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In vitro immunostimulant activity of the aromatic geranyl derivative Filifolinone.

[Actividad inmunoestimulante in vitro del derivado aromático geranilado Filifolinona]

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Abstract
The in vitro effect of the 3 H-spiro [1-benzofuran-2,1'-ciclohexane] derivative (Filifolinone), was evaluated on mouse dendritic cells through the level of expression of MHC molecules class II by flow cytometry. The results show that Filifolinone increases the expression of MHC promoting maturation of dendritic cells. The results suggest that Filifolinone is a potential immunomodulator for veterinary use.

Keywords: immunostimulant activity, immunomodulators, filifolinone, aromatic geranyl derivatives, dendritic cells.

Resumen
La actividad *in vitro* del derivado 3H-espiro [1-benzofurano-2,1'-ciclohexano] (Filifolinona), fue evaluado en células dendríticas de ratón a través del nivel de expresión de moléculas MHC clase II utilizando citometría de flujo. Los resultados muestran que Filifolinona incrementa la expresión de MHC promoviendo la maduración de las células dendríticas. Estos resultados permiten sugerir que Filifolinona es un potencial inmunomodulador de uso veterinario.

Palabras Clave: actividad inmunoestimulante, inmunomoduladores, filifolinona, derivados aromáticos geranilados, células dendríticas.

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INTRODUCTION

Fish farming crops are constantly exposed to infectious diseases due to heavy conditions of production in which microorganisms develop allowing easy dissemination and representing a problem often difficult to control. There is a variety of infectious diseases affecting fish culture which are the leading cause of death. These diseases can be caused by viruses, bacteria, parasites and fungi, and causing each year major production losses. For examples, Bacterial Kidney ***Disease*** (BKD), ***Salmon Rickettsial*** Syndrome (SRS) and Enteric Red mouth ***Disease (ERM)*** (Torres, 2000) and *Caligus,* a parasite that causes sores, stress the fish and are immunosuppressors, that is, weaken its defences leaving them susceptible to bacteria and viruses. In addition, viral infectious agents also affect salmonids, such as the infectious pancreatic necrosis virus (IPNV) (Wolf, 1988) and Infectious ***salmon*** anemia virus (ISAV) (Thorud and Djupvik, 1988). These agents have been a problem for salmon aquaculture in the northern hemisphere, such as Norway, Canada, Scotland, Faroe Islands and the United States, and in Chile (Cottet *et al*., 2011) that high mortalities were observed to cover losses that came in the case of Norway and Chile, almost all production (Mjaaland *et al*., 1997).

Infectious diseases can be controlled and even eradicated by treatments involving the use of vaccines; unfortunately, current vaccines for aquaculture use do not produce the expected level of protection as the achieved efficiencies range from 40 to 70% (Christie, 2004; Salgado-Miranda, 2006; Ramstad *et al*., 2007). One of the most important limitations in the design of current vaccines is the lack of immunostimulants or adjuvants to generate an appropriate and protective response (Sommerset *et al*., 2005), which in the case of viral infection it is associated with a Th1 type of response. Consequently, it is a worldwide priority to be able to develop improved vaccines to protect fish in culture against infectious diseases. One strategy is to be able to modulate and improve the immune response through the administration of a new generation of immunomodulators of plant origin that will be selected because of their ability to initiate cellular immune response. Main effects exercised by these immunomodulators are promote antiviral stage, increasing expression of molecules MHC class I and II, CD80 and CD86, as well as, promote the expression of pro-inflammatory cytokines.

Species of the genus *Heliotropium* (family *Heliotropiaceae*) are characterized by the production of exudates that covers their leaves and stems. Phytochemical research revealed that the resin is constituted mainly by flavonoids, and minor amounts of aromatic geranyl derivatives (Torres *et al*., 1994; Urzúa *et al*., 2000; Urzúa *et al*., 2001; Modak *et al*., 2003; Modak *et al*., 2007; Modak *et al*., 2009). In search for an explanation of the role of resinous exudates, it has been proposed that they may constitute the first stage of protection against predators. This protection could be due to a mechanical effect, associated with a sticky character which causes them to retain predators (Eigenbrode *et al*.,1996) and to a chemical protection due to the presence of secondary metabolites with antimicrobial, antioxidant and cytotoxic properties. In particular, we have demonstrated that aromatic geranyl derivatives have antiviral activity on viruses that infect salmon such as Infectious Pancreatic Necrosis Virus (IPNV) (Modak *et al*., 2010). We thought that the antiviral action may be due to an immunomodulatory effect of these compounds.

Based in the aforementioned facts, we have been looking for new immunostimulants among various naturally occurring substances that can be used in salmon and other species. During the course of our screening tests of compounds extracted from plants, we have studied the immunostimulant activity of 3 H-spiro [1-benzofuran-2,1'-ciclohexane] derivative called filifolinone **1** obtained by semi synthesis from filifolinol **2**, main compound isolated from resinous exudates of *Heliotropium filifolium* (Torres *et al*., 1994). In addition, we have recently identified filifolinone from resinous exudate of *Heliotropium sclerocarpum* (unpublished data).

