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Effects of dihydroquercetin obtained from deodar (*Cedrus deodara*) on immune status of gilthead seabream (*Sparus aurata* L.)

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ABSTRACT

The use of medicinal plants as prophylactic method in fish is considered safe and a very promising alternative to the use of chemicals in aquaculture practices. The prospective mode of action of dihydroquercetin, fraction of the medical plant deodar (*Cedrus deodara),* was evaluated on immune status of gilthead seabream (*Sparus aurata* L.). Fish were divided into 4 groups before being fed for 14 days with commercial diets supplemented with 0% (control), 0.1%, 0.5% and 1% of dihydroquercetin. Cellular (phagocytosis and respiratory burst activities) and humoral (seric complement activity, antiprotease, total protein, peroxidase, bactericidal activity and IgM level) immune parameters were investigated. The results recorded enhancement in all the tested parameters and in all the dihydroquercetin supplemented groups compared to the control. Interestingly, the fish received the lowest dose of dihydroquercetin (0.1%) showed a highly significant difference ($p < 0.05$) in phagocytosis, respiratory burst, IgM level, total protein, complement, antiprotease and bactericidal activities compared to the control. Direct effect of different doses of dihydroquercetin on head-kidney leucocytes was also studied in a previous *in vitro* assay. Again, the lowest doses tested provoked the highest immune cellular activities, where, the highest phagocytic and respiratory bust activities were recorded in leucocytes incubated with 0.025% and 0.0125% doses, respectively. Therefore, the results suggest that low concentrations of dihydroquercetin as food supplements are able to increase the immune status of gilthead seabream.

1. Introduction

Diseases problem in fish farms are dramatically increased as a result of the rapid expansion and high stocking density. The use of antibiotics and other chemotherapeutics to control diseases may led to the development of drug resistant pathogens, environmental pollution and accumulation of residues in fish [1,2]. For this reason, alternative methods using safe natural products have to be explored. In this sense, the use of entire plants or plant products have started to study in fish since few years ago. It is well known that many active plant compounds exert potential immunostimulating activity like alkaloids, terpenoids, flavonoids, quinones glycosides, and phenolic compounds [3,4]. Such those antioxidant compounds work as protective agents that inactivate reactive oxygen species and thus delay or prevent oxidative damage, therefore playing major roles in the prevention of diseases [5]. Furthermore, its use in fish could reduce costs of treatments and be more environmentally friendly as they tend to be more biodegradable than synthetic compounds [6,7]. Therefore, there is a great direction toward using medical plants and its active ingredients as stimulants in order to enhance the immune system of farmed fish and elevate their resistance to infectious diseases [1,2,8–11]. Moreover, besides the plant or the substance itself, the way of administration and the duration of the application are considered as important factors that vary with different compounds and different fish species [12,13] for this reason, much works are needed in this field.

Deodar, *Cedrus deodara* (Roxb.), belonging to the family Pinaceae, is one of the most important medicinal plant which has been used since ancient days in Ayurvedic medical. For example, oil and extracts of this plant are used in treatment of patients from inflammations, dyspepsia, insomnia, cough, fever, urinary discharges,

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bronchitis, itching, elephantiasis, tuberculous glands, leucoderma, ophthalmia, plies, disorders of the mind, diseases of the skin and of the blood, among others [14,15]. Additionally, different parts of the plant have also particular uses: wood extract has been used to treat flatulence, rheumatism, piles, kidney stone, pulmonary and urinary disorder; bark extract is used as astringent and also useful for treating fever, diarrhoea and dysentery [16]. Interestingly, the bark of *C. deodara* contains large amounts of a flavonoid compound known as dihydroquercetin.

