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Evaluation of the bactericidal activity of secondary metabolites isolated from *Heliotropium* genus against *Piscirickettsia salmonis*

[Evaluación de la actividad bactericida de metabolitos secundarios aislados desde especies del género *Heliotropium* contra *Piscirickettsia salmonis*]

Beatriz VALENZUELA[1], Mónica IMARAI[1], Romina CATALÁN[1], Mick PARRA[1], Sarita SOTO[1], Ana María SANDINO[2], René TORRES[3] & Brenda MODAK[3]

[1]*Laboratorio de Inmunología, Centro de Biotecnología Acuícola (CBA), Departamento de Biología,*
[2]*Laboratorio de Virología, Centro de Biotecnología Acuícola (CBA), Departamento de Biología,*
[3]*Labotarorio de Productos Naturales, Centro de Biotecnología Acuícola (CBA), Departamento de Ciencias del Ambiente, Facultad de Química y Biología, Universidad de Santiago de Chile, Av. Bernardo O'Higgins 3363, Santiago, Chile*
Contactos | Contacts: **Brenda MODAK - *E-mail address:*** **brenda.modak@usach.cl**

Abstract: The intracellular bacteria *Piscirickettsia salmonis* is the most prevalent pathogen in the Chilean salmon industry, responsible for 50% of losses in recent years. So far, there are no effective treatments to control infections by this pathogen due to the emergence of antibiotics resistance. Therefore, it is extremely important to conduct research to find successful antibacterial therapies. In this paper, we evaluated the *in vitro* bactericidal activity of flavonoids and aromatic geranyl derivatives isolated from the resinous exudate of species *Heliotropium filifolium*, *H. sinuatum* y *H. huascoense*. The results showed that the compounds Filifolinone, Naringenine and 3-O-methylgalangine cause different percentage of mortality of bacteria and therefore they are good candidates to continue its evaluation *in vitro* and *in vivo*.

Keywords: *Piscirickettsia salmonis*; *Heliotropium*; bactericidal activity; flavonoids; aromatic geranyl derivatives.

Resumen: La bacteria intracelular *Piscirickettsia salmonis* es el patógeno de mayor incidencia en la industria salmonera chilena siendo responsable de un 50% de las pérdidas en los últimos años. Hasta ahora no hay tratamientos efectivos para este patógeno que permitan controlar las infecciones provocadas por él debido a la aparición de resistencia a antibióticos. Por lo tanto, resulta de gran importancia investigar para encontrar terapias antibacterianas efectivas. En este trabajo nosotros evaluamos la actividad bactericida *in vitro* de flavonoides y derivados aromáticos geranilados aislados desde el exudado resinoso de las especies vegetales *Heliotropium filifolium*, *H. sinuatum* y *H. huascoense*. Los resultados mostraron que los compuestos Filifolinona, Naringenina y 3-O-metilgalangina provocan diferentes porcentajes de mortalidad de la bacteria y, por lo tanto, son candidatos para seguir siendo evaluados tanto *in vitro* como *in vivo*.

Palabras clave: *Piscirickettsia salmonis; Heliotropium;* actividad bactericida; flavonoides; derivados aromáticos geranilados.

