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Antimicrobial activity and toxicity *in vitro* and *in vivo* of *Equisetum hyemale* extracts

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ABSTRACT

***Equisetum hyemale* L. (Equisetaceae) species is considered a medicinal plant used in the form of teas to combat infectious or inflammation diseases, presenting several compounds related to these actions. There are no extensive studies about the use against different microbial groups as well as for the toxicity. The objective of these studies was for the first time evaluated the antimicrobial activity against oral microorganisms and the *in vitro* and *in vivo* toxicity of 70% ethanol and methanol *E. hyemale* extracts. Antimicrobial activity assays were performed by broth microdilution technique to determine the Minimum Inhibitory Concentration (MIC) and the cytoxicity was assayed *in vitro* and acute toxicity *in vivo* was performed with mice. The methanol extracts, showed better antimicrobial activity against oral microorganisms whit MIC of 0.5 mg/mL. Both extracts presented low cytotoxicity even in high concentrations and the 70% ethanol extract of *E. hyemale* did not present toxicity inducing significant alterations and/or death in mice. This results suggests that both extracts exhibits great potential to therapeutic applications.**

Keywords: *Equisetum hyemale*. Extracts. Oral microorganisms. Toxicity.

INTRODUCTION

Plants represent a source of interest to search and discovery of new biologically active molecules contributing to new therapeutic options to combat several diseases. Natural products are not completely free from side effects and studies have demonstrated the need to ensure the safety of these products related to its therapeutic properties without taking a risk for the human health (Sahoo *et al*., 2010).

Equisetum hyemale is a cosmopolitan species, popularly used as tea to combat numerous diseases (Canales *et al*., 2005; Park & Jeon, 2008). The chemical composition of this species has important secondary metabolites associated to various biological activities such as ethyl palmitate (Sui *et al*., 1997), kaempferol (Sui *et al*., 1996), glycosides and other phenols compounds (Geiger *et al*., 1982; Milovanović *et al*., 2007), with properties hyperlipidemic, antimicrobial, hepatoprotective, antioxidant and anti-inflammatory (Xu *et al*., 1993; Navarro *et al*., 1996; Eun-Young & Hoon, 2008; Park & Jeon, 2008; Yi *et al*., 2008; Pan *et al*., 2009).

The oral microbiota is influenced by external factors as smoking, alcoholism, nutritional status, oral hygiene, antibiotics and hospitalization, leading to the possibility of selecting bacterial species (Morais *et al*., 2006), with a consequent rise of the resistant. This fact increases the importance of the searches for new therapeutic options to combat infections caused by oral microorganisms.

In parallel of the search for new potential therapies arises the urgent need to study the safety of natural products. The objective of this study was, for the first time, to determine the antimicrobial activity of *E. hyemale* ethanol and methanol extracts against oral microorganisms and establishes the safety of their uses, performing *in vitro* citotoxicity and toxicity tests on animals.

The toxicity tests permit to verify the safety of the use of natural products, representing thus, an important phase in the process of obtaining pharmacologically active

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molecules. The cytotoxicity test *in vitro* is a preliminary assessment which can discriminate vegetal species and its derived compounds that really produces benefits to human health. In addition, it defines the security levels for animal tests in the sequence studies of toxicity (Ngutaa *et al*., 2012).

MATERIAL AND METHODS

Plant harvesting and obtaining extracts

The material of *E. hyemale* was collected in March 2009, of "Horto de Plantas Medicinais e Tóxicas de Araraquara", São Paulo, Brazil and the identification was performed by Prof. Dr. Marco Antônio de Assis. The voucher specimen of *Equisetum hyemale* L. was deposited in the "Herbário Rioclarense do Instituto de Biociências da UNESP" Rio Claro, São Paulo, Brazil under number 51670 HRCB. The aerial parts of *E. hyemale* were dried in an oven with air circulation at 40ºC until stabilization and then pulverized in a balls mill. The plant was submitted to the extraction process with 70% ethanol (v/v) (70% EtOH) and methanol (MeOH) by exhaustive percolation (List & Schmidt, 2000) which was performed with 80 g of the vegetable drug. The extracts obtained were concentrated under vacuum, liophylized and stored in desiccators.

Microorganisms

Oral microorganisms used in the Research Laboratory of Applied Microbiology (LaPeMA), University of Franca were: *Enterococcus faecalis* (ATCC 4082), *Streptococcus salivarius* (ATCC 25975), *Streptococcus sanguinis* (ATCC 10556), *Streptococcus mitis* (ATCC 49452), *Streptococcus mutans* (ATCC 25175), *Streptococcus sobrinus* (ATCC 33478) and *Lactobacillus casei* (ATCC 11578).

