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Immunostimulatory activity in mammalian cells of 3,5-dihydroxy-7-methoxyflavanone ((-)-alpinone) isolated from *Heliotropium huascoense* resinous exudate

[Actividad inmunoestimulante en células de mamífero de 3,5-dihidroxi-7-metoxiflavanona ((-)-alpinona) aislada del exudado resinoso de *Heliotropium huascoense*]

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Abstract: The flavonoid 3,5-dihydroxy-7-methoxyflavanone ((-)-alpinone) isolated from sticky resinous exudate of *Heliotropium huascoense* was evaluated as immunostimulatory in mammalian cells . Preliminary observations had showed that (-)-alpinone had increased the expression levels of pro-inflammatory cytokine transcripts in salmonid. Due to high morbidity and mortality that infectious diseases cause in humans, we evaluate the effect of (-)-alpinone as an immunostimulant in mammalian cells. Reactive oxygen species (ROS) are produced by macrophages activators for the destruction of pathogens; we evaluated (-)-alpinone effect in ROS generation and the proliferation of macrophages. The results showed that proliferation in Raw 264.7 cells treated with 10 and 25 µg/mL of (-)-alpinone had a significant increase in macrophage proliferation. In relation to ROS formation, cells treated with 1 and 5 µg/mL of (-)-alpinone, induce ROS formation in macrophages.

Keywords: Heliotropium, (-)-alpinone, immunistimulant activity.

Resumen: El flavonoide 3,5-dihidroxi-7-metoxiflavanona ((-)-alpinona) aislado del exudado resinoso de *Heliotropium huascoense* se evaluó como inmunoestimulador en células de mamíferos. Resultados preliminares habían demostrado que (-)-alpinona aumentaba los niveles de expresión de transcritos de citoquinas proinflamatorias en salmónidos. Debido a la alta morbilidad y mortalidad que causan las enfermedades infecciosas en los humanos, evaluamos el efecto de (-)-alpinona como inmunoestimulante en células de mamíferos. Dado que las especies de oxígeno reactivo (ROS) son producidas por macrófagos activados para la destrucción de patógenos, se evaluó el efecto de (-)-alpinona en la generación de ROS y la proliferación de macrófagos. Los resultados mostraron que la proliferación en células Raw264.7 tratadas con 10 y 25 µg / mL del flavonoíde tuvo un aumento significativo en la proliferación de macrófagos. En relación con la formación de ROS, las células tratadas con 1 y 5 µg/mL de (-)-alpinona, inducen la formación de ROS en los macrófagos.

Palabras clave: Heliotropim, (-)-alpinona, actividad inmunoestomulante.

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INTRODUCTION

Heliotropium huascoense Johnston (Heliotropiaceae) (The Plant List, 2013), is an endemic Chilean resinous shrub that grows in desert environments. Like all Chilean *Heliotropium* species of Cochranea section, produce resinous exudates (Villarroel *et al.*, 1991; Torres *et al.*, 2002), which next to dense pubescence are complementary adaptations to negative abiotic and biotic factors. (Luebert, 2013). Phytochemical studies of this species have shown that the sticky resin contains 3-O.methylgalangine, 3,7-O-dimethylgalangine, (-)-alpinone and carrizaloic acid (Urzúa *et al.*, 2000; Villarroel *et al.*, 2001). The only known ethnomedicinal use of leaves of this species, is for vaginal washes in Pichasca, Limarí province (Luebert, 2013). Also, previous studies performed in our laboratory showed that (-)-alpinone increased expression levels of pro-inflammatory and inflammatory cytokine transcripts in the SHK-1 cell line derived from salmon head kidney cells and in vivo on Atlantic salmon (Valenzuela, 2013), however, its potential activity in mammals has not been studied.

Today, infectious diseases represent a major public health problem. Although the use of antimicrobials has considerably reduced deaths associated with infections, the massive use of these drugs has resulted in the generation of resistant pathogens. Then, for this reason, use of immunostimulatory compounds that reinforce the immune system, appears as an alternative to control infectious diseases. Based on these findings, in this work we evaluated the immunomodulatory effects of (-)-alpinone in mammals, through macrophage proliferation in the cell line Raw264.7 and on the activation of macrophages by measuring the generation of reactive oxygen species (ROS) in the same cell line, linked to a mechanism of pathogens elimination.