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INTRODUCTION

Aquaculture has been qualified worldwide as the most viable alternative to increase the supply of fishery resources that mankind will demand for the present century. Thus in this regard, Chile has devoted important economic efforts so this activity has become one of the most dynamic and important national economic sectors. In particular, the salmon farming industry has gained an increasing economic, geopolitical and social relevance. It has not only had a strong impact in the southern region of the country, by opening new poles of development, creating jobs and promoting the activity of associated goods and services, but it has also become an important export product (Informe Sectorial de Pesca y Acuicultura 2013, www.subpesca.cl). Together with the increased demand of products from aquaculture, it also emerges with greater interest the need to develop control measures against a series of diseases that cause major crop losses. High-risk pathogens are those that have no therapeutic control and for which no effective vaccines have been developed. This includes viruses and intracellular bacteria. In this regard, the intracellular bacteria *Piscirickettsia salmonis* causing of the Piscirickettsiosis or Salmon Rickettsial Syndrome (SRS) has been responsible for the greatest losses of production in the stage of fattening in the Chilean salmon industry. According to data from the Institute of technology of the Salmon (INTESAL), in recent years the Piscirickettsiosis caused over half (56%) of the total deaths in terms of biomass, taking into account information obtained for Atlantic salmon (*Salmo salar*), coho salmon (*Oncorhynchus kisutch*) and rainbow trout (*Oncorhynchus mykiss*) (Yañez & Martínez, 2010). Although *P. salmonis* is sensitive *in vitro* to some of the antibiotics commonly used to fight other infectious diseases of fish (such as tetracycline, erythromycin and oxolinic acid) these preparations have shown limited effectiveness due to the emergence of resistance to antibiotics, besides the bacteria is kept circulating within macrophages. Plasma levels of the antibiotic cannot get lethal concentrations within the host cell *in vivo*. In addition, there is evidence that the use of antibiotics in aquaculture generates the emergence of antibiotic-resistant bacteria in aquatic environments where this activity takes place and there are epidemiological and molecular evidence, showing that antibiotic resistance genes can be transmitted from aquatic bacteria to bacteria capable of infecting humans and land animals (Cabello, 2004).

Considering the importance of aquaculture in our country, our research group has been dedicated to the research and development of new potential treatments for several years, using of the knowledge, of the molecular biology of pathogens that infect salmon, as well as of compounds able to inhibit pathogen replication. From these studies we have found natural compounds obtained from the resin of species of the genus *Heliotropium* (aromatic geranyl derivatives and flavonoids) that efficiently inhibit the replication of the Infectious pancreatic necrosis (IPN) and Infectious salmon anaemia (ISA) virus (Modak *et al.,* 2010; Modak *et al.,* 2012). We have also shown that the flavonoids Naringenin, 7-O-methyleriodictyol, Pinocembrin, Hesperetin and 3-O-methylgalangin, isolated from the exudate resinous of *H. sinuatum* inhibit the bacterial growth of *E. coli* as well as plant-pathogenic bacteria (Modak *et al.,* 2002; Modak *et al.,* 2003; Modak *et al.,* 2004). Recently, we have also determined the effectiveness of some of these natural compounds as immunostimulants in salmonids. In particular, we demonstrated that the derived terpenoid Filifolinone and the flavonoid Alpinone increased the transcripts of the Th1 type cytokines, IFNγ and IL-12 in the kidneys of Atlantic salmon, important for the control of intracellular pathogens (Valenzuela *et al.,* 2013). Moreover, the derived terpenoid Filifolinol also increased the transcripts of IFNγ (Obreque, 2012). Therefore, due to the need of a continuous search for more effective therapies against *P salmonis*, we explored the possibility that some of the bioactive molecules isolated from *Heliotropium* species can have bactericidal activity against the bacteria. Here, we present the results of the *in vitro* evaluation of potential bactericidal activity of natural flavonoids and aromatic geranyl derivates isolated from resinous exudates of plants of *Heliotropium* genus of compounds on the *Piscirickettsia salmonis*.

MATERIALS AND METHODS

Plant material

Heliotropium sinuatum Miers (ST2563), *H. huascoense* Johnston (ST2580) and *H. filifolium* Reiche (ST2214) were collected in the flowering season, in the north of Vallenar (III region, Chile, 28º45´S,70º49´W). Voucher specimens were deposited in the Herbarium of the National Museum of Natural History, Santiago, Chile.

Isolation of the resinous exudates and the pure compounds tested

The resinous exudates were extracted by immersion of the fresh plant material in dichloromethane (30 s) at room temperature. The extracts were concentrated to a sticky residue and fractioned by column chromatography on silica gel with different solvents. The pure compounds obtained were identified by spectroscopic analysis as Filifolinol; Filifolinone; Naringenine (HS1); 3-O-methylgalangine (HS2); Pinocembrin (HS7); Alpinone. (Torres *et al.*, 1996; Torres *et al.*, 2002; Modak *et al.*, 2004) (Figure 1).

Cell culture

The Chinook salmon embryo cell line CHSE-214 was cultured in supplemented MEM (Corning) containing (CONC) non-essential amino acids, Hepes and 10% fetal bovine serum (Hyclone, Thermo Scientific) at 16 °C.

