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Evaluation *in vitro* of proliferative activity of epithelial cells by flavonoid 3-O-methylgalangine and terpenenic derivative Filifolinone

[Evaluación *in vitro* de la actividad proliferative de células epiteliales del flavonoide 3-O-metilgalangina y el derivado terpénico Filifolinona]

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Abstract: The skin is the largest organ of the human body and its main function is to protect it from the external environment. It is exposed to injuries that require a rapid healing process to recover its functionality. Microorganisms inhabit the skin, which makes up the normal microbial flora, but in situations of injury they can cause infections that slow down the regeneration process. Therefore, there is a great interest in the development of alternative methods to accelerate the regeneration process and prevent infections. In this work, the efficacy of flavonoid 3-O-methylgalangine and the terpenic derivative Filifolinone and its mixtures, isolated from plants of the genus Heliotropium, on the stimulation of cell proliferation was evaluated. The results showed that the mixtures stimulated proliferation and migration in MA104 cells mainly due to the presence of Filifolinone, that together with the known antibacterial activity of 3-O-methylgalangine, opens new alternatives for the use of natural compounds in healing processes.

Keywords: Proliferative activity; 3-O-methylgalangine; Filifolinone.

Resumen: La piel es el órgano más grande del cuerpo humano y su función principal es protegerla del entorno externo. Está expuesta a lesiones que requieren un proceso de curación rápido para recuperar su funcionalidad. Los microorganismos que habitan en la piel, constituyen la flora microbiana normal, pero en situaciones de lesión pueden causar infecciones que retardan el proceso de regeneración. Por lo tanto, existe un gran interés en el desarrollo de métodos alternativos para acelerar el proceso de regeneración y prevenir infecciones. En este trabajo, se evaluó la eficacia del flavonoide 3-O-metilgalangina y el derivado terpénico Filifolinona y sus mezclas, aisladas de plantas del género Heliotropium, en la estimulación de la proliferación celular. Los resultados mostraron que las mezclas estimularon la proliferación y la migración en las células MA104 debido principalmente a la presencia de Filifolinona, que junto con la actividad antibacteriana conocida de la 3-O-metilgalangina, abre nuevas alternativas para el uso de compuestos naturales en los procesos de curación.

Palabras clave: Actividad proliferativa; 3-O-metilgalangina; Filifolinona

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INTRODUCTION

Skin diseases are one of the leading causes of global disease burden, affecting millions of people worldwide (Hay *et al.,* 2014). The skin provides an essential barrier against water, electrolytes and bacteria for the outside world. When this function is lost through burns or chronic non healing ulcers, then patients are susceptible to bacterial infection. In burns patients with extensive skin loss, bacterial sepsis can be fatal (MacNeil, 2008). On the other hand, although mild skin lesions in healthy individuals generally regenerate well, larger lesions or the presence of a variety of common pathological or physiological conditions including age, infection, diabetes, vascular disease and cancer can adversely affect the regeneration process (Eming *et al.,* 2014). Therefore, there is great interest in the search for topical formulations that help or accelerate the regeneration of the skin, to be used as a healing and even as an anti-wrinkle (Moriyama *et al.,* 2016). Opportunistic pathogens, such as Gram negative bacteria *Pseudomonas aeruginosa* or Gram positive bacteria *Staphylococcus aureus* are able to colonize wounds forming biofilms, which are characterized by an aggregation of immobilized bacterial cells in an adhesive matrix of extracellular polymeric substances (Mancl *et al.,* 2013). This bacterial matrix makes it difficult to eradicate these microorganisms from the skin, mainly due to its resistance to antibiotics. In addition, toxins released by bacteria contribute to the recruitment of immune cells resulting in an excessive and damaging inflammatory response (Mangoni *et al.,* 2016). Currently the treatments that are used in case of skin burns are topical products based on silver. However, these treatments, despite effectively controlling infections by microorganisms, delay healing due to their cytotoxicity (Atiyeh *et al.,* 2007).

