

Immunomodulatory effects of *Rhodomyrtus tomentosa* leaf extract and its derivative compound, rhodomyrtone on head kidney macrophages of rainbow trout

(*Oncorhynchus mykiss*)

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Abstract

Rhodomyrtus tomentosa is a medicinal plant that shows biological effects including immunomodulatory activity on human and other mammals, but not in fish. In this study, we evaluated the *in vitro* immunomodulatory effects of *Rhodomyrtus tomentosa* leaf extract and its active compound, rhodomyrtone on the immune responses, using rainbow trout (*Oncorhynchus mykiss*) head kidney (HK) macrophages as a model. The tested immune functions included the expression of genes involved in innate immune and inflammatory responses and the production of reactive oxygen species (ROS). Gene expression was evaluated after exposure to 10 $\mu\text{g mL}^{-1}$ of *R. tomentosa* and 1 $\mu\text{g mL}^{-1}$ of rhodomyrtone for 4 and 24 h. *R. tomentosa* and rhodomyrtone induced changes in the expression of pro-inflammatory cytokines (*il1 β* , *il8*, and *tnfa*), anti-inflammatory cytokines (*il10* and *tgfb β*), inducible enzymes (*inos*, *cox2*, and *arginase*), and an antioxidant enzyme (*gpx1*). Co-exposure of *R. tomentosa* with LPS resulted in a prominent reduction in the expression of genes related to an inflammatory process (*il1 β* , *il8*, *tnfa*, *inos*, *saa*, *hepcidin*, and *gpx1*), suggesting anti-inflammatory effects. Similarly, co-exposure of rhodomyrtone with LPS led to a down-regulation of inflammation-related genes (*il1 β* , *inos*, *saa*, and *hepcidin*). In addition, exposure to both natural plant products caused a reduction in cellular ROS levels by HK macrophages. The present results indicate that *R. tomentosa* and rhodomyrtone exerted immunostimulatory and anti-inflammatory effects on fish macrophages, thus opening up the possibility of using these natural products to further develop immunostimulants for health management in aquaculture.

Keywords Immunomodulatory effects · Macrophages · *Rhodomyrtus tomentosa* · rhodomyrtone · Rainbow trout

Introduction

The use of medicinal plants as an alternative way for disease control and health improvement in aquatic organisms has gain interest, due to increasing concerns on the detrimental effects of antibiotics and chemicals in aquacultured species and the environment. Natural plant products show multiple biological activities, such as antibacterial activities, anti-stress and growth-promoting effects, some of which providing benefits for fish health management (Van Hai 2015). In addition, they may be readily available, inexpensive, and have no hazardous effects because of low side effects on aquatic animals and environment (Van Hai 2015).

Immunomodulatory activity is one of the prevalent properties of medicinal plants that are being widely examined in competition with other immunostimulators like lipopolysaccharides (LPS), β -glucan, chitosan (Sakai 1999), and flagellin (Wangkahart et al. 2016). Previous studies conducted in both *in vitro* and *in vivo* conditions reported that medicinal plants have a positive impact on fish immune responses, which renders higher degree of disease resistance and stress tolerance (Chakraborty and Hancz 2011; Harikrishnan et al. 2011). Experiments on animal models have clearly defined the advantages of plant products as an alternative to the chemical control and treatment of diseases. Nevertheless, variations depending on physical characteristics of animals (e.g. species, size, and sex), environment conditions (e.g. temperature, salinity, and water quality), and occurrence of stressors (e.g. container and restriction effects, catching and capture) need to be assessed in these models.

The use of cell lines and primary cell cultures derived from fish organs has been extensively used as *in vitro* models for determining the toxicity of aquatic pollutants (Fent 2001; Wang et al. 2011), as well as for the study of the immunomodulatory effects of various chemical and biological compounds including natural products (Yin et al. 2011; Picchietti et al. 2013; Villasante et al. 2016). For instance, a *Sparus aurata* fibroblast cell line (SAF-1) has been used to determine the immunostimulatory effects of *Aloe arborescens* at molecular level, evaluating the expression of immune-related genes (Picchietti et al. 2013). Primary myogenic cell preparations of rainbow trout (*Oncorhynchus mykiss*) were used to investigate the effects of anthocyanidins, a flavonoid-polyphenol compound of several vegetables as a promoter of myogenic differentiation and antioxidant defense (Villasante et al. 2016). Recently, two stromal cell lines derived from spleen (TSS), pronephros (TPS-2) and macrophages of rainbow trout have been used as *in vitro* models for immunostimulant studies in aquaculture (Fierro-Castro et al. 2012; Fierro-Castro et al. 2013).

Rhodomyrtus tomentosa (Aiton) Hassk, is a Thai medicinal plant of the family Myrtaceae that can be found in tropical areas of Southeast Asia and some regions of Australia. Its immunopharmacological and chemopreventive properties have been studied and it has been reported to be an antioxidant (Lavanya et al. 2012), anti-inflammatory agent (Jeong et al. 2013) in mice models, while earlier *in vitro* studies reported a stimulatory activity of the plant extract on the differentiation of mouse osteoblastic cell lines (Tung et al. 2009). Rhodomyrtone, a pure compound of the acylphloroglucinol class isolated from *R. tomentosa* leaves, also promoted pro- and anti-inflammatory responses in human monocytes (Srisuwan et al. 2014). While this plant and its compounds have shown encouraging results in human and other mammals such as mouse (Lavanya et al. 2012; Jeong et al. 2013) and cow (Mordmuang et al. 2015), its potential on aquatic animals has not yet been investigated. In this context, the aim of the present study was to determine the immunomodulatory effects of not only the medicinal plant, *R. tomentosa* but also its active compound, rhodomyrtone on macrophages isolated from rainbow trout head kidney (HK) through changes in the expression of target genes related to innate immune responses. The target genes were selected based on the ability of *R. tomentosa* leaf extract or its phytochemical components (e.g. rhodomyrtone, quercetin, ursolic acid, tomentosin etc.) to modulate mRNAs relevant to immune defenses in mammals, as documented in previous studies (Manu and Kuttan 2008; Jeong et al. 2013; Srisuwan et al. 2014). In this study our objective focused on selected indicators of the innate immune function of fish, since the innate immune system of fish responds earlier and non-specifically to any challenge such as the exposure to these plant compounds. Moreover, since the adaptive response is more relevant at systemic level and longer exposure times, it would deserve another experimental approach. The selected genes included pro-inflammatory cytokines, such as interleukin 1 β (*il1 β*), interleukin 8 (*il8*) and tumour necrosis factor- α (*tnfa*); immune-suppressing cytokines, namely interleukin 10 (*il10*) and transforming growth factor beta (*tgfb*); inducible enzymes, such as inducible nitric oxide synthase (*inos*), cyclooxygenase-2 (*cox2*) and arginase; antimicrobial peptides and acute phase proteins, such as serum amyloid A (*saa*) and hepcidin, as well as antioxidant enzymes, namely NADPH oxidase (*nadph*) and glutathione peroxidase 1 (*gpx1*).