Bacterial culture and titration

The strain LF-89 (ATCC VR 1361) isolated from specimens of Atlantic salmon was provided by the Laboratory of Virology at the Universidad de Santiago de Chile. *Piscirickettsia salmonis* was propagated in monolayers of CHSE-214 cells in supplemented MEM (Corning). Infectious titers were evaluated at days 2, 5, and 10 post-infection by the method of Reed & Muench (1938).

Cytotoxic assays

To discard toxicity effects of test compounds, monolayers CHSE-214 cells were incubated with different doses of each compound from 1µg/mL to 1mg/mL in supplemented MEM (Corning) by 12 h at 16 °C. After washing, the cells were resuspended in 300 µL of IF (Phosphate Buffered Saline) and 2 µL of propidium iodide (PI, 1 mg/mL). Viable cells (PI negative cells) were quantified by flow cytometry using a FACSCanto II Flow Cytometer (BD Biosciences). Cells treated with 30% ethanol were used as positive control. The negative control corresponded to untreated cell.

Bactericidal assay

Bacteria from the cell cultures were centrifuged at 200 xg for 5 min to remove CHSE-214.

$1x10^6$ bacteria were incubated with different doses of compounds for 24 h at 16 °C in supplemented MEM. Then, bacteria were centrifuged at 8000 xg for 10 min at 4 °C and the cells were resuspended in 300 µL of IF containing 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). Bacteria treated with 30 % ethanol were used as positive control. The negative control corresponded to untreated bacteria.

Genomic DNA extraction of P.salmonis

Genomic DNA extraction was performed from CHSE-214 cell culture two weeks post infection with *P. salmonis* LF89 and 11085. 75 mL of solution A 5X (3.38 g Tris, 2.98 g EDTA, SDS 1.3 g; β -Me 1.3 mL; 4.0 g NaCl in 100 mL H_2O miliQ) and 3.7 µL Proteinase K (20 mg/mL) were added to 300 µL of solution A. Then the samples were stirred and incubated for 1 h at 50 °C. Following incubation, the samples were treated with 200 µL of basic phenol and 200 µL of chloroform, stirred for 1 min and centrifuged at 6000 xg for 10 min at room temperature. 300 µL of the aqueous phase were transferred to a clean tube and DNA was precipitated overnight at -20 °C with 1 mL of cold absolute ethanol. Finally, the samples were centrifuged at 16000 xg for 30 min at 4 ºC and resuspended with 30 µL of nuclease free H_2O.

16S rRNA gene amplification by PCR

For amplification of the 16S gene a mix performed of 1 µg DNA, 0.5 mM of Primer F 16S (5'-AGG GAG ACT GCC GGT GAT A-3') 0.5 mM of Primer R 16S (5'-ACT ACG AGG CGC TTT CTC A-3') and 25 µL of Gotaq® Master Green Mix, 2X (Promega) in a final volume of 50 µL (Karatas *et al.*, 2008). The thermal profile used consists of an initial denaturation at 95 °C for 2 min followed of 35 cycles at 95 °C for 30s, 55 °C for 30 s and 72 °C for 15s, with a final extension step at 72 °C for 5 min. The PCR product was analyzed by electrophoresis in 2% agarose gel with TAE buffer. The gel was labelled with ethidium bromide (0.5 µg/mL) and the products visualized in a UV transilluminator.

Statistical analysis

Date of the cytokine gene expression levels in whole kidney of fish and surface cellular markers detected for flow cytometry were analyzed and compared using the Mann–Whitney test. 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

Figure 1 shows the chemical structures of the tested compounds isolated from resinous exudates of *Heliotropium sinuatum*, *H. huascoense* and *H. filifolium*: Filifolinol; Filifolinone; (HS1); (HS2); (HS7); Alpinone. These compounds were isolated and purified from the resinous exudates by column chromatography and were identified by spectroscopic analysis (Torres *et al.*, 1996; Torres *et al.*, 2002; Modak *et al.*, 2004).