MATERIALS AND METHOD

Isolation of Alpinone (1)

H. huascoense was recollected in Carrizal Bajo, Chile and identified by Dr. Sebastian Teiller Museo de Historia Natural de Santiago de Chile (number ST 2580). (-)-Alpinone (1) ((2S, 3S)-3, 5-dihydroxy-7-methoxyflavanone) (Figure 1) was obtained from the resinous exudate of *Heliotropium huascoense* as was previously described (Urzúa *et al.*, 2000). Briefly, the resinous exudate was extracted by immersion of the fresh plant material in CH_2Cl_2 for 30 s at room temperature. The extract was concentrated to a sticky residue. The extract was fractionated by CC (silica gel) using a hexane- ethyl acetate step gradient. (-)-alpinone (1) was obtained as a yellow solid, 99.8% purity (HPLC).

Cell culture

Raw264.7 (ATCC TIB-71) cell line corresponding to macrophages of the species *Mus musculus* was maintained in DMEM (Dulbecco´s Modified Eagle´s Medium, Corning) supplemented with 10% fetal bovine serum (FBS, Biowest), with 1% Penicillin/Streptomycin (P/S, Corning) at 37º C with CO_2 5%. The cell line was propagated every 2 days, washing 2 times with 1 X PBS (Phosphate Buffered Saline) and then incubated with trypsin solution 0.25% (v/v) and EDTA 0.1% (v/v) EDTA (Corning).

Cell proliferation assay with MTT

Raw264.7 cells ($2x10^4$) were plated in 96-well and incubated with 1, 5, 10, and 25 µg/mL of (-)-alpinone for 24 h at 37° C. As positive control, ECGS (Endothelial Cell Growth Supplement; Sigma) was used. As negative control cells were incubated with medium only and cells in DMSO (dimethylsulfoxide, Sigma) to 0.4%. Following incubation, the supplemented DMEM culture medium was discarded and replaced with 100 µL IF (1 X PBS with 2% FBS). Next, 10 µL of MTT (Sigma) (5 mg/mL) was added to each well and was incubated for 4 h at 37° C. Subsequently, the solution was removed and 100 µL of isopropanol was added to dissolve the formed crystals. After 30 min absorbance were determined at 570 nm using a microplate reader Tecan INFINITE M200 Pro. The experiments were performed in triplicate, using five replicates for each condition. Cell viability was calculated from the following equation:

$$CV = A_T/A_C \times 100$$

Where CV is cell viability; A_T is absorbance of the treatment group and A_c is absorbance of the control group.

Determination of Intracellular Reactive Oxygen Species by Fluorometry

RAW 264.7 cells were seeded in black 96-well plated (NUNC, Thermo Scientific) at a density 2×10^4 cells per well in culture medium. Twenty four later the culture medium DMEM supplemented with SFB 10% was replaced by medium without supplement. Cells were treated for 8 h with 1, 5 and 10 µg/mL of (-)-alpinone (1). As negative control untreated cells and cells in DMSO (dimethyl sulfoxide, Sigma) to 0.4% were used. As positive control LPS (0.1 µg/mL) and Poly I:C (10 µg/mL) were used. Then, cells were incubated with 2',7'-dichlorodihydrofluorescein diacetate 10 µM (H_2DCFDA, Invitrogen) for 30 min at 37° C. The wells were washed with PBS and fluorescence intensity was measured using a fluorescence microplate reader (Tecan INFINITE M200 Pro) at λex: 485 nm and λem: 530 nm. The experiment was performed 5 time using eight replicates for each condition.

Statistical analysis

All data were expressed as mean ± SEM values. The statistical significance between groups was assessed using ANOVA and Dunnett Tests (Post Hoc Tests algorithms) GraphPad v5.0 for Windows (GraphPadSoftware) was used to calculate the mean and SEM and to perform statistical tests. *P* values < 0.05 were considered statistically significant.

