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Antimicrobial Activity of a Crude Extract and Fractions from *Alternanthera brasiliana* (L.) O. Kuntze Leaves

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SUMMARY. Aqueous EtOH (70%) crude extract of *Alternanthera brasiliana* leaves and dichloromethane, ethyl acetate and butanolic fractions of the crude extract were tested against a panel of microrganisms by the broth microdilution method to determine the Minimal Inhibitory Concentration (MIC). The results demonstrate that the crude extract and some fractions showed moderate activities against *Staphylococcus aureus, Pseudomonas aeruginosa, Saccharomyces cerevisae* and *Prototheca zopffi.* All samples did not present activity against the bacteria *Escherichia coli, Candida albicans* and *Candida glabrata.* The strongest effect occurred with dichloromethane fraction against the algae *P. zopfii,* (MIC= 312.5 μg.mL^{-1}). These results demonstrate that *A. brasiliana* have a weak antimicrobial activity that not support the ethno-pharmacological indication of the plant for the treatment of infection diseases nor it's popular names such as "penicillin" and "terramycin" in Brazil .

RESUMEN. "Actividad Antimicrobiana del Extracto Bruto y Fracciones de las Hojas de *Alternanthera brasiliana* (L.) O. Kuntze". El extracto etanólico crudo (70%) y las fracciones diclorometánica, de acetato de etilo y butanólica de las hojas de *A. brasiliana* fueron analizados en relación a su actividad antimicrobiana. Las concentraciones inhibitorias mínimas (MIC) fueron determinadas usando el método de microdilución en caldo. Los experimentos demostraron que el extracto y algunas fracciones exhibieron moderada acción antimicrobiana, particularmente contra *Staphylococcus aureus, Pseudomonas aeruginosa, Saccharomyces cerevisae* y *Prototheca zopffi*. El efecto más fuerte ocurrió con la fracción diclorometánica frente a *P. zopfii* (MIC 312,5 μg.mL^{-1}). La bacteria *Escherichia coli* y las leveduras *Candida albicans* y *C. glabrata* no fueron inhibidas por ninguno de los extractos probados. La baja actividad antimicrobiana de la planta no respalda las indicaciones planteadas por la comunidad para su uso en el tratamiento de procesos infecciosos, ni tampoco sus nombres populares en Brasil, tales como "penicilina" y "terramicina".

INTRODUCTION

Alternanthera brasiliana (L.) O. Kuntze, commonly known as "Penicilina", "Terramicina" or "Doril" belongs to the Amaranthaceae family. It is a perennial herbaceous plant widely distributed in Brazil's eastern beaches and certain Amazonian's regions [1].The plant is popularly used in Brazilian folk medicine as an analgesic and anti-inflammatory remedy for the treatment of infectious processes [2,3] . Previous phytochemical studies on this plant furnished the presence of triterpenoids and betacianin and triterpenes and steroids such as β-sitosterol, besides phenolic compounds [2,4]. Various bioactivities, including antinociceptive [2], lymphocyte proliferation inhibition [4] and antiviral [5] properties of crude extracts, fractions or isolated compounds from this plant were reported. Regarding antimicrobial activity some controversial results were reported, such as the activity against *Staphylococcus aureus* reported by Caetano *et al.* [6]. and the ineptness of a methanolic extract of the aerial parts of the plant against various microrganisms reported by Souza *et al.* [7] both using the agar-diffusion method. These previous results encouraged us to deepen the studies on antimicrobial properties of *A. brasiliana* by assessing the minimal inhibitory concentration (MIC) using the micro dilution method for the evaluation of the crude extract and fractions against gram-

KEY WORDS: *Alternanthera brasiliana,* Amaranthaceae, Antimicrobial activity.
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positive and gram-negative bacteria, yeasts like fungi such as *Candida albicans* and *Candida glabrata* and the algae *Prototheca zopfii,* the last species also with clinical importance in immunologically suppressed patients [8]. At the same time, the study was conducted with the objective to validate or not the ethnopharmacological claim of this plant concerning the popular names associated to antibiotics (Penicillin and Terramycin) used by the population for this species.

MATERIALS AND METHODS

Plant material

Leaves of *A. brasiliana* were harvested in Santa Maria (State of Rio Grande do Sul, Brazil) on January of 2005. Samples of the collected material were identified by Botanist Dr. Thais Scott do Canto Dorow and archived as voucher specimens in the herbarium of Department of Biology at Federal University of Santa Maria by register number SMD 10038.

Extraction and partition of the leaves

Air dried, powdered leaves of *A. brasiliana* (472.33 g) were extracted with ethanol (70%) at room temperature for seven days with daily agitation. After filtration, the extract was evaporated under reduced pressure to remove the ethanol in order to obtain an aqueous suspension. The aqueous suspension was partitioned successively with dichloromethane, ethyl acetate and *n*-butanol (3 x 100 mL for each solvent), yielding dichloromethane, ethyl acetate, and *n*-butanol extracts, respectively. The extracts were evaporated to dryness under vacuum, to yield dichloromethane (11.80 g, 2.5%), ethyl acetate (2.83 g, 0.6%) and *n*-butanol-soluble (9.44 g, 2.0%) fractions respectively. At the same time 100 g of the dried and powdered leaves was extracted with ethanol (70%) at room temperature for seven days and after filtration the extract was evaporated under reduced pressure to obtain the crude extract.

