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Antiviral activity *in vitro* and *in vivo* of natural flavonoids isolated from *Heliotropium sinuatum* against infectious salmon anemia virus (ISAV)

[Actividad antiviral *in vitro e in vivo* de flavonoides naturales aislados desde *Heliotropium sinuatum* contra el virus de la anemia infecciosa en salmones (ISAV)]

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Abstract
The infectious salmon anemia virus (ISAV) of Orthomyxoviridae family, is responsible for heavy losses in industry aquaculture around the world, affecting several commercial aquatic organisms, mainly *Salmo salar*. Therefore, it is important to find effective antiviral therapies. In this work we evaluated *in vitro* and *in vivo* the antiviral activity of three natural flavonoids isolated from the resinous exudates of the plant *Heliotropium sinuatum* (Heliotropiaceae) against ISAV. The results show that 7-O-methyleriodictyol was able to inhibit the infectivity of ISAV *in vitro* assay with EC 50 of 0.20 µg/mL. Despite having a citotoxicity expressed as CC50 of 12.80 µg/mL, the *in vivo* study showed that this compound protected 100% to the fish infected with ISAV keeping 100% fish viability. These results allow the proposal of 7-O-methyleriodictyol as a good candidate to be used as antiviral therapy for ISAV in salmon industry.

Keywords: ISAV; flavonoids; salmon; antiviral activity; *Heliotropium sinuatum*.

Resumen
El virus de la anemia infecciosa en salmón de la familia Orthomyxoviridae, es el responsable de grandes pérdidas en la industria acuícola alrededor del mundo, afectando diversas especies acuáticas comerciales, principalmente *Salmo salar*. Por lo tanto, es muy importante encontrar una terapia antiviral efectiva. En el presente trabajo, evaluamos la actividad antiviral *in vitro* e *in vivo* de tres flavonoides naturales aislados desde el exudado resinoso de la especie vegetal *Heliotropium sinuatum* (Heliotropiaceae) contra ISAV. Los resultados mostraron que 7-O-metileriodictiol inhibió la infectividad de ISAV *in vitro* con un EC50 de 0.20 µg/mL. A pesar de tener una citotoxicidad expresada como un CC50 de 12.80 µg/mL, el estudio *in vivo* mostró que este compuesto protege en un 100% a los peces infectados con ISAV manteniendo un 100% de viabilidad. Estos resultados permiten proponer que 7-O-metileriodictiol es un buen candidato para ser usado como terapia antiviral para ISAV en la industria salmonera.

Palabras Clave: ISAV; flavonoides; salmón; actividad antiviral; *Heliotropium sinuatum*.

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INTRODUCTION

Infectious salmon anemia virus (ISAV) is a member of genus *Isavirus,* family Orthomyxoviridae. It is segmented, negative-sense and single-stranded RNA virus with a total molecular size of genome of approximately 14.3 kb (Kibenge *et al*., 2006). The virus produces one of the most important viral epidemic diseases in farmed salmon. ISAV initially affected the Atlantic salmon (*Salmo solar),* but now it has been identified in other species of fresh and sea water, such as coho salmon (*Oncorhincus kisutch*) and rainbow trout (*O. mykiss*) among others (Kibenge *et al*., 2004). Since initial isolation in 1984 in Norway (Thorud and Djupvik, 1988), this etiological agent has been a serious problem salmon culture centres of the northern hemisphere, such as Norway, Canada, Scotland, Faroe Islands and United States (Lovely *et al*., 1999; Mjaaland *et al*.,2002) and in the last decade, also in Chile, whit the high mortalities that exceed 90% (Mjaaland *et al*., 1997).

Epidemiologigal studies showed that ISAV can be transmitted by water and or fish secretions, by the close contact between fish (Vike *et al*., 2009). Horizontal transmission can occur in both freshwater and seawater (Scheel *et al*., 2007).

ISAV can be inactived using disinfectants such as sodium hypochlorite, chloramines-T, chlorine dioxide, iodophors, sodium hydroxide and formic acid. This virus is also susceptible to ozonated seawater and ultraviolet irradiation (Smail *et al*., 2004). On the other hand, at present most available virus vaccines based on inactivated virus or recombinant subunit proteins (Sommerset *et al*., 2005) unfortunately, has an estimated coverage in the susceptible species ranked only from 10-80% (Bravo and Midtlyng, 2007). Therefore, due to the ecological and commercial importance of this problem, development of effective vaccines and antivirals is a matter of great importance.