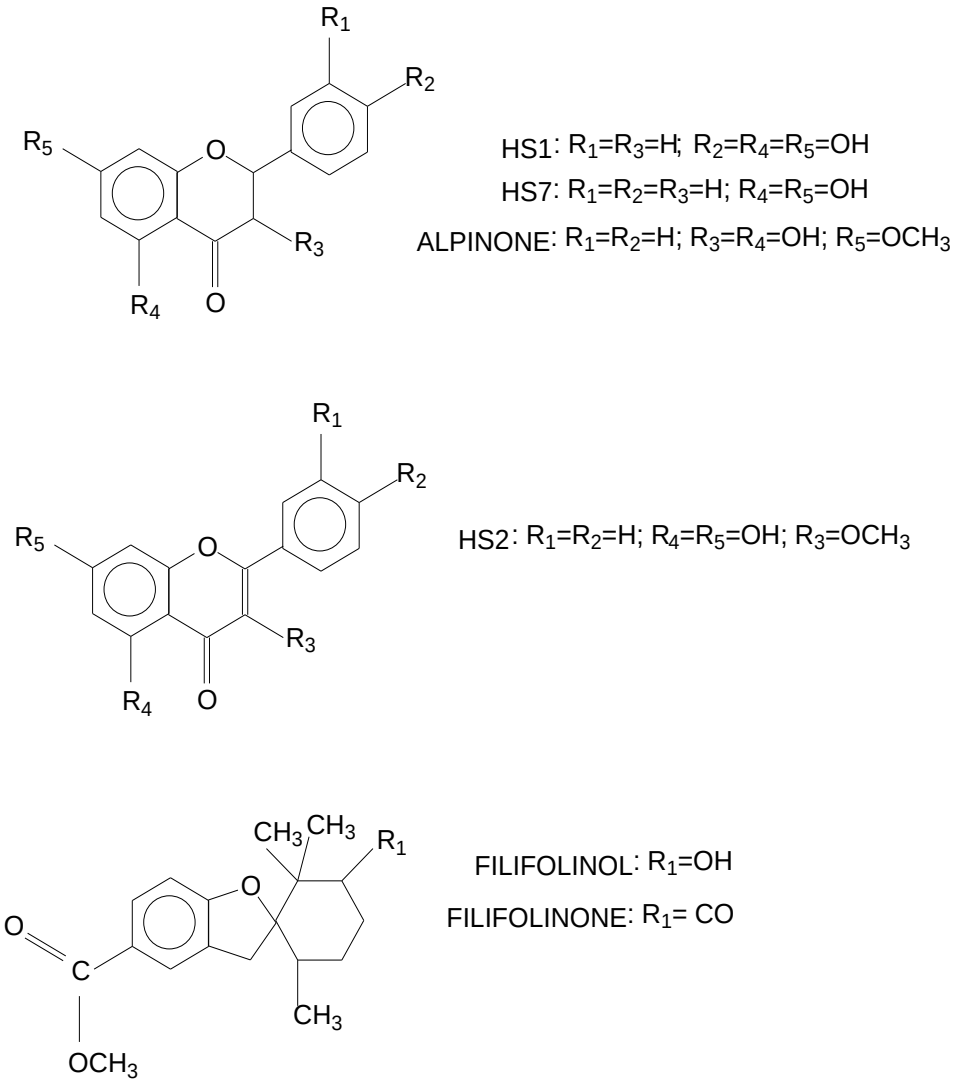

Figure 1
Chemical structures of test compounds isolated of different *Heliotropium* species.

First at all, *Piscirickettsia salmonis* identity was confirmed by the specific amplification of the 16S gene using PCR. The ribosomal RNA 16S is a component of the small subunit 30S of the prokaryotic ribosomes and is used in phylogenetic studies since is highly conserved among different

species of bacteria (Weisburg *et al.*, 1991). As expected, a band of an approximate size of 150 bp was obtained after PCR amplification of samples of CHSE-214 cells infected *P. salmonis* (Figure 2). After sequence analysis of PCR products (not shown) we confirmed that the bacteria grown on CHSE-214 were *P.salmonis* strain LF-89.