Dihydroquercetin, also known as taxifolin, is a flavonoid compound commonly found in many vegetables, fruits and plants. Weidmann [17] showed evidence for a therapeutic promise of dihydroquercetin in major disease states such as cancer, cardiovascular disease and liver disease by reviewing the proposed mechanism(s) of action. Moreover, previous available data shown to exhibit anti-inflammatory effects in protection against oxidative cellular injury in rat peritoneal macrophages [18] and human endothelial cells [19]. Also, oral administration of dihydroquercetin caused decrease in mortality of white mice infected with influenza virus [20]. Injection with dihydroquercetin, shown effectiveness in treatment of atopic dermatitis in mice by preventing the production of inflammatory cytokines and by reducing skin inflammation [21]. Interestingly, a drug known as Ascovertin in Russia (a complex of dihydroquercetin and vitamin C) is a popular treatment for many health conditions that share oxidative stress as an underlying mechanism. It showed improvement in memory, mental performance, relieved vertigo, normalized sleep, and relieved headaches in patients [22]. To the best of our knowledge, the effect of dihydroquercetin as a possible immunostimulant for fish has not been studied yet. Therefore, the current study aims to examine the prospective effect of dihydroquercetin (previously isolated from *C. deodara*) on the immune system of gilthead seabream (*Sparus aurata* L.) with a consider of the effective dose, which could use in fish farms, in order to check its effectiveness to prevent/or treat the fish disease.

2. Materials and methods

2.1. Plant extraction and isolation

The aerial parts of *C. deodara* were purchased as dried material from a local market (Saudi Arabia). One kilogram of dried plant powder of *C. deodara* Roxb was extracted using 95% alcohol (Merck, Germany) by percolation till exhaustion (4 × 4 l) and filtered off. The combined filtrates of the plant were evaporated under reduced pressure and low temperature using rotator evaporator. The obtained residue (250 g) was kept for further phytochemical investigation.

The obtained alcoholic extracts (100 g) was dissolved in methanol and applied on the top of a column (150 × 5 cm) packed with 220 g sephadex LH-20 and methanol was used as an eluent. Two hundred fractions were obtained (50 ml each). All fractions were tested chromatographically investigated on Silica gel TLC using solvent system chloroform-methanol 95:5 and examine under UV lamb before and after spraying with aluminium chlorides spray reagent as described by Markham [23]. Similar fractions were collected together (according to R_f values, number and colour of spots) and concentrated as previously described. For final purification, preparative TLC was used on silica gel G plates (20 × 20 cm), using solvent system ethyl acetate–methanol–water 120:5:4. Thereafter, each band corresponding to the major compound was scratched and eluted using methanol. The elutes were dried and reapplied on the top of silica gel column packed with silica gel G. Elution was proceeded using ethyl acetate–methanol with gradual increasing polarities. The individually collected sub-fraction were dried under reduced pressure at 35 °C and re-crystallized from methanol to yield pure sample of dihydroquercetin.

The dihydroquercetin was obtained as amorphous powder, soluble in methanol, melting point (252–253°C), R_f value = 0.45 in system ethyl acetate–methanol–water (90:5:4: 1 v/v/v). Ultraviolet spectrum in MeOH (log *e)* nm: 232 (4.14), 292 (4.25), 336 sh (3.68); NaOMe. 248, 330; NaOAc 253, 290,330; AlCl 3225, 316, 383; AlCl3/HCl 223, 315, 385; EIMS: *m/z* (rel. int.) 304 (dihydroquercetin [D] +) (40%), 286 [D -HzO]+ (12), 275 [D-CHO]+ (56), 165 (28), 153 (100), 152 (24), 150 (33), 123 (55). ^{1}H NMR (DMSO-d6): The ^{1}H NMR spectrum of this compound in DMSO*d-6*; δ 7.38 (H, *d*, *J* = 2.0 Hz, H-2′), 6.98 (1H, q, *J* = 8.5 Hz, H-6′), 7.11 (1H, *dd*, *J* = 8.4,*J* = 2.1 Hz H-5′). 5.93 (1H, *d*, *J* = 11.2 Hz, H-8) and 5.93 (1H,*d*, *J* = 1.9 Hz, H-6), 4.55(1H, d, *J* = 11.7 Hz, H-2) 4.98(1H, d, *J* = 11.2 Hz). ^{13}C NMR in DMSO *d-6*:(198.1) C-4, at 164.7 (C-7), 169.2 (C-5), 86.1 (C-2),163.2 (C-9), 147.2 (C-4′), 146.1 (C-3′),73.2 (C-3), 122.1 (C-6′), 130.4 (C-1′), 116.3(C-2′), 116.5 (C-5′), 103.4 (C- 10), 96.8 (C-6), 96.7 (C-8).