In this paper we present the results obtained of the *in vitro* and *in vivo* evaluation of antiviral activity on ISAV of natural flavonoids isolated from resinous exudates of the plant *Heliotropium sinuatum* Miers (Torres *et al*., 1996). This plant grows in arid areas with extreme environmental conditions and produce resinous exudates from glandular trichome that covers the leaves and stems as a protection mechanism (Torres *et al*., 1996).Previous studies carried out in our laboratory have showed that these flavonoids have both antimicrobial and antioxidant activities (Torres *et al*., 2008; Modak *et al*., 2004; Modak *et al*., 2005).

MATERIALS AND METHODS

Plant material

H .sinuatum samples were collected in III region, Chile, 29º 57' S, 71º W. A voucher specimen was deposited in the Herbarium of Natural History Museum of Santiago of Chile (ST2563).

Extraction and isolation of the flavonoids

The resinous exudate was extracted by immersion of the fresh plant material in dichloromethane for 60 s at room temperature and was concentred to a sticky residue. The extract was purified by column chromatography (silica gel) using a hexano-ethylacetate step gradient (Torres *et al*., 1996) yielding 3-O-methylgalangin **1**, 7-O-methyleriodictyol **2** and Pinocembrine **3** (Figure 1).

Virus and cells

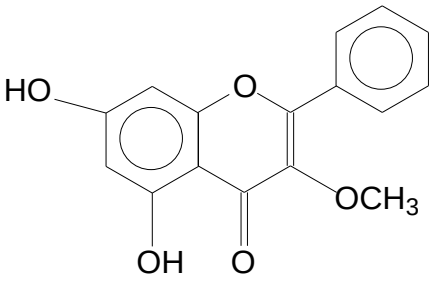
Monolayers of salmon head kidney cells (SHK-1) were grown at 15º C in Leibovitz's 15 medium (L-15) supplemented with 10% fetal bovine serum (FBS), L-glutamine (4 mM), 2-mercaptoethanol (40 μM) and gentamicin (50 μg/mL). The viral inoculum ISAV (HPR1c strain) was propagated in monolayers of SHK-1 cells at 70% confluence with a multiplicity of infection (MOI) of 0.01. Cells were infected 3 days after seeding. After 4 h of absorption at 15º C, the monolayer was washed twice with phosphate-buffered saline (PBS) and supplemented L-15 medium was added. The cells were incubated for 7 days postinfection (dpi). Subsequently, the supernatants of the culture were harvested and stored at -20º C. The viral titer was determined by real-time quantitative reverse transcription-PCR (real time qRT-PCR) (Rivas-Aravena *et al*., 2011).

Evaluation in vitro of antiviral activity of compounds on ISAV

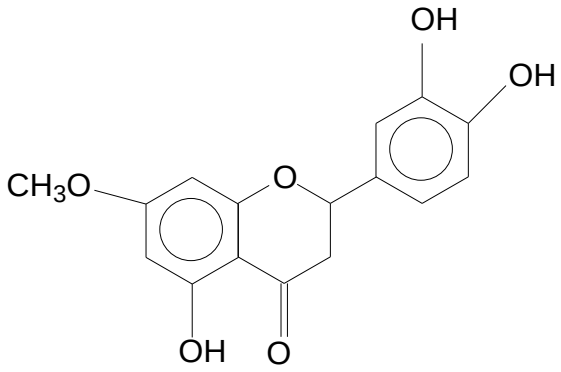
ISAV was incubated for 1 h in L-15 without FBS with increasing concentrations of the different compounds and then added to SHK-1 cells at a MOI of 0.01. After 4 h of absorption at 15º C, the cellular monolayer was washed and supplemented L-15 medium was added with the different compounds at the same concentration as in the preincubation stage. At 7 dpi, the supernatant was recollected and analyzed by real-

time qRT-PCR to determine viral titer according to Rivas-Aravena *et al*., (2011). The results were expressed as EC50 (effective antiviral concentration required to inhibit ISAV replication by 50%).

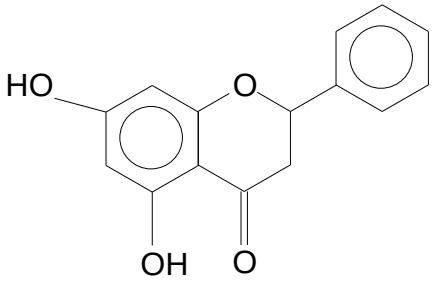
Figure 1
Chemistry structure of test compounds against ISAV



1 3-O-methylgalangine



2 7-O-methyleriodictyol



3 Pinocembrine

Evaluation of the cytotoxic effect of antiviral compounds on SHK-1 cells.
Monolayers of SHK-1 cells were grown in 6-well plates until 90% confluence. Then, increasing concentrations of different compounds were added to each well and incubated for 7 days at 15º C. Subsequently, the supernatant was removed and cells were washed twice in PBS and detached with 200 µL of a solution of 0.5 mM EDTA and 0.02% trypsin. The cells were centrifuged in eppendorf for 10 min at 3000 rpm and resuspended in PBS-2% FBS. Propidium iodide was added at a final concentration of 0.75 µg/mL, and viability was determined by flow cytometry from 100.000 cells. The result was expressed as cytotoxic concentration (CC_{50}), corresponding to antiviral concentration that reduces the cell viability by 50 %.