On the other hand, plant have been traditionally used in the treatment of skin diseases due to their therapeutic activities, including anti-inflammatory, antimicrobial, and cell-stimulating properties (Pereira & Bartolo, 2016). To stimulate the regeneration process and prevent the wound to fail the healing, therapies as plant extracts and natural compounds obtained from them have been used with promising results. *Aloe vera*, a well-known example, has been shown to stimulate cell proliferation and contribute to healing and angiogenesis, has anti-bacterial, anti-fungal and anti-inflammatory activity (Gontijo *et al.,* 2013). Recently, many reports on the potential effectiveness of phenolic compounds in the prevention or attenuation of skin disorder symptoms and reduction of the healing time have been published. Its known properties as antioxidants, anti-inflammatory and antimicrobials, have given them a deserved recognition in natural medicine and can be highly effective in the treatment of various skin problems (Działo *et al.,* 2016). In this regard, we have studied bioactive compounds of plants of the genus Heliotropium section Cochranea, which produce resinous exudates as a defence mechanism against the adverse environmental conditions under which the plant grows such as low temperatures, lack of water, wounding, low nutrients, and presence of pathogen attacks. This resin covers the leaves and stems as a first stage of protection against predators. Protective effects of the resin are due to the presence of secondary metabolites, mainly flavonoids along with aromatic geranyl derivatives in minor quantities, which exhibit antimicrobial and antioxidant activities (Torres *et al.,* 1994; Lissi *et al.,* 1999; Urzúa *et al.,* 2001; Torres *et al.,* 2002, Modak *et al.,* 2003; Modak *et al.,* 2004; Modak *et al.,* 2007; Modak *et al.,* 2009; Modak *et al.,* 2010; Modak *et al.,* 2012; Valenzuela *et al.,* 2015; Parra *et al.,* 2016).

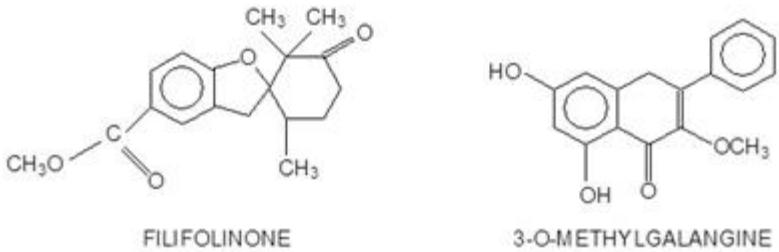

Figure No. 1
Chemistry structure of test compounds. The terpenic derivative Filifolinone isolated from *Heliotropium sclerocarpum* and flavonoid 3-O-methylgalangine obtained from *Heliotropium sinuatum.*

The only known ethnomedicinal use of leaves of these species, is for vaginal washes in Pichasca, Limarí province (Luebert, 2013). In particular, flavonoid 3-O-methylgalangine has shown interesting antibacterial activity in *S.aureus* (Parra *et al.*, 2016).

In this work, the results of the efficacy of mixtures of the flavonoid 3-O-methylgalangine and the terpenic derivative Filifolinone, isolated from *Heliotropium sinuatum* Miers (Parra *et al.*, 2016) and *Heliotropium sclerocarpum* Phil (Parra *et al.*, 2018) plants of the genus *Heliotropium*, on the stimulation of cell proliferation are showed.

MATERIALS AND METHODS

Test compounds

Filifolinone **1** and 3-O-methylgalangine **2** were obtained from resinous exudate of *Heliotropium sclerocarpum* and *Heliotropium sinuatum* as described in Parra *et al.* (2018) and Torres *et al.* (1996), respectively. The compounds have a purity greater than 95%. These compounds were dissolved in dimethyl sulfoxide (DMSO, Merck) at a concentration of 50 mg/mL (stock solution). The work concentrations used were determined from previous research in which the "checkerboard" method was used (White *et al.*, 1996). From these results, the concentrations to which the proliferation was maximum and the executable experiment were selected (Cancino, 2016).

Cell culture

MA104 cells were cultured in Modified Eagle's Medium (MEM; Gibco) supplemented with HEPES (15 mM), sodium bicarbonate (10mM), 10% fetal bovine serum (FBS; Gibco) and gentamicine (50 µg/mL) at 17° C. Cells were propagated every 3 days in a 1: 3 v/v ratio with fresh FBS medium; for this, they were washed 2 times with phosphate-buffered saline (PBS: 1.37 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.27 mM KH_2PO_4, pH 7.2) and recovered with trypsin solution-EDTA (0.1%).

Incubation of compounds

Cells were counted by optic microscopy in a Neubauer chamber, after adding a 0.4% (w/v) trypan blue solution. Then, $1x10^4$ cells/mL were seeded in a 48-well plate with final volume of 2 mL of culture medium. Following this, the cells were treated with the compound **1** [12.5 and 25 µg/mL], with the compound **2** [3.12 µg/mL] and with the mixtures of the compounds **1**:**2** [**M1**; 12.5: 3.12 µg/mL and **M2**; 25: 3.12 µg / mL], according to the previous data of Cancino (2016), at 24, 48 and 72 hours. We used as negative control cells without treatment, as positive control the growth factor Endothelial Cell Growth Supplement (ECGS) and a control with DMSO (solvent used to dissolve the compounds).