Antimicrobial Screening

The crude extract and the fractions were individually tested against a panel of microorganisms including *C. albicans* ATCC 28367, *C. glabrata* (clinical isolate), *Saccharomyces cerevisiae* ATCC 28952, *S. aureus* ATCC 25293, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC2792, *Pseudomonas aeruginosa* ATCC 27853, and *P. zopfii* (clinical isolate). Bacterial strains were cultured overnight at 37 °C in Mueller-Hinton Agar (MHA). Yeasts and the algae were cultured overnight at 30 °C in Sabouraud dextrose agar.

Microdilution Method for MIC Determination

The MICs of the crude extract and fractions against the test microorganisms were determined by the broth microdilution method according to National Committee for Clinical Laboratory Standards [9,10]. Seven different dilutions of each fraction (dichloromethane, ethyl acetate and butanolic) and the crude extract (1.250, 625, 312.5, 156.25, 78.125, and 39.06 $\mu g.mL^{-1}$) in DMSO were prepared. Bacteria were inoculated into Mueller-Hinton agar and, after overnight growth four or five colonies were directly suspended in saline solution so that the turbidity matched the turbidity of the McFarland standard ($\approx 10^8$cfu/mL). By further progressive dilutions with the test medium, the required concentrations were obtained. The suspension was diluted by 1:100 in saline followed by a new dilution of 1:20 in Mueller-Hinton broth, resulting in a final inoculum concentration of 5 x 10^4 CFU per well. Yeasts like fungi were inoculated into potato dextrose agar. Tests were performed in sterile 96-well plates. The first column of the plate was reserved for negative control wells (without inoculants) and the last column, for the positive growth control wells (without antimicrobial agents). The plates were sealed and incubated at 35 °C for 24 h for bacteria and *Candida* and 72 h for *S. cerevisae* and growth or a lack thereof in the antimicrobial agent containing wells was determined by comparing with the growth control, indicated by turbidity. The experiments were repeated twice and the results were determined as an average value. The result readings were made visually. The MIC endpoint was considered as the lowest concentration of the extract or fraction inhibiting the total growth of microrganisms. MIC was detected by lack of visual turbidity (matching the negative growth control). Subcultures were made from the clear wells which did not show any growth after incubation during the MIC assays on Mueller-Hinton agar for bacteria and Sabouraud agar for fungi and algae in order to achieve the Minimal Bactericidal Concentration (MBC) the Minimal Fungicidal Concentration (MFC) and the Minimal Algaecidal Concentration (MAC). The lowest concentration that yielded no growth after this sub-culturing was taken as the MMC, MFC and

MAC. Standard antibiotics (ampicillin, imipenem, and cefoperazone) were used to control the sensitivity of the tested bacteria, whereas fluconazole and amphotericin B were used as control against the tested fungi and the algae.

Determination of Percent activity

The percent activity was determinate according Ellof [11] and Rangasamy *et al.* [12], and demonstrates the total antimicrobial potency of a particular extract or fraction [**Ec. 1**].

$$\text{Activity (\%)} = \frac{\text{100 x nº of susceptible strains to a specific extract}}{\text{Total nº of tested strains}} \quad \textbf{[Ec. 1]}$$

Determination of total activity

Total activity was determinate according Ellof [11] and Rangasamy *et al.* [12]. The value obtained would indicate the largest volume to which biologically active compounds in 1 g of plant material can be diluted and still inhibits the growth of microrganism [**Ec. 2**].

$$\text{Total activity} = \frac{\text{quantity of material extracted from 1 g of plant material}}{\text{MIC}} \quad \textbf{[Ec. 2]}$$

RESULTS AND DISCUSSION

The broth dilution method, standardized by the Clinical and Laboratory Standards Institute (formerly NCCLS) allowed determining the minimal inhibitory concentrations (MICs) and also the MMC, MFC and MAC of the crude extract and different fractions. Results showed that the different extracts differed clearly in their antimicrobial activities (Table 1).