Materials and methods

R. tomentosa leaf extract and rhodomyrtone preparation

R. tomentosa leaves were collected from Khuan Lang, Songkhla Province in the southern part of Thailand. The voucher specimen was identified and deposited at Faculty of Traditional Thai Medicine, Prince of Songkla University, Thailand. An ethanol extract of *R. tomentosa* leaves was prepared according to the method

of Limsuwan et al (2009). Rhodomyrtonone was obtained from Natural Product Research Center of Excellence, Prince of Songkla University, Thailand. Rhodomyrtonone was isolated from the leaves crude extracts of *R. tomentosa* using column chromatography (Hiranrat and Mahabusarakam 2008). A stock solution of *R. tomentosa* extract and rhodomyrtonone was prepared by dissolving in 100% dimethyl sulphoxide (DMSO) and stored at -20°C until use. The stock plant solutions were diluted to appropriate final concentrations in cell culture medium. The final concentration of DMSO in the working solution was 0.4% which did not show any cytotoxic effect on fish macrophages.

Fish

O. mykiss weighing 150–200 g were obtained from a local fish farm, Trout Farm (Oliana, Spain). Fish were maintained in recirculating freshwater tanks, at 14°C , with a 12 h light per 12 h dark photoperiod. Fish were daily fed with a commercial diet (Trouw T6 Classics 3P, Trouw España, Spain). Dissolved oxygen ($<9\text{ mg L}^{-1}$), pH (6.0 – 8.5), nitrite ($<0.05\text{ mg L}^{-1}$), nitrate ($<1.0\text{ mg L}^{-1}$), and ammonia ($<0.07\text{ mg L}^{-1}$) were monitored along the whole experiment. Prior to experiments, fish were acclimatized to these conditions for 2 weeks, and observed during this period for any abnormal clinical sign. All experimental procedures involving fish were submitted and authorized by the Ethical Committee of the “Universitat Autònoma de Barcelona” that agrees with the International Guiding Principles for Biomedical Research Involving Animals (EU 2010/63).

Macrophage isolation and culture conditions

Fish were sacrificed by over-anesthetization with tricaine methane sulphonate (MS222, Sigma-Aldrich). Blood was removed from the caudal vein and HK were excised. HK macrophages were obtained according to the method described by Secombes (1990). Briefly, HK tissue was harvested aseptically and pushed through a nylon mesh ($100\ \mu\text{m}$) with 5 ml of RPMI medium (Gibco) containing 2% fetal bovine serum (FBS), 50 mg mL^{-1} gentamicin, 2 mg mL^{-1} amphotericin B, and 20 units mL^{-1} heparin. HK macrophages were isolated by centrifugation of the cell suspensions on a 34/51% discontinuous Percoll gradient (400 g for 30 min at 4°C). The cells banding in the Percoll interface were collected, washed, and adjusted the cell suspension to 2×10^7 viable cells mL^{-1} with RPMI medium containing 0.1% FCS, 50 mg mL^{-1} gentamicin, and 2 mg mL^{-1} amphotericin B. The resultant cell suspension was seeded in 96-well plate for the cytotoxicity and respiratory burst tests and on 6-well plate for the gene expression study and incubated overnight at 18°C in air containing 5% CO_2 . Purified plastic-adherent cells were cultured in fresh RPMI 1640 medium containing 5% FBS, 50 mg

mL⁻¹ gentamicin, and 2 mg mL⁻¹ amphotericin B for 24 h before use. For the cytotoxicity and respiratory burst activity assays six fish were used (n=6) and for gene expression analysis three fish were used (n=3).

Cytotoxicity assay

Evaluation of cytotoxic activity of *R. tomentosa* and rhodomyrton in fish cells was determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reduction assay (Sigma-Aldrich). The MTT assay is based on the cellular uptake of MTT and its subsequent reduction in the mitochondria of living cells to MTT formazan. Dead cells do not cause this change. Fish cells (2×10^7 cells per well) were seeded in 96-well tissue culture microtiter plates and allowed to adhere for 24 h under the cell culture conditions as described above. Subsequently, adherent cells were exposed to serial concentrations of *R. tomentosa* (2.5–400 $\mu\text{g mL}^{-1}$) or rhodomyrton (0.125–16 $\mu\text{g mL}^{-1}$), and its solvent (0.4% DMSO) and cultured for another 24 h. After incubation, the cells were gently washed with phosphate buffer solution (PBS) and new PBS was replaced into each well. Cell viability was assayed by adding 10 μL of MTT (5 mg mL⁻¹ in PBS) and incubated in the dark at 18 °C for 2 h. Thereafter, supernatant containing MTT was discarded and 100 μL of 100% DMSO were added to solubilize the intracellular formazan crystals. After thorough mixing, the absorbance was recorded at 570 nm using a microplate reader (PerkinElmer, Victor³). Control wells had the same volume of medium without the plant substances. All the experiments were run in triplicate wells for each condition (3 fish per experiment), and independently repeated at least three times.

Percent viability was defined at the ratio of absorbance between the test samples and the positive control using the formula:

$$\% \text{ viability} = (\text{Abs}_{\text{sample}} / \text{Abs}_{\text{control}}) \times 100$$

Treatment of macrophages with *R. tomentosa* leaf extract and rhodomyrton

Two experiments were done; in the first experiment HK macrophages (2×10^7 cells per well) were exposed to *R. tomentosa* (10 $\mu\text{g mL}^{-1}$) or rhodomyrton (1 $\mu\text{g mL}^{-1}$) and sampled after 4 and 24 hours of exposure. These concentrations were chosen regarding the cytotoxicity results. To gain insight into the immunomodulatory effects of the natural plant products in relation with pathogens, a second experiment was carried out in HK macrophages exposed to *R. tomentosa* plus LPS or rhodomyrton plus LPS for 4 and 24 h. The concentration of LPS (5 $\mu\text{g mL}^{-1}$) from *Salmonella typhimurium* (Sigma-Aldrich) was chosen based on previous studies showing that this concentration generates an inflammatory response in HK macrophages

(Fierro-Castro et al. 2012; Fierro-Castro et al. 2013). *R. tomentosa* and rhodomyrton concentrations were chosen based on the results of experiment 1. Unexposed cells were used as control groups. The assays were set up for use in gene transcription analysis as described below. The experiments were carried out in triplicate wells (3 fish per experiment), and independently repeated three times.