MTT assay (Cell viability)

Finished the incubation times (24, 48 and 72 hours) with the compounds, 10 µL of MTT (Sigma Aldrich) solution (5 µg/mL in PBS) was added. The cells were incubated at 37° C for 3 h and the supernatant was removed. Isopropyl alcohol was then added in order to dissolve the formazan crystals and the absorbance was measured at 570 nm. Cell viability was obtained through the following calculation:

$$\text{Cell Viability} = \frac{\text{absorbance sample - absorbance isopropyl alcohol}}{\text{absorbance negative control - absorbance isopropyl alcohol}} \times 100$$

Determination of the migration capacity of the MA104 cells treated with the mixtures of compounds, by means of a wound test.

MA104 cells were grown in 6-well plates to >80% confluence. Then, a wound was made in the middle of the well with a P200 tip and incubated with the mixtures of the compounds **1** and **2** until 8 hours. At 0, 6 and 8 hours the size of the wound closure was recorded and compared with the control without treatment. By MotiConnect application of the microscope, the size in µm of the wounds was calculated.

Statistical analysis

All data are representative of independent experiments performed in triplicate. We used GraphPad v5.0 for Windows (GraphPadSoftware) to calculate the mean and SEM and to perform statistical tests. *P* values less than 0.05 were considered statistically significant.

RESULTS

Evaluation of the proliferative effect of in the MA104 cell line by MTT assay

Figure No. 2 shows the results obtained by incubating MA104 cells with different treatments at 24 (2A), 48 (2B) and 72 h (2C). The Figure 2A shows a significant increase in cell proliferation (40%) using the mixture **M2** (3.12: 25.0 µg/mL of **1**:**2**) with respect to the control at 24 hours ($P<0.05$). This effect is not observed with **M1.** While for pure compounds, an increase in proliferation of up to 50% was observed. In particular, compound **1** at 12.5 µg/mL, proved to be the most active, even surpassing the growth factor. This effect is sustained until 72 hours ($P<0.01$, Figures 2B and 2C). The increase in cellular proliferation with **M2** may be due mainly to the effect of terpenic derivative (**1**), since when using it as a pure compound, the proliferation rate increases better than when used as a mixture. This proliferative characteristic had been observed in flavonoids but has not been described in terpenenic derivative of this type (Xie *et al.,* 2015).

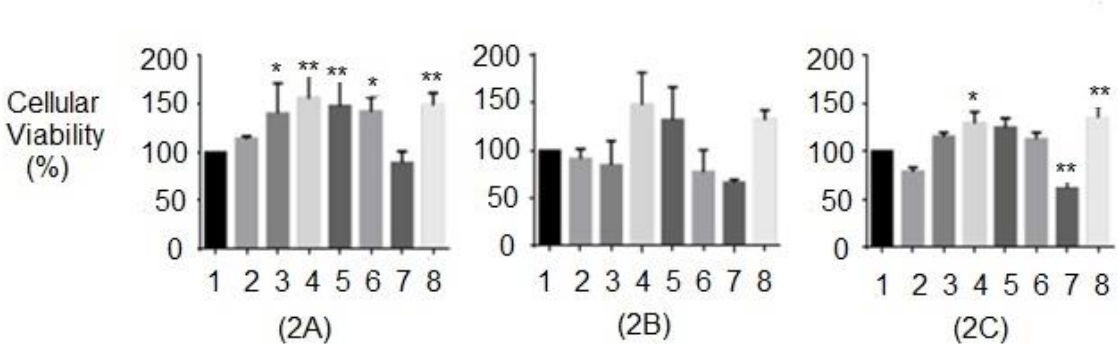

Figure No. 2

Proliferation of MA104 cells exposed to different treatments formed by Filifolinone, 3-O-methylgalangine and mixtures for these, in relation to cells without treatment (negative control) expressed in%. The cells without treatment were assigned 100%, corresponding to the basal percentage of viability. The viability percentages reported for the different treatments were determined in relation to the negative control. The cells were treated with 2 (M1: 12.5 µg/mL:3.12 µg/mL, 1:2); 3 (M2: 25.0 µg/mL: 3.12 µg/mL, 1:2); 4 (1 at 12.5 µg/mL); 5 (1 at 25.0 µg/mL); 6 (2 at 3.12 µg/mL); 7 (DMSO 1%v/v); 8 (positive control, ECGS, 10 µL, 1mg/mL). The data represent the average of three independent trials. Error bars represent the standard deviation. Statistical analysis two-way ANOVA (*) P<0,05 (**) P<0,01.