2.2. Fish, experimental design and sampling

Gilthead seabream (*S. aurata* L.) of average weight 8 ± 0.2 g were obtained from a local farm (Murcia, Spain), and acclimatized for 2 weeks in recirculation systems of seawater aquaria (250 L) at Marine Fish Facility, University of Murcia. The water was maintained at 20 ± 2 °C with a flow rate of 900 l h^{-1} and 28% salinity. The photoperiod was adjusted to 12 h light: 12 h dark. Fish were distributed randomly into 4 groups, each with 21 fish (7 per replicate) and during acclimatization fish were fed twice daily with commercial diet (Skretting) at 3% of body weight. Afterwards, fish in each group fed one of the following experimental diets for 14 days: commercial diet non-supplemented (0%, control), commercial diet supplemented with 0.1 g (0.1%), 0.5 g (0.5%) and 1 g (1%) 100 g^{-1} of dihydroquercetin. At the end of the feeding trial, fish were killed by an overdose of anaesthetic (i.e. 3-amino benzoic acid ethyl ester; Sigma–Aldrich) before blood and head-kidney (HK) samples were obtained.

Blood samples were collected from the caudal vein by insulin syring and left to clot for 2 h at 4 °C, prior to centrifugation (1600 g, 25 min, 4 °C). Serum samples were collected and stored at −20 °C until further analysis. The HK was isolated according to Esteban et al. [24]. Briefly, after fish dissection, HK were removed, cut into small fragments and transferred to 8 ml of sRPMI [RPMI-1640 culture medium (Gibco) supplemented with 0.35% sodium chloride to adjust the medium's osmolarity to gilthead seabream plasma osmolarity of 353.33 mOs], 2% (v/v) foetal calf serum (FCS, Gibco), 10 μl ml^{-1} heparin (Sigma–Aldrich), 100 IU ml^{-1}, penicillin (Flow) and 100 mg ml^{-1} streptomycin (Flow). Then, HK leucocytes were obtained by forcing fragments through a nylon mesh (mesh size 100 mm), before washed twice (400 × g, 10 min) with sRPMI (without heparin) and counted (by Z2 Coulter Particle Counter) to adjusted the cells at 10^7 cells ml^{-1}. Cell viability was greater than 98%, as determined by the trypan blue exclusion test.

2.3. Cellular immune parameters

2.3.1. Phagocytosis

The phagocytic activity of *Saccharomyces cerevisiae* (strain S288C) by gilthead seabream HK leucocytes was studied by flow cytometry *in vivo* and *in vitro* [24]. For *in vivo* assays, samples of 100 μl of HK leucocytes (obtained from fish fed the different experimental diets) were added to 125 μl of heat-killed yeast cells, previously labelled with fluorescein isothiocyanate (FITC, Sigma), washed and adjusted to 5 × 10^7 cells ml^{-1} in sRPMI. Samples were centrifuged (400 × g, 5 min, 22 °C), before being resuspended and incubated at 25 °C for 30 min. At the end of the incubation time, the

samples were placed on ice to stop phagocytosis and 400 μl ice-cold PBS was added to each sample. The fluorescence of the extracellular yeasts was quenched by adding 40 μl ice-cold trypan blue (0.4% in PBS). Standard samples of FITC-labelled *S. cerevisiae* or HK leucocytes were included in each phagocytosis assay. All samples were analysed in a flow cytometer (Becton Dickinson) with an argon-ion laser adjusted to 488 nm. Analyses were performed on 3000 cells, which were acquired at a rate of 300 cells s^{-1}. Data were collected in the form of two-parameter side scatter (granularity) (SSC) and forward scatter (size) (FSC), and green fluorescence (FL1) and red fluorescence (FL2) dot plots or histograms were made on a computerised system. The fluorescence histograms represented the relative fluorescence on a logarithmic scale. The cytometer was set to analyse the phagocytic cells, showing highest SSC and FSC values. Phagocytic ability was defined as the percentage of cells with one or more ingested yeast cells (green-FITC fluorescent cells) within the phagocytic cell population. The relative number of ingested yeasts per cell (phagocytic capacity) was assessed in arbitrary units from the mean fluorescence intensity of the phagocytic cells. The quantitative study of the flow cytometric results was made using the statistical option of the Lysis Software Package (Becton Dickinson).