Gene transcription analysis

Total RNA from HK macrophages was extracted using TRI reagent (Sigma-Aldrich) following the manufacturer's instructions. Purity and concentration of total RNA were measured spectrophotometrically using a NanoDrop Reader (Thermo Fisher Scientific, USA). The verification of RNA integrity was assessed by running an aliquot of the sample pre-stained with bromophenol blue on a 1% agarose gel. One microgram of RNA was used as a template for cDNA synthesis, using iScriptTM cDNA Synthesis Kit (Bio-Rad, USA). The resultant cDNA was stored at -20 °C until use. The GenBank identification and primer sequences are shown in Table 1.

PCR amplification was assayed with the SYBR green method using a iQ5 iCycler thermal cycler (Bio-Rad). The reactions were set up in a 384-well plate according to manufacturer's procedures consisting of 1 µL of cDNA template (1:10 dilution), 5 µL of iTaqTM Universal SYBR Green Supermix (Bio-Rad), 3.6 µL of Milli-Q water, and 0.2 µL of forward and reverse primer (0.1 mM). PCR cycling conditions were 95 °C for 10 s followed by 60 °C for 30 s with an initial denaturation step of 95 °C for 3 min. Fluorescence signal output was monitored at the end of each cycle for 40 cycles. All amplification reactions were run in triplicate for each sample. The relative expression ratios of target gene were calculated using the comparative threshold cycle (Ct) method (Pfaffl 2001). PCR amplification efficiencies (E) for each primer pair were assessed using a five-fold dilution series of pool cDNA. The efficiency was analyzed following the equation: $E = 10^{(-1/s)}$, where s is the slope generated from the serial dilutions of target nucleic acid, when Log dilution is plotted against Ct values. The expression of target genes was normalized against a reference gene (*efla*) following the equation:

$$\text{Ratio} = (E_{\text{target}})^{\Delta C_{\text{t}}^{\text{target}}(\text{control-sample})} / (E_{\text{ref}})^{\Delta C_{\text{t}}^{\text{ref}}(\text{control-sample})}$$

where E_{target} is E of the target gene transcript, E_{ref} is E of a reference gene transcript, $\Delta C_{\text{t}}^{\text{target}}$ is crossing point (CP) deviation of control-sample of target gene transcript, and $\Delta C_{\text{t}}^{\text{ref}}$ is CP deviation of control-sample of reference gene transcript.

Reactive oxygen species (ROS) production

Intracellular ROS production of the fish cells exposed to the plant products was investigated by nitroblue tetrazolium (NBT) reduction assay (Chung and Secombes 1988). In brief, cell monolayers were washed twice with HBSS and 100 μL of *R. tomentosa*; (10, 50, and 100 $\mu\text{g mL}^{-1}$) or rhodomyrtone (1, 2, and 4 $\mu\text{g mL}^{-1}$) dissolved in RPMI medium plus 1 mg mL^{-1} of NBT was added and incubated in the dark at 18 °C under 5% CO_2 for 1 h. Thereafter, the supernatant was removed and the cellular pellet was fixed in methanol (Merck) for 15 min and then washed with 70% methanol. After fixation, the resultant formazan product was solubilized in 120 μL of 2 M KOH and 140 μL of DMSO with thorough mixing. The experiments were repeated two times and the results in each test were based on the mean of three fish per replicate. The absorbance for each sample solution was measured at 620 nm using a microplate reader. The index of exposed cells was calculated using the formula:

$$\text{ROS production (\%)} = [(\text{sample-blank})/(\text{control-blank})]-1 \times 100$$

Statistical analyses

Statistical analyses were performed using SPSS statistical software version 16 (SPSS Inc, IBM, Chicago, USA). Toxicity data was analyzed by one-way ANOVA using Tukey's test for multiple comparisons. For all the other parameters a two-way ANOVA was done considering, as main factors treatment and time. Statistical significance was assumed when $P < 0.01$ or $P < 0.05$.

Results

Cytotoxicity of *R. tomentosa* leaf extract and rhodomyrtone on HK macrophages

The results demonstrated that *R. tomentosa* and rhodomyrtone did not show any significant cytotoxic effect against HK macrophages at concentrations lower than 25 and 1 $\mu\text{g mL}^{-1}$, respectively ($P < 0.05$) (Fig. 1). Meanwhile, *R. tomentosa* at 50 $\mu\text{g mL}^{-1}$ and rhodomyrtone at 2 $\mu\text{g mL}^{-1}$ were slightly toxic (cell viability < 80%) even though there was not statistically significant difference in cell viability between control and treated groups. The plant products exhibited cytotoxicity at higher concentrations in a concentration-dependent manner.

Effects of *R. tomentosa* leaf extract and rhodomyrtone on the expression of immune-related genes

In this study, the variation of immune-related genes was determined in HK macrophages after exposure to 10 $\mu\text{g mL}^{-1}$ of *R. tomentosa* and 1 $\mu\text{g mL}^{-1}$ of rhodomyrtone for 4 and 24 h. Regarding pro-inflammatory cytokines (*il1 β* , *il8*, and *tnfa*) gene expression, the HK macrophages exposed to *R. tomentosa* for 4 h presented

significant upregulation of *il1 β* (Fig.2) ($P < 0.01$). Meanwhile, exposure to rhodomyrtone for 4 h caused a significant upregulation of *il1 β* , *il8*, and *tnf α* ($P < 0.01$).

The expression of immune-suppressing cytokines, *il10* and *tgf β* in HK macrophages was not modified when exposed to *R. tomentosa* for 4 and 24 h in comparison with the control group (Fig. 3). Of the parallel analysis, the mRNA level of these anti-inflammatory cytokines was not significantly changed after exposure to rhodomyrtone for 4 h as seen in *R. tomentosa* treated groups. However, after 24 h, increased expression of both *il10* and *tgf β* was detected in the fish cells ($P < 0.05$).

With respect to immune genes related to inducible enzymes, *inos*, *cox2*, and *arginase*, exposure of HK macrophages to *R. tomentosa* for 4 and 24 h had no significant effect on the alteration of these genes (Fig. 4). Treatment of fish cells with rhodomyrtone for 4 h did not modify the expression of *inos*, *cox2*, and *arginase* expression, whilst treatment with the compound for 24 h resulted in significant upregulation of all the genes ($P < 0.01$).

In terms of genes influencing acute phase protein (*saa*), antimicrobial peptide (hepcidin), and antioxidant enzymes (*nadph* and *gpx1*), *R. tomentosa* failed to alter the expression of the target genes in HK macrophages after 4 and 24 h exposure (Fig. 5). On the other hand, the transcription of those genes was also unaffected by exposure to rhodomyrtone for 4 and 24 h with the exception of *gpx1*. The expression of *gpx1* was remarkably upregulated in response to rhodomyrtone after 24 h exposure ($P < 0.01$).

