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Proliferative effect of plants used for wound healing in Rio Grande do Sul state, Brazil

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ABSTRACT

Ethnopharmacological relevance: Wounds are normally resolved in a few days, but chronic wounds represent a major burden because of economic and social factors. Thereby, the search for new agents is ongoing and natural products become a great target. Also, Brazil as a consumer of herbal medicines with rich social diversity is promising for ethnopharmacological studies.
Aims of the study: The study aims to find the plants popularly used for wound healing purposes in Rio Grande do Sul state, and test the traditional knowledge through an *in vitro* screening.
Materials and methods: Ethnobotanical studies from state of Rio Grande do Sul were analyzed to find the most used plants to treat wounds. The selected species were collected, identified and ethanolic and aqueous extracts were prepared. After, proliferative capacity was accessed by MTT assay in a keratinocyte cell line (HaCaT).
Results: The survey comprehended almost all state regions and led to 117 plant species from 85 genera, from which 14 were selected for *in vitro* testing. Aqueous extracts from *Achyrocline satureioides* DC Lam., *Matricaria recutita* L., *Melia azedarach* L. and *Mirabilis jalapa* L. demonstrated the ability to stimulate keratinocyte growth up to 120% in concentrations of 25 μg/mL and 50 μg/mL. The ethanolic extract of *A. satureioides* was able to stimulate keratinocyte and fibroblast proliferation on the lower concentration tested, 1 μg/mL, being the most promising species.
Conclusions: The traditional knowledge collected from the ethnobotanical studies was accessed by *in vitro* investigation and extracts from *Achyrocline satureioides*, *Matricaria recutita*, *Melia azedarach* and *Mirabilis jalapa* can influence positively cell proliferation.

1. Introduction

Brazil is a great consumer of herbal medicines (Oliveira et al., 2012). Medicinal plants and natural products have already been included in the Brazilian Public Health System (SUS), demonstrating the importance of the traditional medicine to the country. Also, the government implemented a national program to guarantee safe access to medicinal plants, the National Program on Medicinal Plants and Phytotherapy (Figueredo et al., 2014).

The Brazilian culture and biodiversity create a propitious environment to develop ethnopharmacological studies. The Rio Grande do Sul state, South Brazil, target area of this study, has a mixture of different cultures, once it was colonized by several countries that blended their knowledge with the native indigenous groups.

In popular medicine, plants are used to empirically treat a variety of diseases. Wounds and burns are examples of conditions commonly managed with medicinal plants. Although the wound healing process is normally completed in two weeks after the tissue damage, chronic wounds are still a public health problem (Sen et al., 2009). Many factors can influence wound healing, as diabetes mellitus, trauma, kidney and liver insufficiency, smoking, etc., thereby most treatment attempts are unsatisfactory (Tazima et al., 2008).

Wound healing is divided in four phases: hemostasis,

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inflammatory, proliferative and remodeling stages and the healing success depends on the synergic action of blood cells, platelets, cytokines, growth factors, fibroblasts and keratinocytes (Harper et al., 2014). The aims of this study are to find through an ethnobotanical survey, the plants popularly used for wound healing and evaluate their proliferative activity by *in vitro* testing.

2. Materials and methods

2.1. Ethnobotanical Survey

Ethnobotanical studies conducted in the state of Rio Grande do Sul, Brazil, were evaluated to obtain the plants popularly used for wound healing (Somavilla and Canto-Dorow, 1996; Kubo, 1997; Magalhães, 1997; Possamai, 2000; Garlet, 2000; Marodin, 2000; Ritter et al., 2002; Sebold, 2003; Vendruscolo, 2004; Barros et al., 2007; Schwambach, 2007; Ceolin, 2009; Borges, 2010; Battisti et al., 2013). The main descriptors were “wounds”, “wash wounds”, “wound healing” and “burns”, and the selection criteria were the number of citations for each species (minimum of three citations) and the related uses.

2.2. Plant material

The aerial parts of *Achyrocline satureioides* (Lam.) DC, *Bidens pilosa* L., *Chaptalia nutans* (L.) Polak, *Malva parviflora* L., *Matricaria recutita* L., *Melia azedarach* L., *Mirabilis jalapa* L., *Piper regnelli* DC, *Plantago australis* Lam., *Pluchea sagittalis* (Lam.) Cabrera, *Sambucus australis* Cham. & Schltdl., *Sedum dendroideum* Moç. et Sessé ex DC, *Symphytum officinale* L. and *Tanacetum vulgare* L. were collected in the state of Rio Grande do Sul, in Passo Fundo, Carazinho, Porto Alegre and Rondinha cities between April 2014 and January 2015 and identified by a competent botanist. Voucher specimens were deposited in the ICN Herbarium of Universidade Federal do Rio Grande do Sul.