To study the *in vitro* effect of dyhidroquercetin of HK leucocyte phagocytosis, samples of 50 μl of HK leucocytes (isolated from fish eating control diet and previously adjusted to 10^7 cells ml^{-1} in sRPMI) were incubated at 20 °C in eppendorf with 50 μl of 0% (control), 0.0125%, 0.025%, 0.05%, 0.1%, 0.2%, 0.4%, 0.6%, 0.8%, and 1% of dihydroquercetin dissolved in sRPMI. After that, the assay was running as described before.

2.3.2. Respiratory burst

Respiratory burst of gilthead seabream HK leucocytes was studied by a chemiluminescence method [25]. For *in vivo* assay, 100 μl of HK leucocyte suspension (obtained from fish fed the different experimental diets) were placed in triplicate in wells of a 96-well flat-bottomed plate. Then, 100 μl of HBSS (Hank's balanced salt solution, Gibco) containing 1 μg ml^{-1} phorbol myristate acetate (PMA, Sigma–Aldrich) and 10^{-4} M luminol, (Sigma–Aldrich) were added to each well. The plates were shaken and immediately read in a plate reader for 1 h at 2 min intervals. The kinetic of the reactions was analysed and the maximum slope of each curve calculated. Backgrounds of luminescence were calculated using reactant solutions containing luminol but not PMA. To study the *in vitro* effect of dyhidroquercetin of HK leucocytes, samples of 50 μl of HK leucocytes (isolated from fish eating control diet and previously adjusted to 10^7 cells ml^{-1} in sRPMI) were incubated in 96-well flat-bottomed plate with 50 μl of 0%, 0.0125%, 0.025%, 0.05%, 0.1%, 0.2%, 0.4%, 0.6%, 0.8%, and 1% of dihydroquercetine dissolved in sRPMI. The cells were incubated at 20 °C and 5% CO_2 and 85% humidity atmosphere.

2.4. Humoral immune parameters

2.4.1. Alternative complement activity

The alternative complement pathway was assayed using sheep red blood cells (SRBC, Biomedics) as targets according to Ortuño et al. [26]. Briefly, 100 μl of SRBC suspension (6%) in phenol red-free Hank's buffer (HBSS) containing Mg^{2+} and EGTA were mixed with 100 μl of serially diluted serum to give final serum concentrations ranging from 10% to 0.078%. After incubation (90 min, 22 °C), the samples were centrifuged (400 × g, 5 min, 4 °C) to avoid unlysed erythrocytes. The relative haemoglobin content of the supernatants was assessed by measuring their optical density at 550 nm in a plate reader (Omega). The values of maximum (100%) and minimum haemolysis were obtained by adding 100 μl of distilled water or HBSS to 100 μl samples of SRBC, respectively. The volume yielding 50% haemolysis was determined and used for calculating the complement activity of the sample (ACH50) as follows:

$$\text{ACH50 value (units/ml)} = \frac{1}{K} \times (\text{reciprocal of the serum dilution}) \times 0.5$$

where K is the amount of serum (ml) giving 50% lysis and 0.5 is the correction factor since this assay was performed on half scale of the original method.

2.4.2. Total protein content

To measure the total protein content of the sera, a Bradford assay was carried out using bovine serum albumin (Sigma–Aldrich) as the standard according to Bradford [27]. Thus, 2 mg ml^{-1} solution of BSA was prepared and serial dilutions made with PBS (Sigma–Aldrich). One ml of Bradford reagent (Sigma–Aldrich) was added to 20 μl of each dilution and incubated at room temperature for 15 min. The absorbance of each sample was then read at 595 nm in order to obtain the standard curve. Dilutions (1:100) were then made of the sera preparations in PBS and 1 ml of Bradford reagent added to 20 μl of each serum dilution. After 15 min of incubation, the absorbance of the samples was taken and plotted onto the standard curve to obtain the total protein content present on the sera.