To gain insight into the immunomodulatory effects of the natural plant products in relation with pathogen contamination in aquatic ecosystems, the gene expression associated with innate immune response in HK macrophages exposed to a combination of *R. tomentosa* or rhodomyrtone and LPS was investigated. The results demonstrated that exposure to *R. tomentosa* plus LPS caused a significant decrease in the expression of *il1 β* , *il8*, *inos*, and *hepcidin* after 4 h ($P < 0.01$), and then 24 h exposure led to a reduction in the expression of *il1 β* , *il8*, *saa*, and *gpx1*, compared with LPS alone ($P < 0.01$) (Fig. 6). The combined treatment with rhodomyrtone and LPS led to a significant reduction in the expression of *il1 β* , *inos*, and *hepcidin* after 4 h exposure and the expression of *saa*, *hepcidin*, and *gpx1* after 24 h exposure ($P < 0.01$), but resulted in a synergistic upregulation of *tnf α* after 4 and 24 h exposure in comparison with LPS alone ($P > 0.05$).

Effects of *R. tomentosa* leaf extract and rhodomyrtone on ROS production

Concerning intracellular ROS production (superoxide anion), after exposure to *R. tomentosa* (10, 50, 100 $\mu\text{g mL}^{-1}$) and rhodomyrtone (1, 2, and 4 $\mu\text{g mL}^{-1}$) for 1 h, both plant products inhibited NBT reduction in

the HK macrophages, thus showing a significant decrease in absorbance with increasing concentrations when compared with the control (0.4% DMSO) ($P < 0.05$) (Fig. 7). Likewise, the addition of the antioxidant enzyme superoxide dismutase (SOD) (300 U mL^{-1}), to HK macrophages showed a significant decrease in NBT reduction rate ($P < 0.05$).

Discussion

Currently, there has been growing interest in medicinal plants and phytochemical compounds derived from plants as enhancers of the fish immune response against fish pathogens in aquaculture as an alternative to chemicals (Dügenci et al. 2003; Galina et al. 2009; Vaseeharan and Thaya 2014). The understanding of the mechanisms behind the effects of medicinal plants on fish immune response at molecular level are undoubtedly helpful for proposing appropriate applications in fish farming. In this work, the immunomodulatory potential of both natural plant products, the ethanol extract of *R. tomentosa* and its active compound, rhodomyrtone was determined focusing on the mRNA levels of innate immune-related genes in macrophages of rainbow trout and on the functional response of ROS production.

Initially, the cytotoxicity of *R. tomentosa* and rhodomyrtone was preliminary tested on the HK macrophages within a concentration range based on their cytotoxicity on mammalian monocytes/macrophages (Jeong et al. 2013; Srisuwan et al. 2014). The results revealed that doses of *R. tomentosa* that have no cytotoxic effects on HK macrophages were lower than $25 \mu\text{g mL}^{-1}$ in which the solvent (0.4% DMSO) showed absence of cytotoxicity. Previously, Jeong et al (2013) reported no cytotoxic effects of *R. tomentosa* methanol extract on a murine macrophage cell line (RAW264.7 cells) at 2-fold to 16-fold higher than in the present study. Rhodomyrtone did not show any cytotoxic effect on the fish HK macrophages at concentrations less than $1 \mu\text{g mL}^{-1}$, as opposed to a study demonstrating rhodomyrtone toxicity ($1.56 \mu\text{g mL}^{-1}$) on human monocytes (THP-1) (Srisuwan et al. 2014).

Exposition of HK macrophages to *R. tomentosa* resulted in a noticeable upregulation of pro-inflammatory cytokine, *il1 β* gene expression after 4 h, while the fish cells exposed to rhodomyrtone showed an increase in the mRNA levels of *il1 β* , *il8*, and *tnfa* genes. In fish, pro-inflammatory cytokines such as IL1 β , IL8, and TNF α are key players in immune response, stimulating macrophage activation and enhancing the synthesis of inflammatory proteins responsible for antimicrobial defenses and inflammatory reaction (Secombes et al. 2001). Thus, the specific enhancement of the gene expression by the plant substances might promote the activity of the fish macrophages leading to early protective immunity during diseases. In line with our study, Chi et al (2016) observed an upregulation of *il1 β* and *tnfa* mRNA levels in HK macrophages of grass carp (*Ctenopharyngodon idella*) after exposure to a phytochemical compound extracted from *Dryopteris crassirhizoma*. Furthermore, *in vivo* administration of *Ficus carica* polysaccharide induced an increase in the mRNA levels of *il1 β* and *tnfa*, thereby providing resistance to *Flavobacterium columnare* infection in grass carp

(Yang et al. 2015). Reyes-Becerril et al (2013) observed modulatory effects on *il1 β* and *il8* gene expression of gilthead sea bream (*Sparus aurata*) after diet supplementation with lyophilized microalgae *Navicula* sp. Besides, studies with mammalian cells have shown that natural compounds such as anethol, capsicum oleoresin, carvacrol, eugenol, garlicon, and cinnamaldehyde augmented IL1 β and TNF α protein secretion of weaned pig macrophages (Liu et al. 2012), possibly indicating a stimulatory effect of the phytochemical compounds on pro-inflammatory cytokine gene expression in immune cells.

IL10 and TGF β are immunosuppressive cytokines responsible for anti-inflammatory reactions thus restricting the inflammatory response to minimize host damage (Zou and Secombes 2016). In the present study, either *il10* or *tgfb* were unaltered in HK macrophages exposure to *R. tomentosa*. On the other hand, *il10* or *tgfb* mRNA transcriptional levels were obviously increased following rhodomirtone exposure for 24 h. The presence of anti-inflammatory cytokines served as an important negative feedback mechanism to control the expression of pro-inflammatory cytokines, thus balancing stimulatory and inhibitory protein signaling pathways involved in immune responses. Other authors have reported that a recombinant form of IL10 (rgIL10) attenuated the mRNA expression of *tnfa1*, *tnfa2*, *il1 β 1*, *cxcl8*, and *nadph* oxidase component in heat-killed *Aeromonas salmonicida* activated-monocytes of goldfish (*Carassius auratus*) (Grayfer et al. 2011). Likewise, the inhibitory effects of a recombinant TGF β 1 on the expression of *tnfa*, *il1 β* , *il8*, and *inos* via NF- κ B signaling by blocking I κ B α (gcI κ B α ; I κ B inhibitor) protein secretion in LPS-stimulated macrophages of grass carp have been reported (Wei et al. 2015). Therefore, in the present study, rhodomirtone might have positive effects on fish immune response to maintain balance and prevent injuries caused by the over expression of pro-inflammatory cytokines.