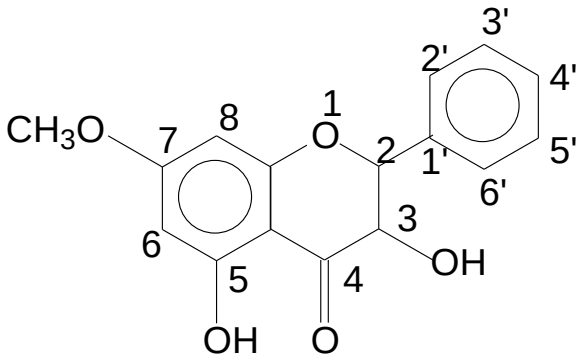

Figure 1
(-)-Alpinone (1) structure

RESULTS

Evaluation of the effect of (-)-alpinone on the proliferation of macrophages

Macrophages together with neutrophils constitute the first host's line of defense against infectious agents. An important function of macrophages in host defense is to ingest and kill pathogens (Struzik *et al.*, 2015). Therefore, macrophages have been used as an *in vitro* ideal model for the detection of new immunostimulatory agents (Li & Xu, 2011; Sun *et al.*, 2015). The effect of (-)-alpinone (1) was evaluated on macrophage proliferation by the cell viability assay with MTT, which indirectly indicates cell proliferation (Mosmann, 1983). Raw264.7 cells were treated with different concentration of (-)-alpinone (1). After incubation, yellow tetrazolium, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added. Metabolically active cells are capable of reducing the tetrazolium dye to insoluble formazan (purple crystals). This dye is solubilized and quantified by measuring absorbance. Results in Figure 2, showed with 10 and 25 µg/mL of (-)-alpinone (1) a significant increase in cell viability. When cultures were treated with 10 µg/mL of (-)-alpinone (1), a 65% increase in cell viability was observed with respect to untreated cells. While cultures treated with 25 µg/mL showed a 69% increase relative to their control. In macrophages treated with lower concentrations of (-)-alpinone (1) (1 and 5 µg/mL), no changes in cell viability were observed. The macrophages treated with ECGS growth factor showed a significant increase in cell viability of 39%, relative to untreated cells, although lower in comparison to cells treated with higher

concentrations of (-)-alpinone (1) . Because cell viability values indirectly represent cell proliferation, the results indicate that macrophages treated with (-)-alpinone (1) at 10 and 25 µg/mL significantly increase cell proliferation.

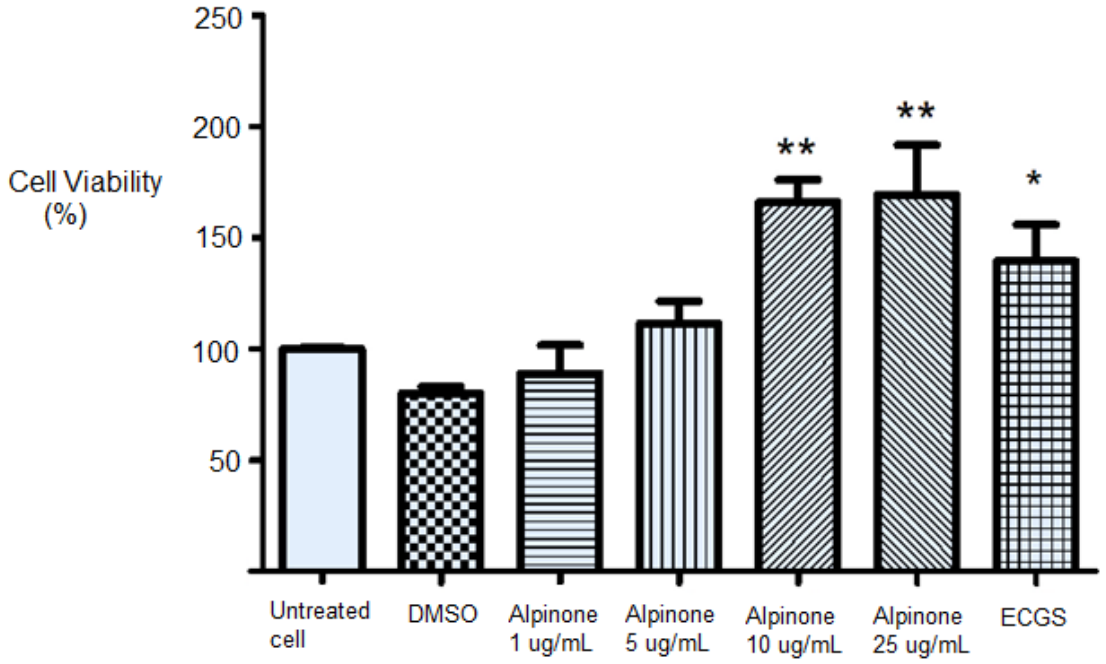

Figure 2
Macrophages proliferation determined by MTT assay
Raw264.7 cells were incubated with (-)-alpinone at different concentrations (1, 5, 10 and 25 µg/mL) for 24 hours at 37° C. As control untreated cell and cells treated with DMSO 0.4% were used. As positive control growth factor ECGS was used. The graph shows the percentage of cell viability compared to the untreated control for each of the conditions. Each value represents the mean and standard deviation of three independent experiments. Statistical differences were determined using the one-way Anova test with a Dunnet post-test in relation to the untreated control (*$P < 0,05$, ** $P < 0,01$).

