A	0,00 C	32,03 B	48,70 A
C.V (%)	32,33			

*Values with the same upper-case letters are not significantly different (P < 0.05) according to Tukey's test.

phytochemical profile of tough lovegrass is not available. Preliminary studies of the plant indicate the presence of tannins, alkaloids, saponins, coumarin, and phenolic acids (caffeic acid, ferulic acid, vanillic, and p-coumaric acid) (Favaretto et al., 2015).

Tannins have antibacterial and antifungal activity (Monteiro et al., 2005) probably by inactivating microbial adhesines, enzymes, and cell envelope transport proteins (Cowan, 1999). The catechins act on membrane disruption (Tada et al., 1992). The bactericidal activity of catechins appears to be higher against Gram-positive bacteria than against Gram-negatives. To epicatechin demonstrated antibacterial activity against S. aureus and *E. coli* (Ikigai et al., 1993).

Alkaloids isolates from barberry (*Berberis vulgaris* L.) display activity against the bacteria *E. coli* and *E. faecalis* (Omulokoli et al., 1997), and alkaloids isolates from black pepper (*Piper nigrum* L.) display activity against fungi (Ghoshal

et al., 1996). The gramine, which is an alkaloid, exhibits high activity against *F. graminearum* (Schereiber et al., 2011). The highly aromatic planar quaternary alkaloids act intercalating DNA (Phillipson & O'Neill, 1987).

Several studies report the antibacterial and/or antifungal activity of coumarins, and the activity was proven against *S. aureus, Bacillus subtilis, Escherichia coli, Salmonella typhimurium, Penicillium chrysogenum* (Jurd et al., 1971), *Pseudomonas aeruginosa* (Tada et al., 2002), *Aspergillus niger*, *Cryptococcus neoformans*, and *Saccharomyces cerevisiae* (Sardari et al., 1999).

The common herbs, tarragon (*Artemisia dracunculus* L.) and thyme (*Thymus vulgaris* L.), both containing caffeic acid, presented effectiveness against bacteria (Brantner et al., 1996) and fungi (Duke, 1985). The caffeic acid isolated from coffee (*Coffea* sp.) has displays an antimicrobial activity against *Legionella pneumophila* (Furuhata et al., 2002). Herald & Davidson (1983) observed the antimicrobial activity of caffeic, p-coumaric, and ferulic acids against *E. coli* and *S. aureus.* These acids inhibited the growth of the fungus *Penicillium notatum* (Leifertova et al., 1975). Phenolic compounds affect the cytoplasmic membrane by altering their structure and function, changing the active transport, and coagulating the cellular content (Sikkema et al., 1995; Burt, 2004).

Due to the high MIC required for containing sensitive bacteria in the microdilution assay, it is possible the association of an antibacterial property of the extracts with a compound(s) present(s) in low concentration in crude extract. For a better understanding of this property, it would be necessary to concentrate the metabolites of interest by partitioning the crude extract, which would facilitate the study of biological and chemical activities.

Most existing plants are unknown from a scientific point of view. Only about 5% of 250,000-500,000 species have been studied by phytochemistry, and a smaller percentage have been evaluated based on the biological aspects (Cechinel Filho & Yunes, 1998). The reduced percentage of analysis of plant species indicates the relevance of these studies. The first evidence about the antimicrobial activity of tough lovegrass both against human and phyto-pathogenic microorganisms indicates that the compounds produced by this plant may be useful in pharmacology and in the agronomic field. The continuity of these studies is essential in seeking an applicability for this species, which, for now, only creates problems and damage for RS ranching.

Conclusions

Tough lovegrass produces compounds with antimicrobial activity, which is a property that was previously suggested by its resistance to pathogens and the class of compounds produced. Until now, it has not been scientifically proven. The leaves showed a broader spectrum of action than the roots, which attests the importance of studies with different parts of the plant. Improving the process of obtaining tough lovegrass leaf extract could result in an effective natural product to contain the wheat disease caused by *Dreschlera tritici-repentis*. The determination of the active compound(s) against the pathogen must be investigated, which could increase the antifungal spectrum.

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