To further address the immunomodulatory effects of *R. tomentosa* and rhodomirtone on innate immune defense, we examined the expression patterns of genes involved in inducible enzymes (*inos*, *cox2*, and *arginase*), *saa*, *hepcidin*, and antioxidant enzymes (*nadph* and *gpx1*) of rainbow trout HK macrophages. In the present study we observed that *R. tomentosa* had no effect on mRNA abundance of all inducible enzymes and antioxidant enzymes, whereas rhodomirtone obviously upregulated the expression of *inos*, *cox2*, and *arginase* including *gpx1* gene in the fish cells after 24 h exposure. This data supports the findings on THP-1 human macrophages exposed to rhodomirtone, which have shown the potential of this compound to induce nitric oxide production through enhanced expression of the *inos* gene (Srisuwan et al. 2014). In addition, the immunomodulatory effects of ginsan (*Panax ginseng*) by increasing *inos* mRNA level and other major pro-inflammatory cytokines (e.g. *il1 β* , *il6*, and *tnfa*) in comparison with LPS/IFN γ stimulation in murine peritoneal macrophages have been reported (Song et al. 2002). In animal models, ginger (*Zingiber officinale*) has been

found to possess an antioxidant protective efficacy for chemoprevention of liver diseases by enhancing mRNA expression of antioxidant genes such as *gpx1*, *gstA1* (glutathione S transferase $\alpha 1$), and *cat* (catalase) in rats (Mohamed et al. 2015).

We found that after co-exposition of HK macrophages to *R. tomentosa* and LPS, mRNA levels of *ill β* , *il8*, *inos*, *tnfa*, *saa*, *hepcidin*, and *gpx1* were dramatically down-regulated, pointing out the anti-inflammatory properties of this plant on the fish immune cells. Previous studies with mammalian cells indicated that pro-inflammatory cytokine production such as IL1 β , IL6, and TNF α can be inhibited by quercetin, a flavonoid found in many plants when co-exposed to immunostimulators (e.g. LPS, phorbol 12-myristate 13- acetate) (Cho et al. 2003; Nair et al. 2006). Quercetin acts as an anti-oxidant and anti-inflammatory agent by reducing the phosphorylation and activation of ERK and p38 MAP kinases and inhibiting NF- κ B system through the I κ B kinase complex, thus blocking pro-inflammatory cytokines and *inos* expression (Cho et al. 2003). Intriguingly, this compound has also been detected in the methanol extract of *R. tomentosa* (Jeong et al. 2013). Analogous results observed in murine macrophages exposed to *R. tomentosa* demonstrated that the plant extract suppressed the activation of NF- κ B pathway via Syk/Src and AP-1 pathway via IRAK1/IRAK4 signaling, responsible for nitric oxide and prostaglandin E2 productions (Jeong et al. 2013).

In co-exposition to rhodomyrtone and LPS, the plant compound noticeably induced pro-inflammatory gene expression, *tnfa* in HK macrophages, indicating a synergistic effect between both stimuli, while the mRNA levels of other pro-inflammatory and immune-related genes including *ill β* , *inos*, *saa*, *hepcidin*, and *gpx1* were down regulated. Conversely, in human macrophages, rhodomyrtone did not show any stimulatory effects on *ill β* and *tnfa* expression, but this compound synergistically enhanced other pro-inflammatory mediators such as *il6* and *inos* in THP-1 human monocytes exposed to heat-killed multidrug-resistant *Staphylococcus aureus* (Srisuwan et al. 2014). These contradictory results might be attributed to species differences, co-stimulating sources, and sampling times. In other fish cells, elevated gene expression of *tnfa* with other pro-inflammatory mediators (e.g. *ill β* , *cox2*) was found in LPS-activated sea bream fibroblasts during co-incubation with *Aloe arborescens* leaf extract (Picchiatti et al. 2013).

In line with our findings in other animals, oleifolioside A, a triterpenoid compound from *Dendropanax morbifera* was found to attenuate the expression of *inos* and *cox2* gene in LPS-activated murine macrophage (Yu et al. 2012). Other authors have reported the anti-inflammatory effects of a flavonoid antioxidant silymarin, which depressed the level of *inos* gene transcription and its protein expression in LPS-induced murine macrophages (Kang et al. 2002). The extracts of *Echinacea pallida* (Zhai et al. 2009), *Grindelia robusta*, *Salix*

nigra, *Arnica montana*, and *Quassia amara* (Verma et al. 2010) suppressed the production of iNOS and COX2 protein in LPS-stimulated macrophages. Recently, an *in vivo* study revealed that feeding rats with *Cichorium intybus* root extract caused increase in oxidative stress and hepatotoxicity resulting in suppression of the antioxidant gene transcripts, *gpx1*, *cat*, and *pon1* (El-Sayed et al. 2015). There was no information about the effect of natural products on *saa* gene transcription, but a reduction in SAA protein in activated-mouse liver cells by colchicine, a secondary metabolite originally isolated from plants of genus *Colchicum*, has been reported (Tatsuta et al. 1984).

Our results suggest that *R. tomentosa* and rhodomyrtonone can inhibit the production of superoxide anion in HK macrophages of rainbow trout. Respiratory burst is a vital mechanism of innate immune defense that occurs in phagocytes, in particular neutrophils and macrophages (Neumann et al. 2001). Superoxide anion is one of the reactive respiratory burst activation byproducts that exert antimicrobial actions against invading pathogens and also play a cytotoxic role in the development of inflammatory response. Superoxide dismutase is an antioxidant enzyme that removes superoxide anion. In this study, it was expected that both *R. tomentosa* and rhodomyrtonone would induce SOD production by regulating the expression of *sod* gene in the fish cells. This might be a protective mechanism to avoid cell damage caused by oxidative stress during upregulation of cytokines and other immune mediators involved in inflammatory response under immunostimulation with the plant substances. A previous study has reported protection against ischemia in rats by upregulation of gene expression of antioxidant enzymes (*cat*, *gpx*, and *sod*), while increasing pro-inflammatory cytokine genes (*il1 β* , *il6*, and *tnfa*) during myocardial ischemia/reperfusion process in animal models (Chandrasekar et al. 1997). Similar results were observed in mouse peritoneal macrophages following intraperitoneal administration of *Hippophae salicifolia* bark extract (Chakraborty et al. 2016). This plant extract also prevents oxidative DNA damage, lipid peroxidation, and reduces glutathione in the mouse macrophages.

Conclusions

The present results show, for the first time in fish, the intrinsic immunomodulatory effects of the natural plant products, *R. tomentosa* and rhodomyrtonone on the expression of genes related to innate immune response in rainbow trout HK macrophages by inducing the mRNA levels of certain pro-inflammatory cytokines, anti-inflammatory cytokines, inducible enzymes, and antioxidant enzymes. In LPS-triggered HK macrophages, *R. tomentosa* and rhodomyrtonone possessed an anti-inflammatory effect by acting as an inhibitor of the gene expression of pro-inflammatory cytokines, acute phase proteins, and oxidant enzymes. In addition, both

plant substances significantly decreased the production of ROS in the fish cells. According to their pronounced effects, this study expands the information on the phyto-immunomodulatory effects of specific plant products as possible enhancers of fish immunity, being valuable useful scientific data and a potential tool for further utilization in aquaculture.