The cytotoxic effects of the compounds on a model host cell (CHSE-214 cells) were evaluated in order to establish the concentration ranges to further examine bactericidal activity. We used propidium iodide staining and flow cytometry analysis to measure the number of viable and non-viable cells. Figure 3 shows the percentage of CHSE-214 viability after incubation in the presence of the compounds using concentrations between 30 and 1000 µL/mL. Filifolinone, Filifolinol and Naringenine (HS1) showed no cytotoxic effects at any of the concentrations studied, establishing that the concentration required to reduce the cell viability by 50% (CC_{50}) is > 1000 µL/mL. On the other hand, the compounds 3-O-methylgalagine (HS2) and Pinocembrine (HS7) showed a CC_{50} value of 300 µg/mL and Alpinone a value of 500 µg/mL.

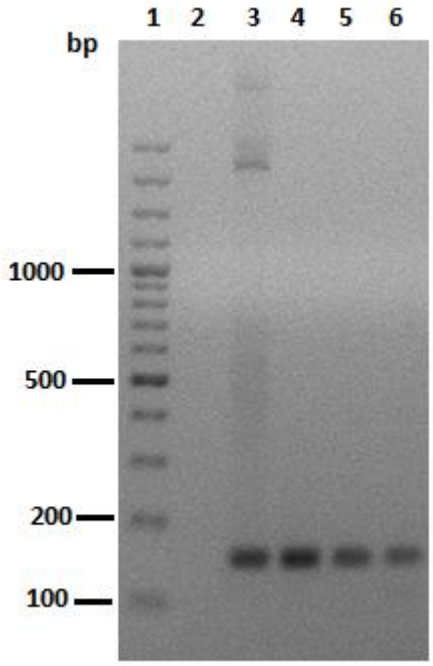

Figure 2
PCR 16S rRNA gen of *P. Salmonis*. Lane 1, molecular weight marker (0'GeneRuler 100 bp DNA Ladder Plus); lane 2, PCR negative control; lane 3, positive control, original stock of *P. Salmonis* LF89 Cepa ATCC; lane 4-6, CHSE-214 cells cultured for 2 weeks with *P. salmonis*-strain LF89.

