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Cytotoxic profile of the hydroalcoholic extract of *Sonchus oleraceus L*. in NHI/3T3 Lines of Fibroblast Cells

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Abstract: Fibroblasts, the main cells involved in re-epithelialization, undergo very marked phenotypic changes, taking on the specific function of protein synthesis. The advances in the use of *in vitro* tests relative to cell viability assays enabled such tests to become the first step in the analysis of the biological compatibility of a compound, in order to prove the death and dysfunction of cell metabolism. For the analysis of cytotoxicity, cell culture was utilized and the cell lines used were: MDCK and NIH-3T3, measured though MTT. After 60 hours of exposure of normal NHI/3T3 fibroblast cell lines, it was found that, according to the EC_{50}, the extract at concentrations ≥2,057µg/mL stimulate cell viability. It is concluded that the hydroalcoholic extract of *S. oleraceus L.* can stimulate the proliferation of fibroblasts and increase collagen production, aiding in tissue regeneration.

Keywords: Cytotoxicity, Fibroblasts, *S. oleraceus.*

Resumo: Os fibroblastos são as principais células envolvidas na reepitelização, e estas passam por mudanças fenotípicas bastante acentuadas, passando a ter a função específica de síntese proteica. Contudo os avanços na utilização de testes *in vitro*, por meio de ensaios de viabilidade celular, se tornou um primeiro passo para analisar a compatibilidade biológica de um composto, com o intuito de comprovar morte e disfunção do metabolismo celular. Para a análise da citotoxicidade utilizou-se cultivo celular das linhagens celulares utilizadas foram: MDCK e NIH-3T3, medida melo MTT. Após o tempo de 60 horas de exposição das linhagens de células normais de fibroblastos NHI/3T3, verificou-se que de acordo com o EC_{50} o extrato em concentrações ≥2, 057µg/mL estimulam a viabilidade celular. Conclui-se que o extrato hidroalcoólico de *S. oleraceus L.* pode estimular a proliferação de fibroblastos e aumenta produção de colágeno auxiliando na regeneração tecidual.

Palavras-chave: *Citotoxicidade, fibroblastos, S. oleraceus*

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Introduction

The skin is the largest organ in the body and performs several functions, such as protection against water loss; storage of fats, carbohydrates, and proteins; assistance in the protection against friction; thermoregulation; and production of vitamins. Moreover, it belongs to the immune system due to its abundance of immunocompetent cells (SARANDY, 2007).

The extracellular matrix (ECM) is the largest component of normal skin, and the synthesis of its components is essential for the effectiveness of the wound healing process. These components provide the characteristics of elasticity and resistance to skin traction; the main ones among these are collagen, elastin and fibronectin (SHULTZ, LADWING and WYSOCKI, 2005). In general, the tissue healing process is composed of three phases: the inflammation phase, the granulation tissue phase and the tissue remodeling phase (SINGER and CLARK, 1996). Rocha Junior et al. (2006), however, classifies the existence of five phases: hemostasis (clotting), inflammation, proliferative, wound contraction and remodeling.

Remodeling is the last phase of the healing process. This phase involves the organization of the collagen deposited in the matrix and of the collagen fibers and the consequent increase in tensile strength. There is also an improvement in the reabsorption of water, as well as a tendency towards the diminishment of scar thickness (MANDELBAUM, DI SANTIS and MANDELBAUM, 2003). In this phase, lymphocytes are attracted to the lesion, as well as macrophages; macrophages are, however, found in greater numbers (HATANAKA and CURI, 2007).

Among the cells involved in the regeneration of injured tissue, circulating cells (neutrophils and monocytes) and cells from adjacent areas such as epithelial cells, keratinocytes and fibroblasts are highlighted. To the latter is attributed a central function in fibroplasia, with fibroblasts being collagen producers and depositing extracellular matrix, acting directly on angiogenesis, wound healing and re-epithelialization (SINGER and CLARK, 1999).