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Table 1 Primers used for mRNA analysis by real-time PCR

Gene name	Acronym	GenBank accession	Forward	Reverse
Interleukin 1 β	<i>il1β</i>	AJ223954	GGATTCACAAGAACTAAGGAC	ACTGTGATGTACTGCTGAAC
Interleukin 8	<i>il8</i>	AJ279069	GAATGTCAGCCAGCCTTGTC	TCCAGACAAATCTCCTGACCG
Tumor necrosis factor- α	<i>tnfa</i>	AJ277604	AGCATGGAAGACCGTCAACGAT	ACCCTCTAAATGGATGGCTGCTT
Interleukin 10	<i>il10</i>	AB118099	CGACTTTAAATCTCCCATCGAC	GCATTGGACGATCTCTTTCTT
Transforming growth factor beta	<i>tgfβ</i>	OMY7836	CTCACATTTTACTGATGTCACTTCCTGT	GGACAACCTGCTCCACCTTGIG
Inducible nitric oxide synthase	<i>inos</i>	AJ295231	CCAACCATGCACATCAAAGTT	CCTGAGGTAGGATTTCAAGAGTAGAAA
Cyclooxygenase 2	<i>cox2</i>	AJ238307	ATCCTTACTCACTACAAAGG	GCTGGTCCTTTCATGAAGTCTG
Arginase	<i>arginase</i>	AY056477	TGGGAGCCGGACACACTAC	GCCTTCCACTGAACCAATGG
Serum amyloid A	<i>saa</i>	AM422447	GGTGAAGCTGCTCAAGGTGCTAAAG	GCCATTACTGATGACTGTTGCTGC
Hepcidin	<i>hepcidin</i>	CA369786	GCTGTTCCCTTCTCCGAGGTGC	GTGACAGCAGTTGCAGCACCA
Nicotinamide adenine dinucleotide phosphate oxidase	<i>nadh</i>	AB192465.1	CATCGCCCACCTGTTTAACT	GTATGACCTGCGGATGACCT
Glutathione peroxidase 1	<i>gpx1</i>	BG934453	GATTCGTTCCAAACTTCCTGCTA	GCTCCAGAACAGCCTGTTG
Elongation factor-1 α	<i>ef1α</i>	AF498320	CAAGGATATCCGTCGTGGCA	ACAGCGAAACGACCAAGAGG

Figure legends

Fig. 1 Cytotoxicity of *Rhodomyrtus tomentosa* leaf extract (RT) and rhodomyrtone (RD) on rainbow trout head kidney macrophages. Control cells were exposed to medium containing 0.4% DMSO. Results were expressed as mean values of triplicate independent experiments \pm SD (n = 3 biological replicates). Single asterisks indicate significant differences from 0.4% DMSO (Control) at $P < 0.05$ (*), $P < 0.01$ (**). There were no significant difference between non treatment (100% viability) and DMSO treatment

Fig. 2 Relative mRNA expression levels of interleukin 1 β (*il1 β*), interleukin 8 (*il8*), and tumour necrosis factor- α (*tnf α*) in rainbow trout head kidney (HK) macrophages exposed to *Rhodomyrtus tomentosa* leaf extract (RT; 10 $\mu\text{g mL}^{-1}$) and rhodomyrtone (RD; 1 $\mu\text{g mL}^{-1}$) alone or combined with LPS (5 $\mu\text{g mL}^{-1}$) for 4 and 24 h. Bars represent the mean \pm SD of relative mRNA expression as a fold change relative to elongation factor 1 α (*ef1 α*) (n = 3 biological replicates). Significant differences are: * vs. control (* $P < 0.05$, ** $P < 0.01$), \blacktriangle vs. difference between 4 and 24 h ($\blacktriangle P < 0.05$, $\blacktriangle\blacktriangle P < 0.01$)

Fig. 3 Relative mRNA expression quantities interleukin 10 (*il10*) and transforming growth factor beta (*tgfb*) in rainbow trout head kidney (HK) macrophages exposed to *Rhodomyrtus tomentosa* leaf extract (RT; 10 $\mu\text{g mL}^{-1}$) and rhodomyrtone (RD; 1 $\mu\text{g mL}^{-1}$) alone or combined with LPS (5 $\mu\text{g mL}^{-1}$) for 4 and 24 h. Bars represent the mean \pm SD of relative mRNA expression as a fold change relative to elongation factor 1 α (*ef1 α*) (n = 3 biological replicates). Significant differences are: * vs. control (* $P < 0.05$, ** $P < 0.01$), \blacktriangle vs. difference between 4 and 24 h ($\blacktriangle P < 0.05$, $\blacktriangle\blacktriangle P < 0.01$)

Fig. 4 Relative mRNA expression quantities of nitric oxide synthase (*inos*), cyclooxygenase-2 (*cox2*) and arginase in rainbow trout head kidney (HK) macrophages exposed to *Rhodomyrtus tomentosa* (RT; 10 $\mu\text{g mL}^{-1}$) and rhodomyrtone (RD; 1 $\mu\text{g mL}^{-1}$) alone or combined with LPS (5 $\mu\text{g mL}^{-1}$) for 4 and 24 h. Bars represent the mean \pm SD of relative mRNA expression as a fold change relative to elongation factor 1 α (*ef1 α*) (n = 3 biological replicates). Significant differences are: * vs. control (* $P < 0.05$, ** $P < 0.01$), \blacktriangle vs. difference between 4 and 24 h ($\blacktriangle P < 0.05$, $\blacktriangle\blacktriangle P < 0.01$)

Fig. 5 Relative mRNA expression quantities of serum amyloid A (*saa*), hepcidin, NADPH oxidase (*nadph*), and glutathione peroxidase 1 (*gpx1*) in rainbow trout head kidney (HK) macrophages exposed to *Rhodomyrtus*

tomentosa leaf extract (RT; 10 $\mu\text{g mL}^{-1}$) and rhodomyrtone (RD; 1 $\mu\text{g mL}^{-1}$) alone or combined with LPS (5 $\mu\text{g mL}^{-1}$) for 4 and 24 h. Bars represent the mean \pm SD of relative mRNA expression as a fold change relative to elongation factor 1 α (*efl1 α*) (n = 3 biological replicates). Significant differences are: * vs. control (0.4% DMSO) (* P < 0.05, ** P < 0.01), \blacktriangle vs. difference between 4 and 24 h ($\blacktriangle P$ < 0.05, $\blacktriangle\blacktriangle P$ < 0.01)