In the healing process, after the inflammation stage, intense cell proliferation begins in order to regenerate the wound area. *In vitro* model was chosen because MA104 cells are epithelial as well as skin cells. The *in vitro* model studied in this research (MA104 cells) corresponds to epithelial cells as well as those of the skin (Smith *et al.,* 2006), so that compound **1** at non-cytotoxic concentrations, shows an excellent proliferative capacity, equal to or greater than the growth factor, that remains in time.

Determination of the migration capacity of the MA104 cells treated with the treatments by means of a wound test.

Wound healing is a complex event that develops in three phases: inflammatory, proliferative and remodeling (Serra *et al.,* 2017). The proliferative phase progresses with an intense proliferation and migration of fibroblasts, endothelial cells and keratinocytes, therefore, it was considered very interesting to be able to evaluate the migration capacity of MA104 cells with pure compounds and in mixtures. The images in Figure No. 3 shows the results obtained from the migration assay of MA104 cells in a wound treated with the test samples. The size of wound closure was measured at 0, 6 and 8 hours of treatment. The results indicated that with the negative control (without treatment) the wound closure was 75% at 8h (figure 3A). While with pure compound **2** 86% closure of the wound was achieved (Figure No. 3B) and compound **1** at a concentration of 12.5 µg / mL, caused 85% wound closure (Figure No. 3C). When these compounds were mixed at the concentrations used in the proliferation assay, this effect was enhanced achieving 88.7 and 93.0 % closure of the wound for the mixture **M1** and **M2** respectively (Figures 3D and 3E). It is observed that for migration effects, the mixtures of **1** and **2** work in a synergistic way, enhancing the effect of the pure compounds.