Evaluation of the effect of (-)-alpinone on the activation of macrophages through the measurement of reactive oxygen species (ROS)

Reactive oxygen species (ROS) have been identified as one of the major effector molecules produced by activated macrophages (Fialkow *et al.*, 2007). These molecules are oxidants that destroy microbes and other cells (Turrens, 2003). Based on this background, the formation of ROS in macrophages was measured by fluorometry. The results are shown in Figure 3. Cells treated with 1 and 5 µg/mL of (-)-alpinone showed a significant increase in ROS formation, increasing 1.8 and 1.7 times the fluorescence intensity respectively. Cells treated at a concentration of 10 µg/mL of (-)-alpinone (1) did not show changes in fluorescence intensity relative to untreated cell cultures. On the other hand, cells treated with Pathogen-Associated Molecular Patterns (PAMPs) as LPS and Poly I:C, caused a significant increase in the formation of ROS, with notorious differences between them. Cells treated with LPS showed a double increase in fluorescence intensity. While the cultures treated with Poly I:C increased 4.5 fold the fluorescence intensity. These results indicate that cells treated with 1 and 5 µg/mL of (-)-alpinone (1) induce the formation of ROS in macrophages. The results are shown as mean of fluorescence intensity (MFI) of each condition relative to untreated cells.

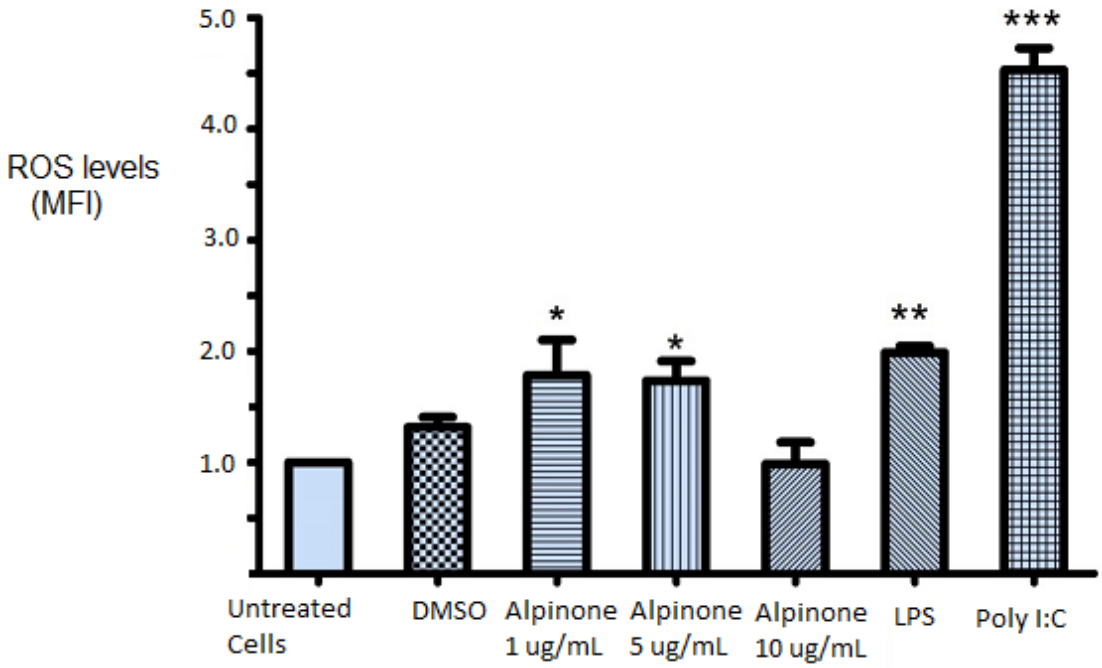

Figure 3
Formation of intracellular ROS in macrophage cells determined by fluorometry
Raw264.7 cells were incubated with (-)-lpinone at different concentrations (1,5 and 10 µg/ml) for 8 hours at 37 ° C. As control untreated cell and cells treated with DMSO 0.4% were used. LPS (0,1 µg/mL) and Poly I:C (10 ug/mL) were used as positive control. The graph shows the ROS levels expressed as mean IF (fluorescence intensity) measured for each treatment as compared to the untreated control. Each value represents the mean and standard deviation of five independent experiments. Statistical differences were determined using the one-way Anova test with a Dunnet post-test in relation to the untreated control (*P < 0,05, ** P < 0,01, *** P < 0,001).