Both EC_{50} as CC_{50} concentrations were calculated from concentration-effect curves after linear regression analysis. The results represent the mean ± standard deviation of the mean values of three different experiments.

Evaluation in vivo of antiviral activity of the test compounds on Atlantic salmon.
A group of 55 healthy postmolt Atlantic salmon (*Salmo solar*) with an average weight of 35 g was kept in 200-liter tanks with 25 ppt of salinity at a density of 22 kg/m^3, at temperature of 14 to 18º C, and oxygen rate of 5.8 to 7.1 mg/L. After 7 days of acclimatization a group of 15 fish were injected with the different compounds used: 3-O-methylgalangin **1** (5 fish), 7-O-methyleriodictyol **2** (5 fish) and Pinocembrine **3** (5 fish) by two times (every one day) by intraperitoneal

injection of concentration of 200 mg/kg by injection. The behaviour was observed for one week. After 8 days, the fish that survived to the treatment were infected with ISAV together with other 20 fish without treatment. The infection was realized by cohabitation with fish infected (Trojans) by intraperitoneal injection of 0.2 mL from $1x10^5$ copies/mL of virus. A third group of 20 fish were inoculated with culture medium without virus as control.

The fish were distributed in the following way:
Tank 1: 15 fish infected with test compounds (5 with **1**; 5 with **2** and 5 with **3**).
Tank 2: 20 fish infected without treatment.
Tank 3: 20 non-infected fish and without treatment.
Daily physical-chemical parameters were monitored: nitrite, nitrate, ammonium, pH, temperature, and dissolved oxygen. Also, daily mortality, activity and appetite of the fish were registered. After infection, the fish kept for 16 days to monitor performance.

RESULTS

The results of the *in vitro* antiviral activity of the natural flavonoids 3-O-methylgalangin **1**, 7-O-methyleriodictyol **2** and Pinocembrine **3** (Figure 1) using a concentration range between 0.1 and 100 μg/mL are shown in Table 1. All the above mentioned compounds were active against ISAV on the *in vitro* evaluation. However, 7-O-methyleriodictyol **2** and Pinocembrine **3** showed greater antiviral activity with EC_{50} at 0.20 μg/mL and 0.25 μg/mL respectively, unlike to 3-O-methylgalangin **1**, which needed an effective antiviral concentration to inhibit ISAV replication by 50% much greater (EC_{50} 2.99 μg/mL).

Table 1
Evaluation of the antiviral activity of test compounds 1-3 on ISAV infection in SHK-1 cells

Compounds	EC_{50} (μg/mL) ± SD
1	2.99 ± 0.51
2	0.20 ± 0.03
3	0.25 ± 0.05

EC_{50}: Effective antiviral concentration required to inhibit ISAV grow by 50%.
SD: Standard deviation.
As control were used infected cells without test compounds.

As the three compounds showed antiviral activity, the cytotoxic effect on SHK-1 cells was studied. For this, we used flow citometry, which allowed determining the number of viable and non-viable cells at different concentrations of test compounds. The results were expressed as test compounds concentration required to reduce the cell viability by 50% (CC_{50}) (see Table 2).