Fig. 6 Relative mRNA expression quantities of interleukin 1 β (*il1 β*), interleukin 8 (*il8*), tumour necrosis factor- α (*tnf α*), interleukin 10 (*il10*), transforming growth factor beta (*tgf β*), inducible nitric oxide synthase (*inos*), cyclooxygenase-2 (*cox2*), arginase, serum amyloid A (*saa*), hepcidin, NADPH oxidase (*nadph*), and glutathione peroxidase 1 (*gpx1*) in rainbow trout head kidney (HK) macrophages exposed to *Rhodomyrtus tomentosa* leaf extract (RT; 10 $\mu\text{g mL}^{-1}$) and rhodomyrtone (RD; 1 $\mu\text{g mL}^{-1}$) alone or combined with LPS (5 $\mu\text{g mL}^{-1}$) for 4 and 24 h. Bars represent the mean \pm SD of relative mRNA expression as a fold change relative to elongation factor 1 α (*efl1 α*) (n = 3 biological replicates). Significant difference are * vs. control (* P < 0.05, ** P < 0.01), \blacktriangle vs. LPS ($\blacktriangle P$ < 0.05, $\blacktriangle\blacktriangle P$ < 0.01)

Fig. 7 Percent of NBT reduction in rainbow trout HK macrophages after exposed to *R. tomentosa* leaf extract (RT; 10, 50, and 100 $\mu\text{g mL}^{-1}$) and rhodomyrtone (RD; 1, 2, and 4 $\mu\text{g mL}^{-1}$) for 1 h. Results were expressed as mean values of duplicate independent experiments \pm SD (n = 3 biological replicates). The cells treated with SOD (300 U mL^{-1}) were used as a positive control. Single asterisks indicate significant differences from 0.4% DMSO (Control) at P < 0.05 (*), P < 0.01 (**). There were no significant difference between non treatment (100% NBT reduction) and DMSO treatment