Fibroblasts are the main cells involved in re-epithelialization and undergo very marked phenotypic changes. Immature migratory cells that emerge at the beginning of the healing process change their phenotype, which becomes characteristic of cells that act in protein synthesis. Thus, their cytoplasm becomes voluminous and presents an abundant rough endoplasmic reticulum. After this change, fibroblasts begin to secrete large amounts of collagen, which gradually replaces proteoglycans and fibronectin until it becomes the main component of the cicatrization process (BALBINO et al., 2005).

The use of topical formulations to aid in the healing process has been studied over time (SANTOS et al., 2006). The most tested and applied formulation is the topical administration of compounds to the skin, since local action is more effective and safer, as it does not cause systemic effects (PRAUSNITZ, MITRAGOTRI and LANGER, 2004).

In this context, the advances in the use of *in vitro* tests relative to cell viability assays enabled such tests to become the first step in the analysis of the biological compatibility of a compound. The cytotoxicity test aims to assess whether the tested substance causes cell death or affects their metabolic functions (DAGUANO et al., 2007). It is important to verify the toxicity and applicability as a therapeutic agent of new compounds (MELO et al., 2000).

Studies confirm the healing action of *S. oleraceus* and suggest that this effect is related to its high concentration of flavonoids (anthocyanins). These metabolites are known to act as mediators of inflammation, stimulating the production of inflammatory cytokines and tumor necrosis factor. In nature, they demonstrate the peculiarity of characterizing the color in several plant species (NONATO et al., 2015; NONATO et al., 2015).

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The characterization of the components of the extract of *S. oleraceus*, allied to the clarity of the effect regarding the biocompatibility with fibroblasts, can be of aid in the understanding of its effects or validate the effects already found in previous researches. This information is important to guide the establishment of safer and more effective doses and indications for this species.

In light of this, the objective of this work was to evaluate the cytotoxicity of the lyophilized hydroalcoholic extract of *Sonchus oleraceus L*. in cell cultures of the MDCK and NIH-3T3 lines.

Material and methods

"*In vitro*" assay of the cytotoxic activity of the extract of *S. oleraceus L*.

Cell culture

The protocol used in this work is determined for NIH/3T3 (ATCC® CRL-1658™). The cell lines used were: MDCK and NIH-3T3 (Appendix 4a and 4b). All cell lines were grown in complete media, that is, media supplemented with 10% fetal bovine serum and 1% antibiotics (amphotericin B; penicillin and streptomycin) in 25 cm² polystyrene culture flasks in an incubator at 37ºC, 5% CO2 and 95% relative humidity in Dulbecco's Modified Eagle's Medium (DMEM). When the cultures reached semiconfluence, the cells were trypsinized, replated and maintained under the conditions described above. Cultures were periodically observed under an inverted light microscope, and aliquots were frozen and thawed, according to the necessities of the experiment.

Trypsinization

After the culture medium was discarded, subconfluent cell cultures were washed with PBS and incubated at 37ºC with a 0.25% trypsin and 0.05% ethylenediamine tetraacetic acid (EDTA) solution, until, under microscopic observation, it was possible to detect the dissociation of the cells from each other and from the substrate. The trypsinization reaction was stopped by the addition of 5.0 mL of complete medium. The cell suspension was then collected in a centrifuge tube and centrifuged at 600 G for 5 minutes at room temperature. The supernatant medium was carefully discarded and the cells resuspended in complete medium. After carrying out the cell count, with a viability test, volumes of the cell suspension containing the desired number of cells were subcultured.

Viable cell count

Viable cell counts were performed in a Neubauer hemocytometer after both chambers were loaded with the cell suspension, obtained through trypsinization of the desired cell culture.

To calculate the concentration of cells, the following formula is used:

$$\mathcal{N}umber\ of\ Cells\ mL = Total\ number\ of\ cells \times 10^4\ /Number\ of\ counted\ quadrants$$

In order to determine the number of viable cells, the dye exclusion test was used. This consists of the incubation of an aliquot of cells with a 0.1% Trypan Blue solution (1:1 dilution) for ten minutes at 37° C. Healthy cells exclude the dye. To calculate the percentage of viable cells, the formula is used:

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% *of viable cells = Number of viable cells x* 100 /*Total number of cells*

Cryopreservation and thawing

When necessary, aliquots of cells were frozen after routine trypsinization of semiconfluent cultures. The interruption of the trypsinization reaction was carried out by adding complete medium and washing the cells by centrifugation at 600 G for 5 minutes. The cells were then resuspended in medium supplemented with 40% fetal bovine serum and containing 10% dimethyl sulfoxide (DMSO). The cell suspensions were placed in cryotubes of up to 1.8 mL, which remained in the freezer overnight and were later transferred to liquid nitrogen (-196ºC), where they were stored.