In this study we have used mouse dendritic cells and tested the level of expression of MHC molecules class II by flow cytometry to evaluate immunostimulant activity of filifolinone **1** from *Heliotropium filifolium*. We used mouse dendritic cells (DCs) and not fish DCs, because these antigen presenting cells have not been characterized in most teleost fish species. Dendritic cells are professional antigen presenting cells that play pivotal roles in the induction of protective immunity. In peripheral epithelial tissues, DCs are immature and, upon encountering microbial products or proinflammatory mediators, they undergo dynamic and coordinates reprogramming of gene expression, surface phenotype and cellular function, the process known as dendritic cell maturation. For example, DCs differentiate into fully potent T-cell stimulators by increasing surface expression of major histocompatibility complex class II (MHC II) molecules and co stimulatory molecules,

and produce a wide variety of cytokines, which regulates immune response, when antigen presentation takes place (Hugues *et al*., 2006).

MATERIALS AND METHODS

Plant Material

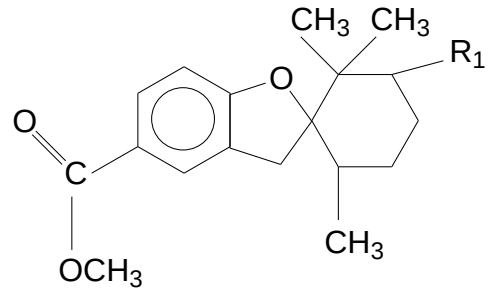
Heliotropium filifolium (Miers) Reiche samples were collected in IV region, Chile, 28º45'S, 70º49'W. A voucher specimen was deposited in the Herbarium of the Faculty of Biological Science of the Catholic University of Chile (ST-2214 SSUC).

Extraction and isolation of the natural compounds

The resinous exudate was extracted by immersion of the fresh plant material in dichloromethane for 30 s at room temperature and was concentred to a sticky residue. The extract was purified by column chromatography using hexane- ethyl acetate step gradient (Urzúa *et al*., 2001) yielding filifolinol **2** (Figure 1).

Synthesis of Filifolinone 1

Filifolinone **1** (Figure 1) was obtained from filifolinol by conventional oxidation with CrO_3 and was purified by column chromatography using benzene-ethyl acetate (Torres *et al*., 2002).



1 R_1: =O

2 R_1: —OH

Figure 1

Structures of 3 H-spiro [1-benzofuran-2,1'-ciclohexane] derivatives Filifolinone **1** and Filifolinol **2.**

Dendritic cells

Female mice BALB/C of 6 weeks old were sacrificed by cervical dislocation. After removing all muscle tissues with gauze from the femurs and tibias, the bones were placed in 70% alcohol, washed with PBS and transferred into RPMI-1640 without supplement for extract bone marrow. Red cells were lysed with buffer ACK pH 7.2 (NH_4Cl; $KHCO_3$;EDTA) before plating cells.$1x10^6$ cells were placed by plate in medium RPMI-1640 supplemented with 10% SFB, 4mM de L-Glutamine and 10ng/mL of GM-CSF (granulocyte/macrophage colony-stimulating factor). The cells were cultures by 6 days at 37º C with CO_2 5%. The medium were changed in day 2 (with cytokine) and day 4 (without cytokine), aspirating 75% of the medium (Inaba *et al*., 1992).

Determination of citotoxicity

To discard toxicity effects of filifolinone **1** on cells toxicity, dendritic cells ($1x10^6$) were incubated with 5.0 and 0.5 µg/mL of filifolinone for 6 h at 37º C in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 4mM of L-glutamine, 1% penicillin/streptomycin and 10.0 ng/mL of granulocyte/macrophage colony-stimulant factor (GM-CSF). After washing, the cells were resuspended in 300 µL of IF (2% FBS and PBS 1X) and 2 µL of propidium iodide (PI, 1mg/mL).Viable cells (PI negative cells) were quantified by flow cytometry using a FACSCanto II Flow Cytometer (BD Biosciences).

Evaluation of the immunostimulant activity on mouse dendritic cells

Dendritic cells ($1x10^6$) in 96-well plates were incubated with 5.0 µg/mL of filifolinone **1** for 24 h at 37º C under 5% CO_2 in RPMI supplemented medium. As positive control dendritic cells in RPMI supplemented medium were treated with LPS (100 ng/mL) and as negative control, cells were incubated with medium only. The effect of the filifolinone on dendritic cells was determined measuring the level of expression of the MHC class II. For this, the cells were washed and resuspended in 300 µL of IF and incubated for 30 min at 4º C. Then, the FITC conjugated anti-mouse MHCII antibody was added (Pharmingen, BD, USA) and incubated for 1 h at 4º C. After incubation time, the cells were centrifuged, resuspended in 300 µL of IF and analyzed using a FACSCanto II Flow Cytometer (BD Biosciences). The level of dendrtic cell maduration was determined through the median of fluorescence intensity (MFI).

Data analysis:

All data were analyzed using GraphPad software and are shown as mean ± standard error. P values less than 0.05 were considered significant.