Evaluation of antimicrobial activity

The determination of Minimum Inhibitory Concentration (MIC) of oral microorganisms was performed using the microdilution technique in 96-well plates, according to the Documents M7-A7 of the Clinical and Laboratory Standards Institute (CLSI, 2006). Stock solutions of the extracts were dissolved in DMSO (50 mg/mL) and evaluated at concentrations between 1.25 to 0.12 mg/mL, in the range from 0.016 to 0.00001 mg/mL. Chlorhexidine dihydrochloride was used as positive control against oral microorganisms in a concentration of 0.0059 to 0.00001 mg/mL and DMSO was used as solvent control (1:5 (v/v). Growth inhibition tests to oral microorganisms used BHI in microplates which were incubated at 37 °C for 24 h. Oral microorganisms MICs were determined as the lowest concentration presenting blue coloration after 2 h at 37 ºC of 0.01% resazurin addition (Gabrielson *et al*., 2002). All the tests were performed in triplicate.

Cytotoxicity evaluation

Cytotoxicity tests were performed with two eukaryotic cells: the rabbit corneal fibroblasts (SIRC, CCL-60) and murine macrophages (J774). Fibroblast cells were maintaining in culture flasks in Eagle's medium (pH 7.0) supplemented with 50% Leibovitz L-15 medium, 10% fetal calf serum and 0.2% sodium bicarbonate. Macrophage cells were maintaining in culture flasks in RPMI-1640 (pH 7.0), prepared with 10% fetal bovine serum, 0.2% sodium bicarbonate and 10 mM Hepes with addition of 10 µg/mL of. 0.1% streptomycin and 0.1% amphotericin B. Both cells types were incubated under the same conditions at 37°C under an atmosphere of 5% CO2.

The cytotoxicity technique consisted in collecting the cells by trypsinization, centrifuge at 1500 rpm, 10 minutes, and adjusting the concentration for 105 cells/mL in culture medium. The cells were incubated in 96-well microplates at 37°C, 5% CO2 for 24 h and 72 h for macrophages and fibroblasts, respectively. After the observed confluence of the cells, the medium were removed and a new one with the extracts serially diluted in concentration ranging from 2000 µg/mL to 15.65 µg/mL was added. The tests were performed in triplicate. After 24 h incubation, 15 µL of resazurin aqueous solution (0.1 mg/mL) was added, mantaining for additional 3 h in the same conditions. There were a positive and negative control of cell growth and a control of extract samples. The reading of the results was done visually by the differentiation between the blue color (absence of living cells) and pink (living cells) and by the fluorescence of the reduced product resorufin which was read using a microplate spectrofluorometer (Spectra Fluor Plus, Tecan) with excitation wavelength of 530 nm and 590 nm emission (Takahashi *et al*., 2008). The 50% cytotoxic concentration (CC50) was calculated based on fluorescence analyzed by Magellin software for correlation and regression (Perrot *et al*., 2003).

Acute oral toxicity test in vivo

The use of animals testing was approved by the "Comitê de Ética em Pesquisa da Faculdade de Ciências Farmacêuticas de Araraquara- UNESP" (protocol number 17/2010). Following the guidelines of the OECD (Organization for Economic Co-operation and Development, Guideline 423) (OECD, 2001), 10 mice (*Mus musculus*) Swiss adult male, weighing between 29-35 g were maintained in cages, in a room with light-dark cycle of 12 hours and 20ºC submitted to food and water deprivation. For the test, the animals were kept in same conditions and three hours before the extract administration they returned to receive food and water. A single dose of 70% ethanol extract (1 mL) was administered at a concentration of 100 mg/kg by gavage, using distilled water for the control group. They were observed in the first 30, 60, 120, 240 and 360 minutes and every 24 hours for 14 days, for parameters changes such as locomotion, heart and respiratory rates, piloerection, diarrhea, excessive salivation, hypnosis, seizures and number of deaths.

RESULTS

Antimicrobial activity

MIC results of the 70% ethanolic extract showed a range between 1 to 2 mg/mL and the methanolic extract showed a range between 0.5 to 2 mg/mL (Table 1) against oral microorganisms.

Table 1 Minimum Inhibitory Concentration (MIC) against oral microorganisms.

Microorganisms	70% Ethanolic Extract (mg/mL)	Methanolic Extract (mg/mL)	Control Chlorhexidine (mg/mL)
E. faecalis	2	2	0.014
S. salivarius	1	1	0.007
S. sanguinis	1	1	0.007
S. mitis	1	0.5	0.014
S. mutans	2	1	0.001
S. sobrinus	1	1	0.001
L. casei	2	2	0.003

Values means the triplicate average.

Cytotoxicity

The cytotoxicity effect for the 70% ethanolic and methanolic extracts of *E. hyemale* was determined to strains of fibroblast and murine macrophage. The 70% ethanol extract showed no cytotoxicity effect to fibroblasts at the highest concentration tested and the cell viability was reduced 50% of in the range of 1000 to 2000 µg/mL to murine macrophage while as methanolic extract was cytotoxic in this concentration range for both cells (Figure 1).