DISCUSSION

Recognition of pathogens by immune cells induces an increase in cell proliferation at the site of infection (Abbas *et al.*, 2014). Among the most abundant cells of the immune system at the site of infection are the macrophages (Davies *et al.*, 2013). These cells play a key role in the defense mechanism against host infection, one of its functions is to phagocyte and kill microbes, through different mechanisms such as; the enzymatic generation of reactive oxygen species (Fujiwara & Kobayashi, 2005). In addition to ingesting pathogens, macrophages also ingest dead host cells, this is part of the cleaning process following infection. At the same time, activated macrophages secrete different cytokines that act on endothelial cells lining blood vessels to enhance the recruitment of more monocytes and other leukocytes from the blood to areas of infection, amplifying the protective response against pathogens (Janeway *et al.*, 2001). Therefore, an increase in cell proliferation is important, since it would allow a greater recruitment of macrophages at the site of infection. The results obtained in the cell proliferation assay showed that Raw264.7 cells treated with 10 and 25 µg/mL of (-)-alpinone (1) showed a significant increase in macrophage proliferation compared to their control without treatment.

Different flavonoids increase cell proliferation in some cell types of the immune system. For example, naringenin (2) (Figure 4) possess immunomodulatory properties, since stimulates the activation of natural killer (NK) cells inducing proliferation of T and B lymphocytes (Maatouk *et al.*, 2016).

Other studies have shown that hesperetin (3) and chrysin (4) (Figure 4) modulate the immune

response, since increase the proliferation of T and B lymphocytes (Sassi *et al.,* 2017). Although the mechanism by which some flavonoids exert their proliferative action is unknown, there is evidence that could exert their effects through modulating actions in different components of cascades of cellular signaling of protein kinases (Williams *et al.,* 2004) such as mitogen-activated protein kinase (MAPK), which regulate cellular functions including cell survival, apoptosis and proliferation. For example, it has been proposed that different cell lines treated with low concentrations of quercetin (5) (Figure 4) may activate the MAPK pathway, resulting in increased cell proliferation (Dihal *et al.,* 2006).