Table 2
Viability of SHK-1 cells treated with test compounds 1-3

Compounds	CC_{50} (μg/mL) ± SD
1	0.20 ± 0.05
2	12.80 ± 2.30
3	> 100

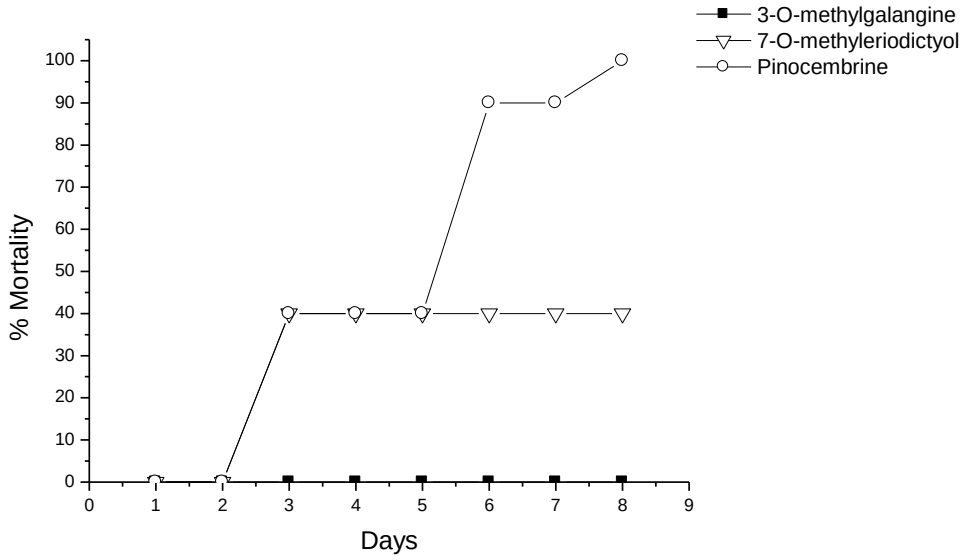
CC_{50}: Test compounds concentration required to reduce the cell viability by 50 %.
DS: Standard deviation.
Ethanol was used as control of death

Table 2 shows that the CC50 of 7-O-methyleriodictyol **2** and Pinocembrine **3** were 12.80 and > 100 μg/mL respectively, therefore, there is sufficient difference with EC_{50} values and it allows that both compounds are suitable candidates for *in vivo* studies. Although 3-O-methylgalangin **1** showed a CC_{50} very small (0.20 μg/mL) similarly it was included *in vivo* studies.

For *in vivo* studies, healthy fish were treated with the flavonoids **1-3.** For this purpose, a group of 15 fish were injected with 3-O-methylgalangin **1** (5 fish), 7-O-methyleriodictyol **2** (5 fish) and Pinocembrine **3** (5 fish) on two occasions, with a day of difference, by intraperitoneal injection at a concentration of 200 mg/kg and observed daily for 8 days. The results are showed in Figure 2. Is possible to observe that 3-O-

methylgalangin **1**, although presented a low value of CC50, there was no mortality in fish treated with this compound was observed. However, with Pinocembrine **3**, which had a low toxicity in vitro test, here showed a mortality of 100%. On the other hand, 7-O-methyleriodictyol **2**, presented a mortality of 40%, which was observed beginning on the third day, but post infection remained constant until the end of the experiment. Based in these results, we continued working with compounds **1** and **2** only.

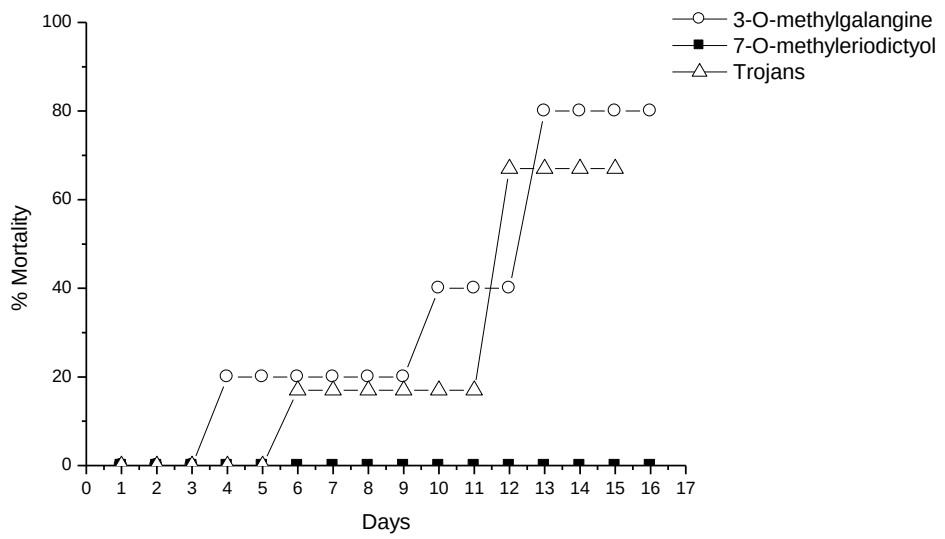
Figure 2
Effect of treatment with test compounds (Flavonoids 1 – 3) on Atlantic salmon.



Percentage of mortality of fish in tank 1 after 8 days of treatment with flavonoids 1-3.