2.3. Plant extracts

Plants were dried at room temperature and hide from sunlight. Dried and powdered samples (0.5 g) were extracted with ethanol by maceration (3×24 h) and by decoction or infusion with distilled water (1:50), according to traditional use. The extracts were evaporated until dryness at 50 °C under reduced pressure and stocked for further analyses. The yields varied around 16% for the ethanolic extracts and 21% for the aqueous extracts.

2.4. Methods of cell biology

2.4.1. Cell culture

Immortalized Human Keratinocytes (HaCaT) cell line was kindly provided by Luisa L. Villa PhD (ICESP, School of Medicine, University of São Paulo) and Silvya S. Maria-Engler PhD (Faculty of Pharmaceutical Sciences, University of São Paulo). Human Lung Fibroblasts (MRC-5) cell line was kindly provided by Jennifer Saffi (Department of Basic Health Sciences, Federal University of Health Sciences of Porto Alegre). The cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium) high glucose, with 10% FBS (Fetal Bovine Serum) and maintained at 37 °C, in 5% CO_2 humidified atmosphere.

2.4.2. MTT assay

In order to evaluate cell proliferation, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay was performed, in which only viable cells are able to reduce MTT salt to purple formazan crystals. The intensity of purple color corresponds directly to the number of viable cells.

HaCaT keratinocytes were seeded at a density of 1×10^4 cells per well in 96-well plates and incubated for 24 h before treatment. A stock solution was prepared by dissolving the plant extract in a mixture of 75% sterile distilled water and 25% DMSO (Dimethyl sulfoxide), and following test samples were obtained by dilution with DMEM (1, 5, 10, 25 and 50 μg/mL). The final DMSO concentration was lower than 0.5% (v/v). Culture medium was used as negative control. Cells were treated with test samples and incubated for 24 h, then the treatment was withdrawn and the MTT solution (0.5 mg/mL) was added and incubated for 3 h at 37 °C. The formazan crystals formed were solubilized with DMSO and the absorbance was measured using a micro-plate reader at 570 nm with background subtraction at 630 nm. The assay was performed in three independent tests, with five replicates for each concentration. Cell viability was calculated as percentage in relation to control.

2.4.3. Cell counting

Cell proliferation was confirmed by counting of viable cells. HaCaT keratinocytes and MRC-5 fibroblasts were seeded in 48-well plates (1×10^4 cells/well), and after 24 h treated with *Achyrocline satureioides* ethanolic extract at concentrations of 1, 5, 10, 25 and 50 μg/mL. After 24 h of incubation, the treatments were removed, cells were washed with phosphate-buffered saline (PBS) and 0.25% trypsin/EDTA solution was added to detach the cells. Then, DMEM/10% FBS was added, and the cell suspension was analyzed by flow cytometry (FACSVerse, BD Biosciences, San Jose, CA, USA), where events were counted over a period of 30 sec and the volume analyzed at this time was also determined, generating a value of events/μL of cell suspension. The results were expressed compared to control (cells treated with DMEM/10% FBS) that represents 100% of viability.

2.4.4. Ki-67 proliferation assay

Ki-67 proliferation assay was performed, once is considered gold standard to evaluate cell proliferation. HaCaT keratinocytes and MRC-5 fibroblasts were seeded in 24-well plates (2×10^4 cells/well), and after 24 h the cells were treated with *Achyrocline satureioides* ethanolic extract at concentrations of 1, 5, and 10 μg/mL. Control cells were treated with only DMEM/10% FBS. After 24 h of incubation, the treatment was withdrawn; the cells were trypsinized and centrifuged to obtain a pellet of cells. Then, cells were permeabilized with a 1% SFB and 0.09% NaN_3 PBS solution, and 10 μL of Ki-67 antibody (sc-23900 PE, Santa Cruz Biotechnology) was added to each tube. After 20 min of incubation at room temperature, protected from light, the cells were centrifuged and resuspended in PBS. In parallel, a sample was processed in the same way, except for the addition of the Ki-67 antibody (unlabelled control). The fluorescence intensity of the samples was analyzed using a FACSVerse flow cytometer (BD Biosciences, San Jose, CA, USA).

2.5. Statistical analysis

Data were expressed as mean ± standard deviation for each analysis. Statistical analysis for MTT assay results was performed by Generalized Estimating Equations (GEE) in a factorial model, where all the interactions were analyzed and multiple comparisons were performed by Bonferroni Test. Additionally, for the cell counting and Ki-67 proliferation assay, statistical analysis was done by One-way ANOVA followed by Tukey's Test. Data were analyzed with SPSS 18.0 Software. Differences were considered significant if $p < 0.05$.