For the thawing stage, cryovials with cells of the cell line in analysis were kept at 37ºC for 5 minutes. The cell suspension was diluted in complete medium in a 25 cm² culture flask and the medium was changed to a new complete medium the following day.

Cytotoxic activity of the S. oleraceus *L.* extract measured through MTT assay

Cells were plated in flat-bottom 96-well plates at a concentration of 1x 10^4 cells/mL. Each well received 100 µL of medium and cell suspension. The plates were incubated for 24 hours at 37ºC, 5% CO2 and 95% humidity in their respective culture media. After this period, the extract concentrations were added to the cells, which were incubated for 60 hours at 37ºC, 5% CO2 and 95% humidity. The compound was diluted in DMSO and concentrations were made between 12.5µg/mL and 1600 µg/mL. The DMSO concentration did not exceed 0.5% in each well. Controls for this assay (100% viable cells) were performed with culture medium containing 0.5% DMSO (v/v) and without the extract.

After this period, the medium containing the drugs was removed; all wells were washed with PBS and 50 µL of a MTT solution (3-{4,5-dimethylthiazol-2-yl}-2,5 -diphenyltetrazolium bromide) at 0.5 mg/ml and the plates were again incubated under the conditions described above for 4 hours. After this period, MTT was removed from all wells and then 100 µL of DMSO was added. The absorbance rates were read in a spectrophotometer with a wavelength of 570 nm. Results were expressed as percentage of viable cells compared to control (no treatment).

Data analysis

For the *in vitro* analysis, the results of the inhibition coefficients in 50% (IC_{50}) of the cells were analyzed and determined in the programs Graph Pad Prism® 5.0 and Microsoft Office Excel 2013®, according to the methodology indicated in the program manual. Statistical differences between experimental groups were evaluated by one-way ANOVA ($p<0.05$).

Results and discussion

Determining cell viability of cells NIH/3T3 treated with *S. oleraceus L.* extract (MTT)

After 60 hours of exposure of the normal cell lines of NHI/3T3 fibroblasts, it was found that, according to EC_{50}, the extract kills cells at lower concentrations (< 2,057µg/mL); however, at concentrations ≥ 2,057µg /mL, it stimulates cell viability (Figure 1).

Supposedly, in a lower concentration, some chemical compounds present in the extract, which inhibit cell growth, prevail in relation to those that stimulate it; when the concentration is

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increased this role is reversed. Thus, the compounds that stimulate cell growth incessantly prevail.

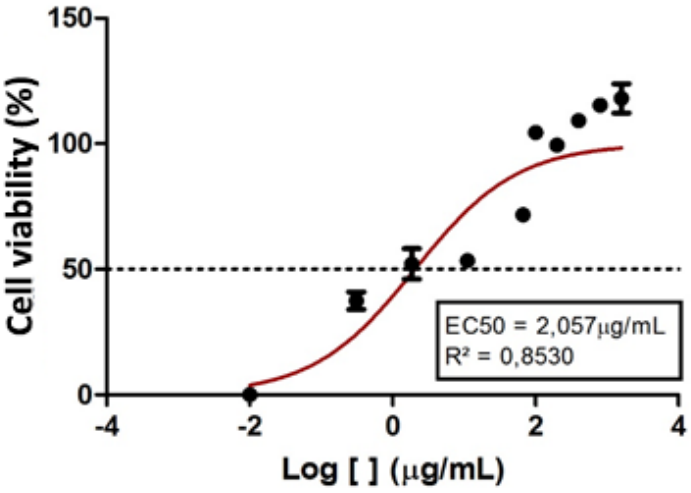

Figure 1: Cytotoxic effect of the *S. oleraceus L.* extract versus NIH/3T3 cells, after 60 hours of incubation.