The surviving fish in the previous experiment were inoculated with ISAV by cohabitation with fish infected denominated Trojans (corresponding to 40% of the fish) by intraperitoneal injection of 0.2 mL from $1x10^5$ copies/mL of virus, in this way, infection is similar to what happens on field. After ISAV infection, the fish were daily observed for 16 days. Daily mortality, activity and appetite of the fish were observed. The results are shown in Figure 3. The results indicated that the totality of the fish infected with ISAV and treated with 7-O-methyleriodictyol **2** survived to the infection with a 0% of mortality after 16 days post infection. In the case of 3-O-methylgalangin **1**, was observed mortality of fish from fourth day post infection, reaching 80% of mortality from 13^{th} d.p.i. A similar behaviour was observed in the Trojans fish with a 67% of mortality.

Figure 3
Effect of 3-O-methylgalangine 1 and 7-O-methyleriodictyol 2 on Atlantic salmon infected with ISAV



Percentage of mortality of fish infected with ISAV after 16 days of infection.
The infected fish correspond to those that survived after treatment with flavonoids

Finally, Table 3 shows a summary of percentage cumulative mortality in the three tanks. It notes that the Trojans fish of both tanks have percentage of mortality equal or higher than to 60%, similar to infected fish without treatment, validating the effectiveness of the experiment. On the other hand, the control fish in tank 3, corresponding to healthy fish, showed a 0% of mortality, showing that the experimental conditions for the development of the fish were the appropriate. To evaluate the effectiveness of the treatment, the following criteria were applied:

The nonspecific mortality percentage must be less than 5%. Mortality in infected fish with ISAV without treatment must be equal or greater than 50%.

Therefore, this experiment showed that the flavonoid 7-O-methyleriodictyol **2** could be used as an antiviral against ISAV.

Table 3
Total mortality of infected fish completed the test period

	Tank1			Tank 2		Tank 3
	Fish treated with 3-O-methylgalangine	Fish treated with 7-O-methyleriodictyol	Trojans	Fish infected untreated	Trojans	Control
% Mortality	80	0	67	60	69	0

The tank 1 corresponds to the test realized with fish inoculated with ISAV, surviving the treatment with flavonoids 3-O-methylgalangine 1 and 7-O-methyleriodictyol 2.
The tank 2 corresponds to the test realized with fish inoculated with ISAV but untreated with flavonoids.
The tank 3 corresponds to control group with healthy fish.

DISCUSSION

The heavy losses caused by the action of infectious diseases in aquaculture industry have led to the search for solutions to control its progress or eradicate them. In the case of ISAV, also has been demonstrated that orthomyxovirus possess a high rate of mutations and recombination giving rise to new strains, impeding prevention by vaccines. Therefore, therapies with natural compounds that possess antiviral activity may be a fundamental tool for its control. Then we evaluate the antiviral activity against ISAV of flavonoids isolated from exudates resinous of *Heliotropium sinuatum*. It was possible to observe that results obtained in vitro cannot be extrapolated to what may occur in vivo, because on many occasions the same trend is not maintained. For example, 3-O-methylgalangin **1**, that showed high toxicity in vitro, however, it did not cause mortality of fish treated with this compound. On the contrary, Pinocembrine **3**, that showed light toxicity *in vitro*, it resulted a 100% mortality in fish treated with it. In the case of 7-O-methyleriodictyol **2**, was observed partial toxicity in vitro and excellent results in vivo giving 100% protection to fish infected with ISAV, although the selectivity index (CC_{50}/EC_{50}) is low compared with other proposed antivirals to be used against ISAV as rivabirin (Rivas-Aravena *et al*., 2011).

On the other hand, 7-O-methyleriodictyol **2** possesses a significant antioxidant activity evaluated through of the bleaching of stable free radicals ABTS and DPPH (Lissi *et al*., 1999). The evaluation of structure-antioxidant activity relationships, showed by this flavonoid, that the good antioxidant activity is attributed to the presence of a catechol system in its B ring, which makes it best antioxidant due to a better radical stabilization caused by H-abstraction at position C-4' forming a hydrogen bond with the phenolic hydroxyl group at C-3' (Modak *et al*., 2005). Different kinds of evidence suggest that modulation of the intracellular redox balance might be helpful to control the innate and the adaptive immune response at different levels. Therefore, the antiviral activity of this compound may be associated directly with its antioxidant activity.

In conclusion, the results obtained shows that the flavonoid 7-O-methyleriodictyol **2** is an efficient antiviral against ISAV and could be used in treatments in *Salmo salar*. It is of great interest to continue the studies to determine the mechanism of action of this compound.

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