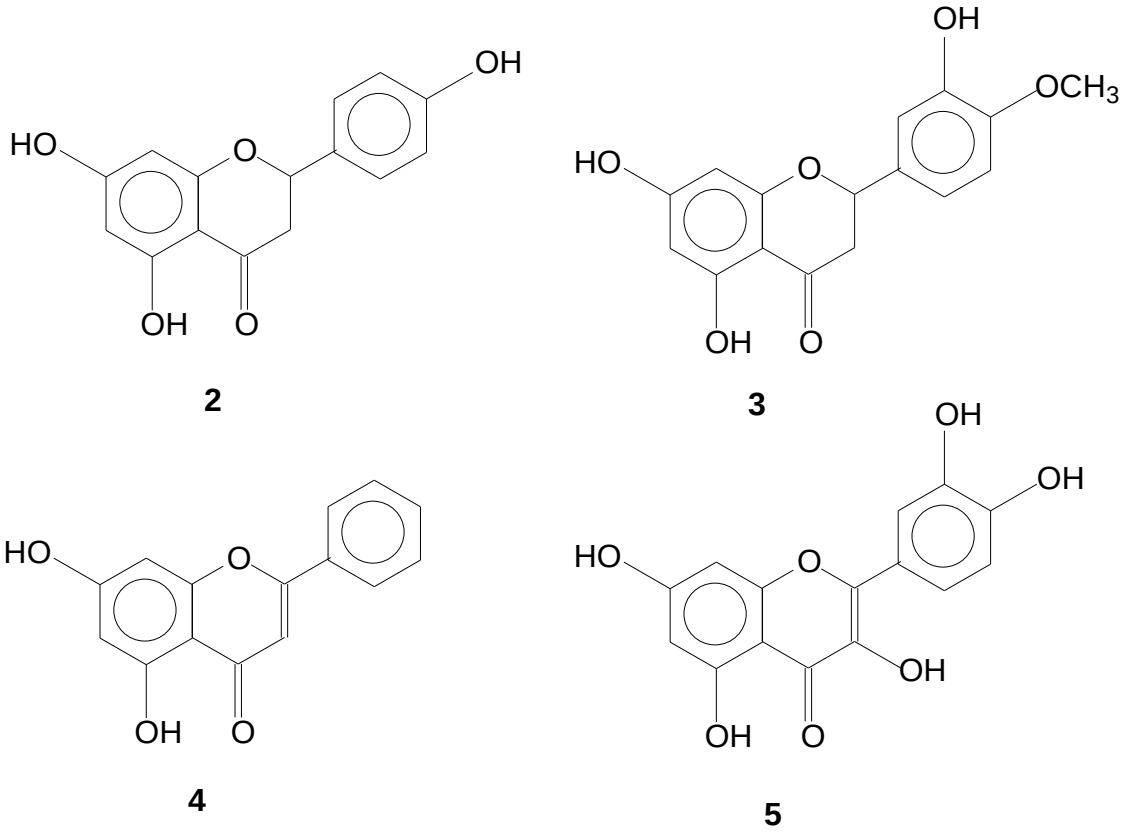

Figure 4
Structures of flavonoids with immunostimulant activity
(2) Naringenin; (3) Hesperetin; (4) Chrysin; (5) Quercetin.

On the other hand, activation of macrophages occurs after recognition of PAMPs, and aims to eliminate pathogens by microbicide mechanisms such as the formation of reactive oxygen species generated by the enzymatic complex NADPH oxidase (Fang, 2004). This process of free radical formation is known as respiratory burst, during which the production of free radicals such as superoxide anion (O_2^-) and hydroxyl radical ($OH^\cdot$) is induced, and non-radical species such as hydrogen peroxide (H_2O_2) (Nathan & Shiloh, 2000). Therefore, the production of ROS in macrophages reflects the activation of the microbicide mechanisms characteristic of the innate immune response. The results obtained with the macrophages of the cell line Raw264.7, when treated with concentrations of 1, 5 and 10 µg/mL of (-)-alpinone (1), showed that this flavonoid is capable to increasing intracellular ROS levels, however, not all the concentrations of Alpinone used stimulated the generation of ROS in these cells. These results indicate that (-)-alpinone (1) at low concentrations induces the formation of ROS, microbicide mechanism related to an activation of macrophages. Flavonoids are known mainly for their antioxidant

properties; however it has been shown in some studies, which can also act as pro-oxidants. Some of the mechanisms through which the flavonoids exert their pro-oxidant actions include the temporary reduction of Fe^{+3} to Fe^{+2} that produces ROS by the Fenton reaction, in which Fe^{+2} is oxidized by hydrogen peroxide to Fe^{+3}, forming a hydroxyl radical and a hydroxide ion in the process. Fe^{+3} is then reduced to Fe^{+2} by another molecule of hydrogen peroxide, forming a hydroperoxyl radical and a proton.

The positive LPS and Poly I:C controls, these molecules had a significantly increase intracellular ROS levels with respect to untreated cells. Lipopolysaccharide (LPS) is a potent activator of the innate immune system, since it modulates different effector mechanisms of macrophages, including ROS formation through binding to TLR4 membrane receptor (Hsu & Wen, 2002). In relation to Poly I:C, it has been shown to induce the formation of ROS by binding to receptors TLR3, RIG-1 and MDA-5 that recognize double-stranded RNA (Harashima *et al.,* 2014). The results showed that there is a large difference in the formation of ROS between the molecule present in LPS bacteria and the structural component of some Poly I:C viruses, which could indicate that the activation of macrophages is more potent for antiviral responses. Therefore can be deduced that (-)-alpinone (1) at low concentrations increases the formation of ROS, however, this increase is generated by a small population of macrophages.

CONCLUSION

The flavonoid (-)-alpinone (1) increased proliferation of macrophages important for start and development the innate immune response in mammals. Also, Raw264.7 cells treated with (-)-alpinone (1) at low concentrations, increase intracellular ROS formation, mechanism associated with macrophage activation. Therefore, (-)-alpinone (1) could function as an immunostimulatory in mammals against infectious diseases caused by extracellular pathogens.

Finally, the obtained results indicate that (-)-alpinone (1) is a good candidate to test their immunomodulatory activity *in vivo* in the search of flavonoids with novel mechanisms of action.

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