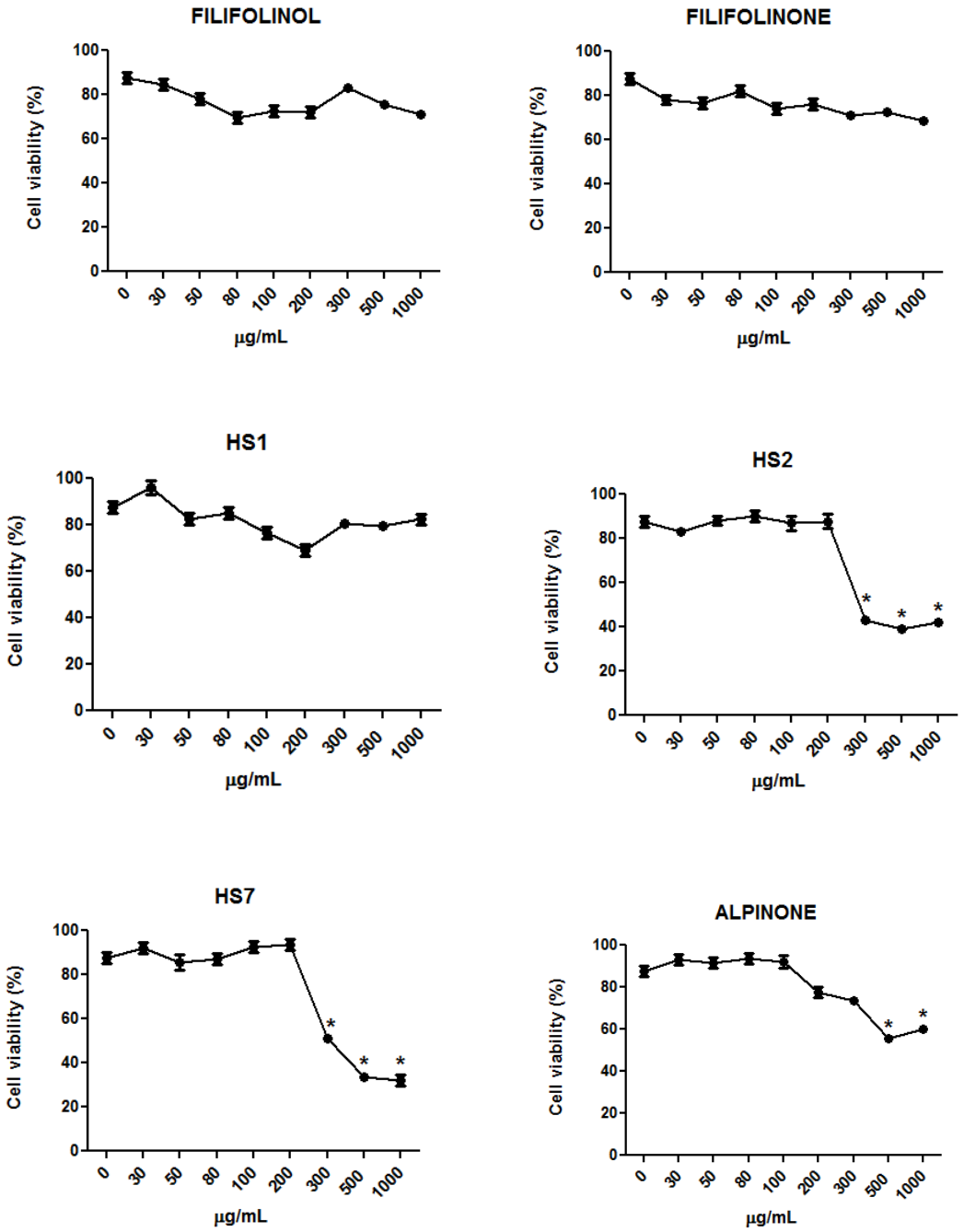

Figure 3
Viability of CHSE-214 cells treated with the tested compounds. Filifolinol, Filifolinone, Naringenine (HS1), 3-O-methylgalangine (HS2), Pinocembrin (HS7) and Alpinone. Cells treated with 30% ethanol as positive control showed 10% of viability.

Once the cytotoxic effect was tested, we proceeded to evaluate the bactericidal effect caused by the compounds on *P. salmonis*. Propidium iodide staining and subsequent FACS analysis were used to quantify the viable bacteria. The results were expressed as percentage of viability of *P. salmonis* (Figure 4).