Fig. 1

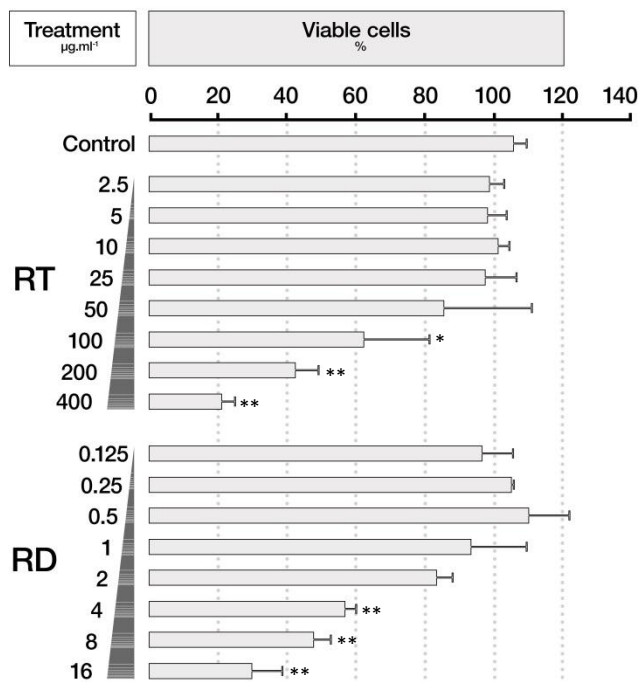


Fig. 2

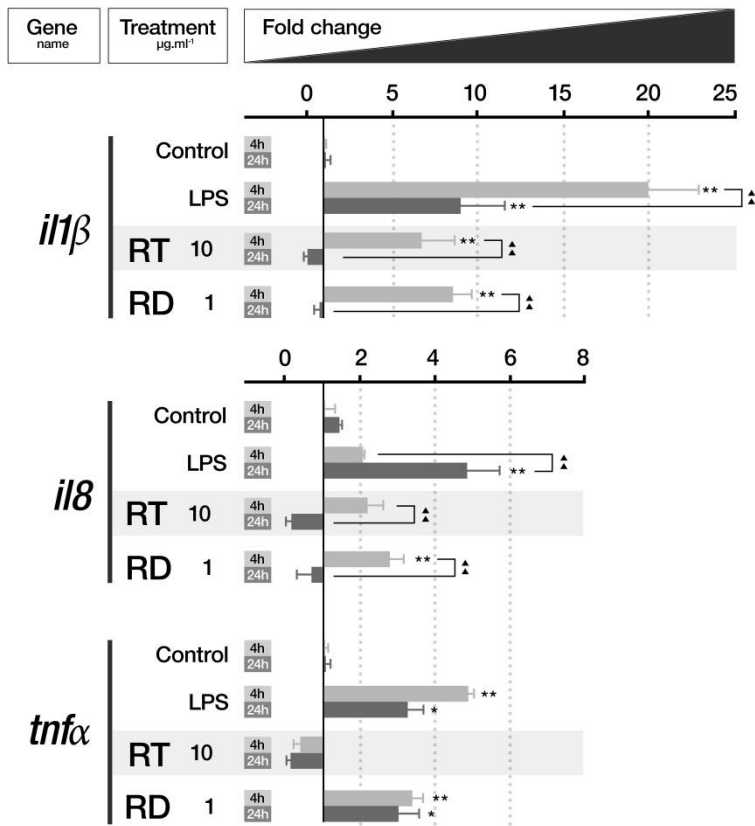


Fig. 3

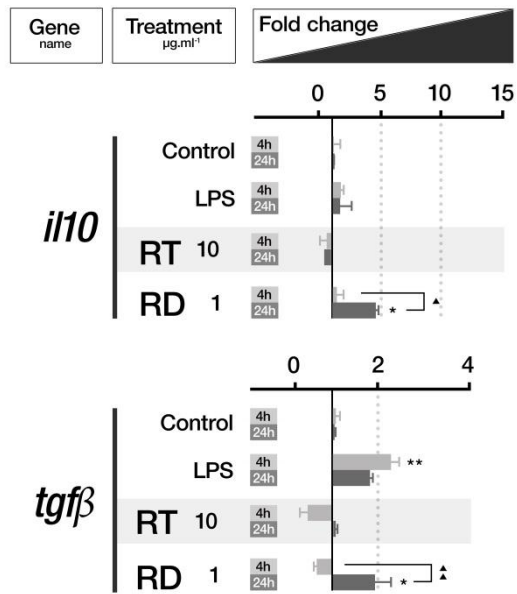


Fig. 4

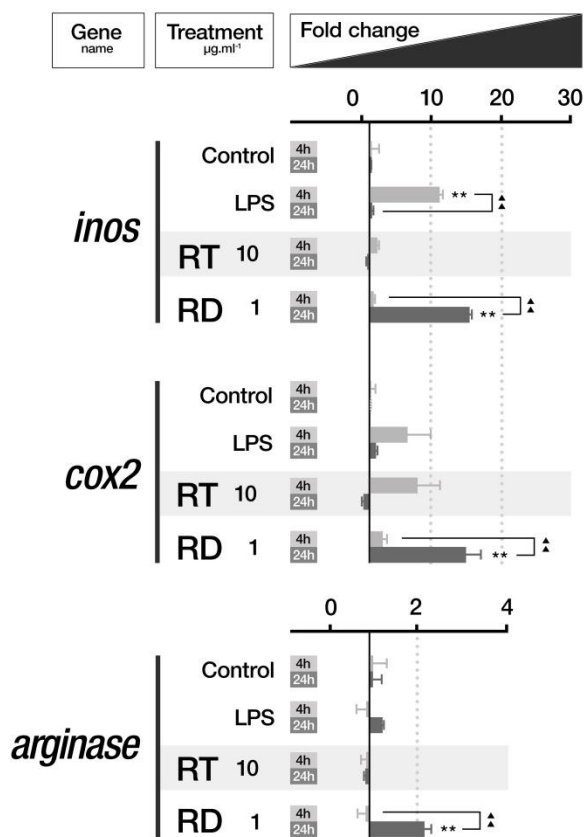


Fig. 5

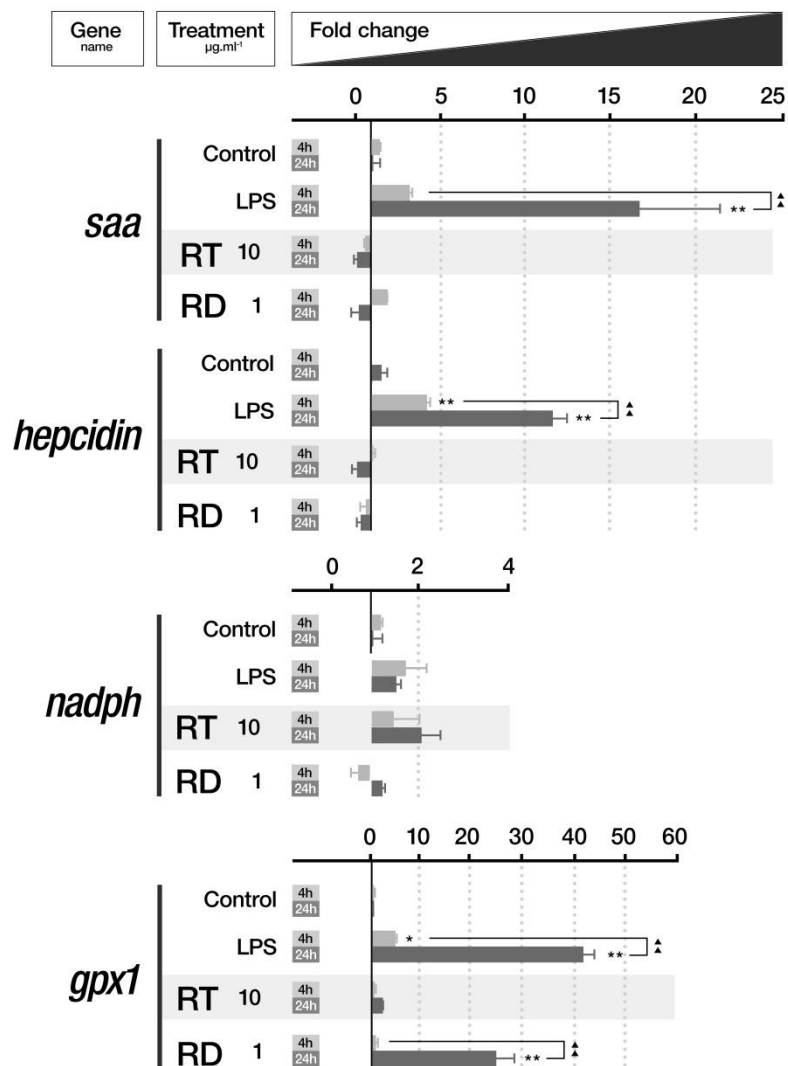


Fig. 6

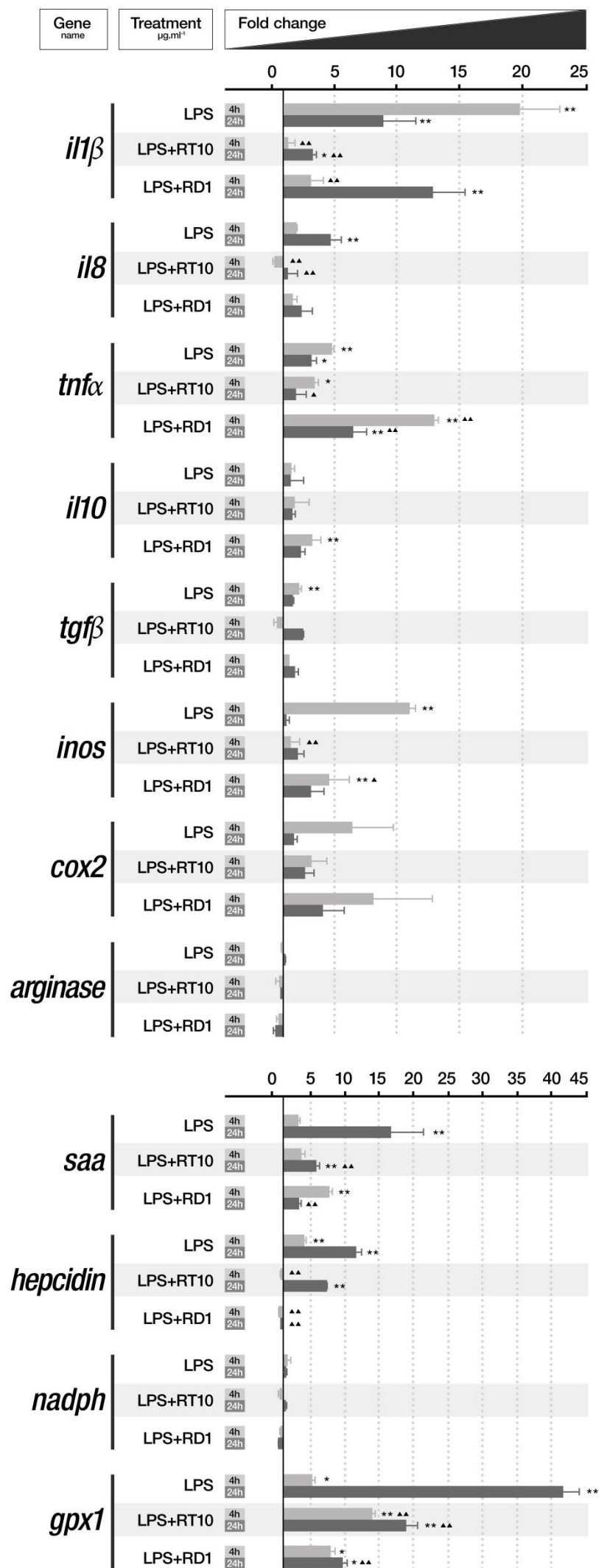


Fig. 7