RESULTS AND DISCUSSION

The citotoxicity activity of 3 H-spiro [1-benzofuran-2,1'-ciclohexane] derivative called filifolinone **1** (Figure 1**)** on dendritic cells, was evaluated to concentration 0.5 and 5.0 µg/mL by measuring the percentage of cell viability after 6 h of incubation. The results showed that this compound is not toxic to any of the concentrations tested as viability of the control and treated cells was the same and reached 100% (Figure 2). Therefore, for the evaluation of the immunostimulant activity, we used 5.0 µg/mL.

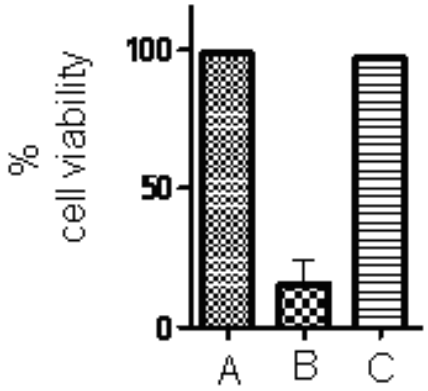


Figure 2
Percentage of cell viability obtained by flow cytometry, after treating dendritic cells with different tests samples. **A** corresponds to dendritic cells without filifolinone. **B** is ethanol 30%, which was used as a control of cell death. **C** corresponds to filifolinone (5 µg/mL). Dendritic cells were incubated for 6 h in the presence of test compounds. Values represent mean ± SE of three independent experiments.

Dendritic cells have the function of capture and present bacterial and viral antigens, activating T lymphocytes, mechanism necessary by initiate the cellular immunity against the pathogen. There are different stages of differentiation of dendritic cells (Fajardo-Moser *et al*., 2008) functionally distinct. Before of the recognition of a microbial agent, dendritic cells are in immature stage and have a phagocytic capacity, but they have low capacity to active T lymphocytes. Mature dendritic cells have high levels of major histocompatibility complex (MHC) class II molecules, which have a key role in the antigen recognition by lymphocytes T (Iruretagoyena *et al*., 2005). For this reason, an analysis of expression of MHC class II is a good indicator of the promotion of dendritic cells maturation. Therefore, the effect of Filifolinone **1** on the maturation of dendritic cells through of the level of expression of MHC molecules class II was evaluated. The results are shown in figure 3.

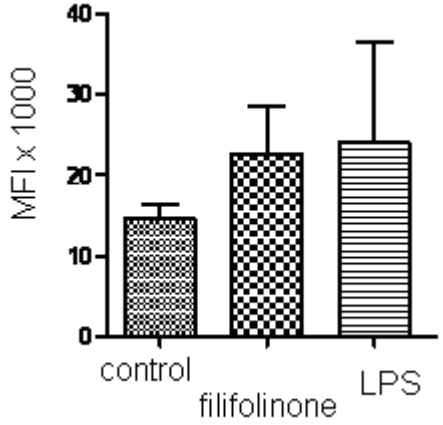


Figure 3
Mean fluorescent intensity (MFI) obtained for the expression of MCH class II in presence of different tests samples. Dendritic cells were incubated for 24 h in the presence of Filifolinone and LPS. Values represent mean ± SE of three independent experiments.

Figure 3 shows that mean of fluorescence intensity (MFI) obtained with Filifolinone ($21.2 \times 10^3 \pm 3.9 \times 10^3$) is significantly higher than control ($15.9 \times 10^3 \pm 2.5 \times 10^3$) increasing expression the activation in 25% ($p = 0.05$). Values are similar to those obtained with lipopolysaccharide (LPS) used as activation control ($23.9 \times 10^3 \pm 10.1 \times 10^3$), a component of the Gram negative microbial cell-wall product, widely used to induce dendritic cells maturation (Matsunaga *et al*., 2002). Thus results indicate that filifolinone increased the expression of class II MHC in dendritic cells. This is the first time such immunostimulant activity of compounds 3 H-Spiro [1-benzofuran-2, 1'-ciclohexane] derivatives is reported.

As mentioned above, dendrite cells play a fundamental role in immune responses against pathogens. Particularly, dendrite cell maturation is necessary to induce activation of T lymphocytes and immune response thereafter. The maturation process is translated into a significant increase in the density of molecular signals on the surface of dendritic cells, necessary for T lymphocytes activation (Iruretagoyena *et al*., 2005). These signals are antigens as bacterial or viral peptides linked to molecules MHC, which are recognized by T lymphocytes receptor; and molecules

co-stimulators antigen-non-specific. Since filifolinone demonstrated their ability to increase expression of molecules MHC class II results suggest that it may promote dendritic cells maturation, which has to be confirmed evaluating the capacity of filifolinone to induce expression of CD80, CD86 and some cytokines. Further functional studies will be also needed.

These results, along with the antiviral activity shown by filifolinone for IPNV, make it be a good candidate for use in salmon farms, helping protection against pathogens that cause great economic losses.

CONCLUSION

This result allows to state that Filifolinone increases the expression of MHC Class II promoting maturation of dendritic cells. Therefore, Filifolinone is a good candidate to study in vivo immunostimulant activity.

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