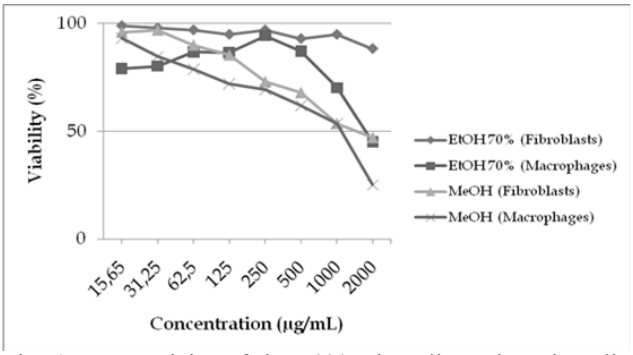

Fig. 1. Cytotoxicity of the 70% ethanolic and methanolic extracts of *E. hyemale*.

Acute oral toxicity

The acute toxicity assay performed with 70% ethanol extract showed changes in the piloerection pattern during the initial minutes after the administration, but any other alterations were observed throughout the 24 h of experiment or during the subsequent 14 days with survival of all treated animals.

DISCUSSION

The search for new therapeutic agents is an urgent need because the resistance of anaerobic microorganisms has increase significantly; and the high frequency of resistance has contributed to reduction the efficiency of commercially available antimicrobial, becoming a serious public health problem (Sweeney *et al*., 2004).

Equisetum sp extracts presented antimicrobial activity in higher concentrations by different analysis and against several microbial strains (Navarro *et al*., 1996; Lee *et al*., 2001; Uzun *et al*., 2004; Canales *et al*., 2005; Kloucek *et al*., 2005; Robles-Zepeda *et al*., 2011). However, no other previous study tested the ethanol and methanol extracts of *E. hyemale* against oral microorganisms. *E. hyemale* that is commonly used as tea in several regions of the world, showed in our study, good action against oral microorganisms.

The cell viability was determined as preliminary assessment of toxicity of the extracts and show that that the methanolic extract of *E. hyemale* did not show cytotoxicity effect at the maximum concentration tested of 1000 µg/mL performed by the method using MTT (Park & Jeon, 2008). Our results showed low levels of cytotoxicity and corroborate this study since no cytotoxic effect, in addition to the antimicrobial properties evaluation of the extracts of *E. hyemale*, suggesting their safety. The high concentration required to produce cytotoxicity effect to the cell population is indicative of the safety of these extracts.

In this study was not observed any toxic effects and similar results were observed in previous studies for other species of the genus, in which oral treatment with *E. arvense* extracts was not able to produce hepatic changes in rats (Baracho *et al*., 2009) and the extract of *Equisetum* myriochaetum demonstrate no genotoxicity either *in vivo* or *in vitro* (Téllez *et al*., 2007). The safe use of natural products is a factor of great importance nowadays. As an option to investigate the possible presence of toxic agents in plant extracts, the method of single dose acute toxicity represents an estimative as preliminary evaluation, providing introductory information about the risks to health resulting from a short-term exposure to the selected via of administration.

CONCLUSION

The methanol extracts, showed better antimicrobial activity against oral microorganisms and low toxicity was observed even when tested at high concentrations of the 70% ethanolic and methanolic *E. hyemale* extracts, showing a potential for safe use for different applications.

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RESUMO

Atividade antimicrobiana e toxicidade in vitro e in vivo de extratos de Equisetum hyemale

***Equisetum hyemale* L. (Equisetaceae) é considerada uma planta medicinal por ser utilizada sob a forma de chás para combater doenças infecciosas ou inflamatórias, apresentando vários compostos relacionados a essas ações. Não existem estudos extensos sobre a utilização contra diferentes grupos de micro-organismos, bem como para a toxicidade. O objetivo desse estudo foi avaliar a atividade antimicrobiana contra micro-organismos orais e a toxicidade *in vitro* e *in vivo* dos extratos etanólico (70%) e metanólico de *E. hyemale.* A avaliação da atividade antimicrobiana foi realizada pela técnica de microdiluição em caldo para determinar a Concentração Inibitória Mínima (CIM), a citotoxicidade foi realizada *in vitro* frente a linhagens de macrófagos e fibroblastos e a toxicidade aguda foi realizado *in vivo*. O extrato metanólico apresentou melhor atividade antimicrobiana contra micro-organismos orais, com CIM de 0,5 mg/mL. Ambos os extratos apresentaram baixa citotoxicidade, mesmo em altas concentrações e o extrato etanólico (70%) não apresentou toxicidade *in vivo* capaz de induzir alterações e/ou morte significativa em camundongos. Estes resultados sugerem que ambos os extratos apresentam potencial para aplicações terapêuticas.**

Palavras- chave: *Equisetum hyemale*. Extratos. Micro-organismos orais. Toxicidade.

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