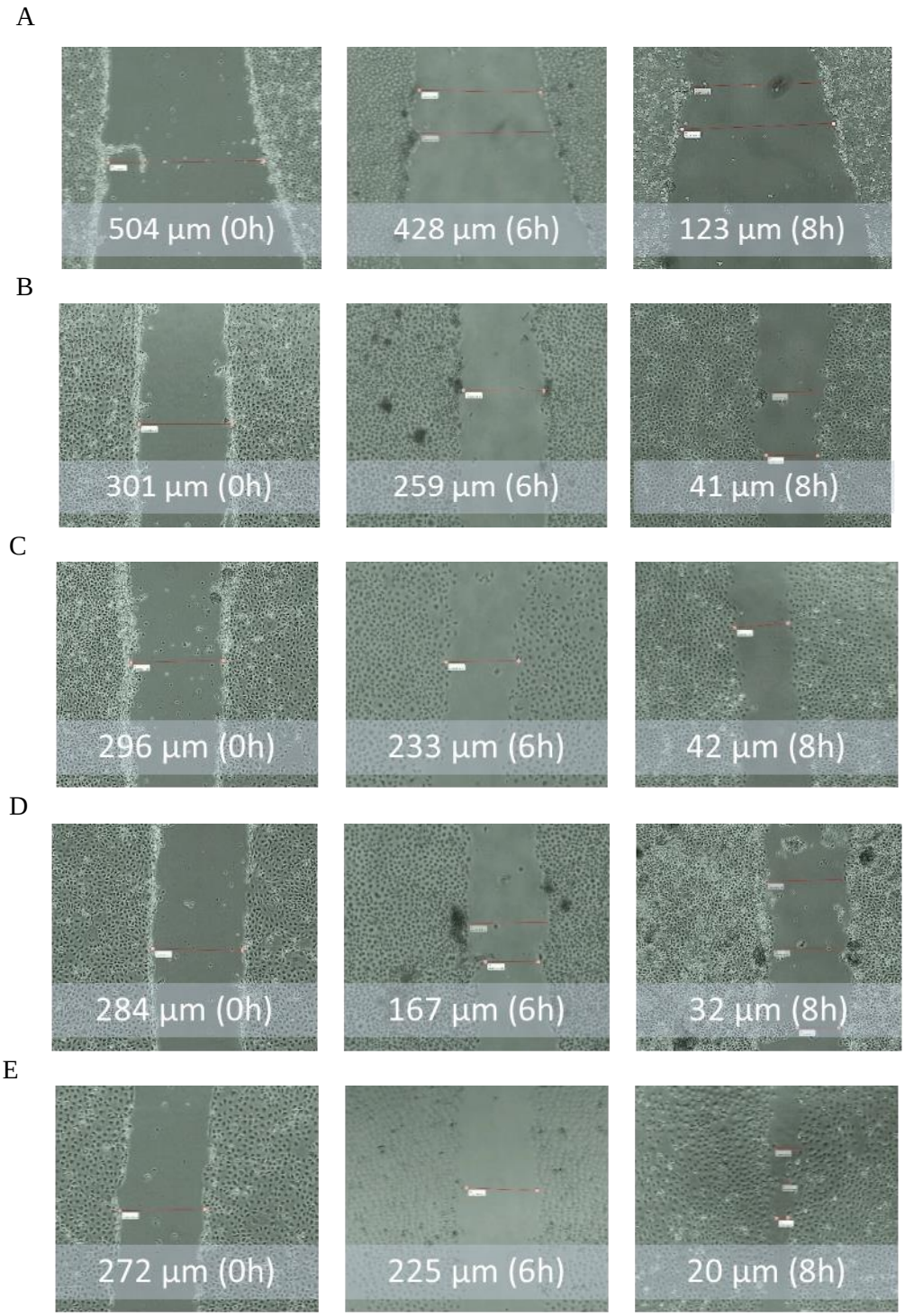

Figure No. 3

Effect of the treatments with Filifolinone, 3-O-methylgalangine and mixtures for these on the migration of MA104 cells. (A) Confluent cultures of MA104 cells were wounded with a micropipette tip (P200) and incubated at 37°C until 8h without treatment (negative control). (B) MA 104 cells incubated with 3-O-methylgalangine 3.12 µg/mL for 0, 6 and 8 h. (C) MA 104 cells incubated with Filifolinone 12,5 µg/mL for 0, 6 and 8 h. (D) MA 104 cells incubated with M1 (12,5 µg/mL:3,12 µg/mL) for 0, 6 and 8 h.(E) MA 104 cells incubated with M2 (25 µg/mL:3.12 µg/mL) for 0, 6 and 8 h. Microscopic observations were conducted by inverted microscope with built-in camera and using the MotiConect application. The values represent the length of the wound measured in µm for each treatment. The measurements were made in triplicate and the percentages represent the average of the measurements. The figure corresponds to the photograph of one of the experiments.

DISCUSSION

The plants are a well-known source of natural of new therapeutic agents for the human healthcare. In this study, terpenic derivative Filifolinone (**1**) and the flavonoid 3-O-methylgalangine (**2**) isolated from resinous exudate of *Heliotropium sclerocarpum* and *Heliotropium sinuatum* respectively, were evaluated *in vitro* as activators of cell proliferation in MA104 epithelial cells. Compounds **1** and **2** showed an excellent proliferative activity, even when being mixed, highlighting the capacity of the terpenic derivative Filifolinone (**1**), which showed an activity that is maintained over time, equal to or higher than the growth factor used as control. On the other hand, the mixtures of the flavonoid and the terpenic derivative studied, **M1** and **M2**, showed accelerating the migration process to close a wound over the pure compounds, regardless of the concentration. Several studies have shown that flavonoids accelerate greatest migratory response improving the healing process by increasing the migration of human epidermal keratinocytes (Seo *et al.,* 2017). Other studies showed that flavonoids naringin and hesperidin, induced the promotion cell migration in nontumorigenic colon epithelial cells (Fenton & Hord, 2004). Nevertheless, terpenic derivatives similar to Filifolinone, have not been studied in this regard, representing a novel contribution. The improvement of the results obtained with the mixtures seem to depend on the terpene derivative in a greater proportion, since when increasing its concentration, the closing of the wound accelerates.

Previous studies carried out in our laboratory have shown that 3-O-methylgalangine has an interesting antioxidant effect, evaluated by measuring the bleaching of stable free radicals as ABTS and DPPH (Lissi *et al*., 1999). Besides, this flavonoid showed antibacterial activity against *Staphylococcus aureus* (Parra *et al.,* 2016) opportunistic pathogen that is characterized by colonizing wounds. In that work, treatment with 3-O-methylgalangine, showed a decrease in size and shape of the cells and the total disappearance of the bacterial populations was observed at 1000 µg/mL, measured by flow cytometry.

Accordingly, Filifolinone as a promoter of cell proliferation, linked to 3-O-methylgalangine, which on the one hand is antioxidant and could help to prevent cell aging and could also help to prevent contamination by *S. aureus*, shows that **M2**, it is a promising tool to be used in the manufacture of creams and lotions for skin with potential health benefits, especially as wound healing, due to the dual capacity that the mixture would have as an accelerator of wound closure and antibacterial at the same time. In an independent way, Filifolinone could also be used in anti-aging creams, helping to eliminate wrinkles and facial lines.

CONCLUSION

This results provide a scientific basis in the detection of promising natural resources as a basis for the manufacture of medicines and the potential use of plant resources.

ACKNOWLEDGMENTS

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