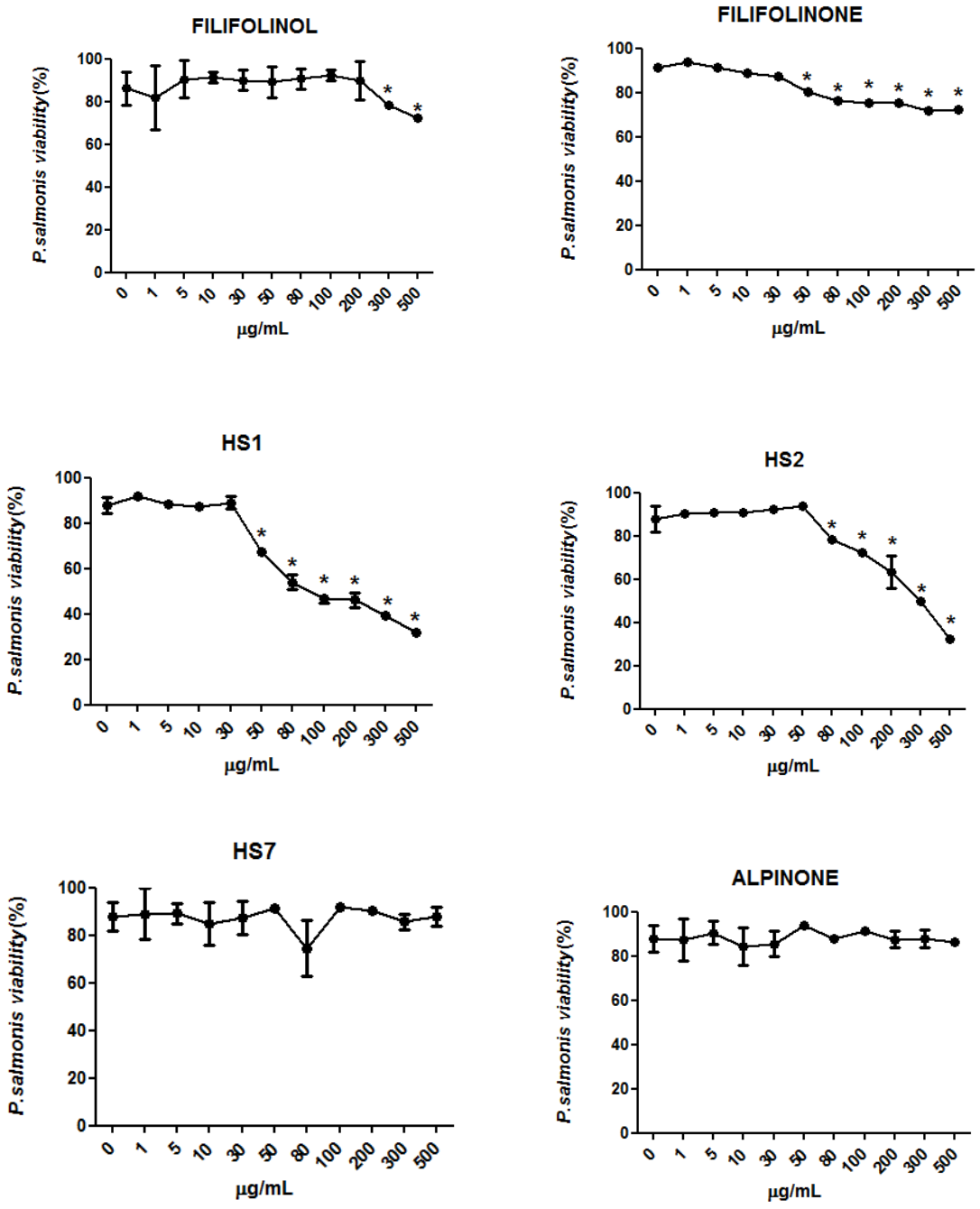

Figure 4
Evaluation of the bactericidal activity of test compounds on *Piscirickettsia salmonis* Filifolinol, Filifolinone, Naringenine (HS1), 3-O-methylgalangine (HS2), Pinocembrin (HS7) and Alpinone. Bacteria treated with 30% ethanol as positive control showed 20% of viability.

Results in Figure 4 showed that Filifolinone, Naringenine (HS1) and 3-O-methylgalangine (HS2) possess significant bactericidal effects on the *P. salmonis* from 50 μg/mL. Filifolinone produces a mortality of *P. salmonis* close to 20% at a concentration of 50 μg/mL. Considering that the CC_{50} for this compound was higher than 1000 μg/mL, it seems interesting to continue testing at higher concentrations to determine if it is possible to increase mortality of the bacteria and determine the value of EC_{50} (effective bactericidal concentration required to inhibit *P. salmonis* grow by 50%). The flavonoid Naringenine (HS1) produces significant mortality from 50 μg/mL with a EC_{50} value between 200 and 300 μg/mL (Figure 4). For Naringenine, it is also possible to test activity at higher concentrations, since this compound has a CC_{50} higher than 1000 μg/mL (Figure 3). As the concentrations showing bactericidal activity are sufficiently different in relation to CC_{50}, both compounds are suitable candidates for *in vivo* studies. Other two compounds showed bactericidal activity (Figure 4). Filifolinol that only showed a lower bactericidal effect (15% of bacteria die) at high concentrations and 3-O-methylgalangine (HS2) that produces 50% bacterial mortality similar to HS1 (Figure 4). In this latter case, activity is observed at concentrations similar to those inducing cytotoxicity (Figure 3). Although this would lead us to discard HS2 as a good candidate, as the *in vivo* behaviour can be different we will also continue evaluation of this candidate in the *in vivo* analyses.