The crude extract and ethyl acetate fraction showed a discrete activity against *S. aureus* with MIC and MMC at 1250 µg.mL^{-1} whereas only the dichloromethane fraction presented MIC and MMC at 1250 µg.mL^{-1}, against *B. subtilis*. Antibacterial activity against various strains of *S. aureus* has been reported for the crude ethanolic extract from *A. brasiliana* by Caetano *et al.* [6] at a concentration of 65 µg.mL^{-1} determined by the agar diffusion technique. Although using different methods, the results reported here are near 50 times better than those reported above. Concerning the gram-negative bacteria, the results obtained were modest, with a reasonable activity of the dichlorometane fraction against *P. aeruginosa* at 1250 µg.mL^{-1}, as this microrganism exhibits resistance to many antimicrobial agents [13], whereas against *E. coli,* all samples were ineffective even at the higher concentration tested (2500 µg.mL^{-1}). In respect *of S. cerevisae,* the crude extract and the fractions tested were effective, with better results obtained with crude extract (MIC and MFC at 625 µg.mL^{-1}).

No activity was observed for all fractions against *C. albicans and C. glabrata,* with MIC above 1250 µg.mL^{-1}. Only the dichloromethane fraction showed good activity against the algae *P. zopfii,* with MIC and MAC at 312.5 µg.mL^{-1}. To our knowledge, this is the first assay involving this plant and the algae *P. zopfii*. Protothecosis is especially difficult to eradicate with

	Crude extract MIC	**Crude extract MBC MFC or MAC**	**CH_2Cl_2 MIC**	**CH_2Cl_2 MBC MFC or MAC**	**AcOet MIC**	**AcOet MBC MFC or MAC**	**BuOH MIC**	**BuOH MBC MFC or MAC**	**Control µg.mL^{-1}**
E. coli	>2500	*	>2500	*	>2500	*	>2500	*	Amp 8.0
P. aeruginosa	>2500	*	1250	1250	>2500	*	>2500	*	Cpz. 16.0
S. aureus	1250	1250	1250	>1250	1250	1250	>2500	*	Amp 2.0
B. subtilis	2500	2500	1250	1250	2500	2500	2500	2500	Imi 0.06
C. albicans	>1250	*	>1250	*	>1250	*	>1250	*	Flu 16
C. glabrata	>1250	*	>1250	*	>1250	*	>1250	*	Flu 32
S. cerevisae	625	625	625	>1250	625	1250	>1250	*	Flu 2.0
P. zopfii	>1250	*	312.5	312.5	>1250	*	>1250	*	Amph 0.5

Table 1. Minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC), minimal fungicidal concentration (MFC) and minimal algaecidal concentration (MAC) for crude extract and fractions of *Alternanthera brasiliana* leaves. Amp = ampicillin, Cpz = cefoperazone, Imi = Imipenem, Flu = fluconazole, Amph = Amphotericin B. * not tested because the MIC was not determined. Tests were carried out twice. Values are expressed in µg ml^{-1}.

drugs and there is no defined treatment protocol [14,15].

The discovery of new plant products with potential antimicrobial application is of considerable interest in view of the increasing antibiotic resistance observed in many bacteria such as *S. aureus, E. coli,* and *P. aeruginosa* and the use of ethnopharmacological knowledge is one striking way to reduce empirism and enhance the probability of success in new drug active development. Partition between immiscible solvents is an adequate approach for the preliminary separation of complex matrices because this procedure permit discrimination between polar and non-polar fractions activities. The importance of a preliminary fractionation could be seeing in the case of *P. zopffi,* in view of the fact that the crude extract was ineffective against this algae and the dichloromethane fraction showed a very good activity (Table 1). It is mainly probable that the low concentration of the active(s) compounds may prejudice their detection in crude extracts. In this study, dichlorometane fraction was found to be more effective than the crude extract and ethyl acetate and butanolic fractions against all the microrganisms tested, suggesting that the active(s) substance(s) are concentrated in the less-polar fraction. Total activities values revealed that the dichloromethane fraction had a high magnitude of antimicrobial activity, as the active component(s) from this fraction can be diluted in ca 80 mL of solvent and still inhibits growth of *P. zopfii* (total activity= 79.93 mL g^{-1}) and in ca 40 mL for the inhibition of *S. cerevisae* (total activity = 39.96 mL g^{-1}). Also, the Percent Activity (A%) for the crude extract and fractions against the panel of microrganisms tested was: dichloromethane (62.5%), crude extract and ethyl acetate fraction (37.5%) and butanolic fraction (12.5%), the less active fraction.

On the other hand, it is moreover of fundamental importance to clarify the ethno-pharmacological claim regarding the plant employ in infectious processes, because the results obtained in this work, even though a reasonable antimicrobial activity against some bacteria's and a algae were not sufficient to justify the plant use in such diseases especially when we consider Cos *et al.* [16] guiding principles and the MICs of the antibiotics used as reference standards (Table 1). Additionally, the local names of *A. brasiliana* such as "Doril", "Penicilina" and "Terramicina" may be conduct to a misuse of the plant and may also encourage self medication, because in Brazil, "Doril" is the trade name of a pharmaceutical speciality commercialised as analgesic (contains acetilsalicilic acid) whereas "Penicilina" is a short name commonly used to designed the β-lactam antibiotic benzylpenicillin (Penicillin G) and "Terramicina" (Terramycin) is a registered trade mark of the antibiotic oxytetracycline.

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