Studies with plant extracts have been evaluated in recent years with the aim of demonstrating the intrinsic capacity that can lead to a metabolic change in cells in culture, injuring them or leading to cell death.

In a previous study, Nonato et al. (2018) and collaborators demonstrated that the use of a product (ointment) based on *S. oleraceus L.* extract, at a concentration of 15%, used in surgical wounds in Wistar rats, was effective in the healing process, identifying the total retraction of the wound in 10 days. In the present work, cell viability was demonstrated in the time of 60 hours, which is equivalent to two and a half days. These are good results, since they demonstrate that, both in *in vivo* and *in vitro* tests, fibroblast stimulation prevails and can be of help in inflammatory processes and wound healing in both animals and humans.

CAVALARO (2016) worked with the species *Calendula officinalis L.,* a plant that belongs to the same family as the species *S. oleraceus L*., and verified, in the MTT test using human fibroblasts (CCD1072SK), a peak of cell proliferation and growth in the dose of 30µL/mL. These results are similar to those of the present study, which shows continuous growth above the starting concentration (≥ 2,057µg/mL) of *S. oleraceus L*.

Santos (2017) also evaluated cell viability of NIH-3T3 fibroblasts from mice treated with extract of the *Psidium guajava L*. plant, which belongs to the Myrtaceae family. It was observed that the cells remained viable at a concentration of 6,25 to 25 µg/mL, in exposition after 24 hours. Even though *Psidium guajava L.* belongs to a different family from *S. oleraceus L*., both show biological activity to stimulate cell growth. Thus, it can be said that the use of *S. oleraceus L*. can generate a product that stimulates the proliferation of fibroblasts, increasing collagen production and thus assisting in tissue regeneration, maintaining the stability of the extracellular matrix.

There are several studies on the topic of tissue repair with phytotherapics, used from formulations, due to their healing action and consequent stimulation of fibroblast proliferation. In recent years, the highlighted plants were: *Aloe vera (L.) Burm. f.* (Aloe), *Coronopu didymus* ("Mastruz"), *Tabebuia avellanedae* (Purple Ipe), *Arnica Montana L.* (Wolf's bane), *Orbignya*

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phalerata (Babassu palm), *Stryphnodendron adstringens Martius* ("Barbatimão"), *Caesalpinia ferrea Martius* (Brazilian ironwood), *Chenopodium ambrosioides L.* (Jesuit's tea), *Triticum vulgare* (Wheat), *Tabernaemontana catharinensis* (Jasmine), *Calendula officinalis* (Marigold) and *Helianthus annus* (Sunflower), *Catharanthus roseus L.* (Pink Periwinkle) and *Schinus terebinthifolius Raddi* (Brazilian Peppertree). In all of these, flavonoids are found in their respective chemical compositions (MORESKI, MELLO and BUENO, 2018), in the manner of the biological activity proven in a formulation containing *S. oleracaeus L*. extract in wound cicatrization.

NONATO (2019) analyzed dose-effect relationship of *S. oleraceus L*. extracts in rats by evaluating hematological profile and histopathological analysis. In the treated groups, no tissue damage was observed in liver, kidneys, ovary, intestine, or testicles, and there was no significant changes in liver enzymes. These results guarantee safety and reliability when using products based on S. oleraceus L. extract, as it does not cause damage to the organ parenchyma nor relevant changes in hematological and biochemical parameters.

In this context, it can be said that products based on *S. oleraceus L*. extract stimulate the proliferation of fibroblasts, thus increasing collagen production, and can be used not only in wound treatment but also as cosmetics.

Conclusion

The extract of *S. oleraceus L.* is rich in phenolic compounds: arjunic acid, maslinic acid, betulinic acid, lupeol, β-amyrin, α-amyrin, stigmasterol, sitosterol, polyphenols, and flavonoids. The S. oleraceus L. extract stimulates the growth of NIH3T3 and MDCK fibroblasts. A perspective on formulations derived from a natural base of the hydroalcoholic extract of S. oleraceus L. can generate a commercial product that is characterized by being a product capable of promoting quality of life for patients with persistent ulcerative lesions, as it has an antioxidant action, healing, and fibroblast proliferation activity.

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