DISCUSSION

The high losses in the salmon industry caused by *P. salmonis* infection and low effectiveness of current treatments due to the emergence of antibiotic resistance, have led to the search for new compounds of natural origin having bactericidal activity. The natural compounds being new can be administered in mixture to decrease the probability of resistance, they are biodegradable and do not generate unauthorized waste in fillet fish. Based in this, we evaluate the bactericidal activity of flavonoids and aromatic geranyl derivatives isolated from exudates resinous of different species of *Heliotropium* genus against *P. salmonis*. The results obtained from evaluation *in vitro* showed that the compounds Filifolinone and Naringenine (HS1) possess an interesting bactericidal activity with high selectivity index (CC_{50}/EC_{50}), results justify continuing with the evaluation of the in vitro and *in vivo* activity. The bactericidal activity of Filifolinone can synergist efficacy of a potential treatment because Filifolinone also increases the transcripts of the Th1 type cytokines, IFNγ and IL-12, important for the control of intracellular pathogens as *P. salmonis* (Obreque, 2012). In addition, 3-O-methylgalangine (HS2) also showed a significant bactericidal activity although at concentrations where cytotoxicity is observed. However, there are studies that demonstrate that often the activity *in vitro* does not have a perfect correlation with the *in vivo* displayed activity (Modak *et al.*, 2012) therefore, it is advisable to perform both tests independently.

In regard to the structural requirements for the antibacterial activity of flavonoids, it has been described that activity of flavonols isolated from resinous exudates of *Haplopappus* against Gram positive bacteria, is related to the presence of free hydroxyl groups and to the degree of lipophilicity (Urzúa *et al.*, 2012). The flavonoids exert their toxicity through the acidity of the hydroxyl group by uncoupling oxidative phosphorylation (Franklin & Snow, 2005). However, the results obtained with these flavonols do not agree with the general statement that lipophilic flavonoids foundinplant surface extracts are antibacterial (Wollenweber & Dietz, 1981). Other studies of antibacterial activity with Filifolinol derivatives, showed that Filifolinol has a low activity, consistent with our results and in this case a positive correlation between antibacterial activity and the degree of lipophilicity was observed (Urzúa *et al.*, 2008).

According to the literature, there are four antibiotics in aquaculture that are used mainly to treat fish pathogens: florfenicol (only for salmon), oxolinic acid (salmonids) and sulphadiazin/trimethoprim (finfish and salmon). In regard to *P. salmonis* infection, orally administered oxolinic acid is the drug of choice, even though the response is slow and the antibiotic has to be given repeatedly (Fryer & Hedrick, 2003). Moreover, there are many antibiotics that have been tested *in vitro* but cannot be used in aquaculture because they are used to treat human diseases. Their use in aquaculture may lead to increased of bacterial resistance, which will undermine the effectiveness of those antibiotics in treating human illnesses. On the other hand, at present, there are several injectable vaccines against piscirickettsiosis available in Chile, where most corresponds to inactivated vaccines (Rozas &

Enriquez, 2013). These vaccines have variable long-term efficacy, but apparently, they only protect the fish after transfer of fish from fresh water to seawater. After this the fish are susceptible to a second, more aggressive form of piscirickettsiosis, which has proved to be much more difficult to protect the fish against (Tobar *et al.,* 2011). This is because at this stage of the life cycle of salmon, when fish are close to being harvested, is when more effective treatment is required. However, due to multiple treatment of antibiotics that have previously received in their life cycle and for the short immunological memory that present to the vaccines, adult fish are the most vulnerable to treatments. Therefore, the active compounds isolated from *Heliotropium* have several advantages in regard to their potential use in aquaculture: they are new and therefore have low chances to encounter resistance mechanisms in bacteria including *P. salmonis*, they are not prohibited because of human consumption as many current antibiotics are, there are no restrictions of use or waiting periods for harvesting the fish before selling them, although of course potential human toxicity has to be discard, as more than one compound is active, they could be applied alone or in mixture to improve its potential as bactericide. It is also important to consider that these compounds have other biological activities, for example they are antioxidants (Lissi *et al.,* 1999) and immune-stimulants (Obreque, 2012) what can further enhance its effectiveness.

Finally, the results obtained indicate that these compounds are good candidates to test their antibacterial activity *in vivo* in the search of new compounds with novel mechanisms of action without having resistance and biodegradable.

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