2.4.3. Antiproteases activity

The serum anti-trypsin activity was measured by established methods [28]. Thus, samples of 20 μl of standard trypsin solution (Sigma–Aldrich, 5 mg ml^{-1}) were incubated with 20 μl of serum (10 min, 22 °C). Subsequently, 200 μl of 0.1 M PBS (PH 7.2) and 250 μl of 2% azocasein solution (20 mg ml^{-1} PBS) were added and samples were newly incubated (1 h, 22 C). The reaction was then terminated with the addition of 500 μl of 10% (v/v) trichloro acetic acid (TCA, Sigma–Aldrich) and incubation for 30 min at 22 C. The mixture was centrifuged (6000 × g, 5 min) and 100 μl of the supernatants were transferred to a flat-bottomed 96-well plates containing 100 μl of 1 N NaOH per well. The absorbance was read in the spectrophotometer (Omega) at 410 nm, and the percentage inhibition of trypsin activity was calculated by comparing with a 100% control sample, in which buffer replaced the serum. For a negative control, buffer replaced both serum and trypsin.

2.4.4. Peroxidase

Total peroxidase content present in serum was measured according to Quade and Roth [29]. Briefly, 50 μl serum was diluted with 135 μl of Ca^{+2} and Mg^{+2} free HBSS (Sigma–Aldrich) in flat-bottomed 96-well plates. Then, 50 μl of 20 mM 3,3′,5,5′- tetramethylbenzidine hydrochloride (TMB, Sigma) and 5 mM H_2O_2 (Sigma–Aldrich) were added (both substrates of peroxidase). The colour-change reaction was stopped after 2 min by adding 50 μl of 4 M sulfuric acid (H_2SO_4). The optical density was read at 450 nm in a plate reader. Standard samples without serum were also analysed.

2.4.5. Immunoglobulin M (IgM)

In order to optimize the indirect enzyme-linked immunosorbent assay (ELISA), serial dilutions of serum (from 1/1 to 1/1000) and the commercial monoclonal antibody were tested following the manufacturer's instructions. The 1/100 serum dilution gave an OD in the linear range of the serum dilution versus absorbance curve and was chosen to compare the total immunoglobulin M (IgM) level in samples. Thus, 20 μl of diluted serum (1/100) were placed in flat-bottomed 96-well plates in triplicate and coated by overnight incubation at 4 °C with 200 μl of carbonate–bicarbonate

buffer (35 mM $NaHCO_3$ and 15 mM Na_2CO_3, pH 9.6). The plates were rinsed three times with low salt buffer (LSB, 20 mM Tris–HCl, 380 mM NaCl and 0.05% Tween 20, pH 7.3) before blocking for 2 h at room temperature with blocking buffer (3% Bovine serum albumin, BSA in LSB). After three rinses with LSB and five with high salt buffer (HSB, 20 mM Tris–HCl, 500 mM NaCl and 0.1% Tween 20, pH 7.7), the plates were then incubated for 1 h with 100 μl per well of mouse anti-gilthead seabream IgM monoclomonoclonal antibody (1/100 in blocking buffer, Aquatic Diagnostics, Scotland), washed and incubated with the secondary antibody anti-mouse IgG-HRP (1/1000 in blocking buffer, Aquatic Diagnostics, Scotland). After exhaustive rinsing with HSB the plates were developed using 100 μl of a 0.42 mM solution of 3,3,5,5-tetramethylbenzidine hydrochloride (TMB, Sigma) prepared daily in a 100 mM citric acid/sodium acetate buffer, pH 5.4, containing 0.01% H_2O_2. The reaction was stopped after 10 min by adding 50 μl of 2M H_2SO_4. The plates were read at 450 nm in a plate reader. Negative controls were samples without serum or without primary antibody. The results are expressed as the stimulation index (mean value ± S.E.), which was obtained by dividing each sample value by its respective control value. Values higher than 1 express an increase and lower values a decrease in the serum content of IgM.

2.4.6. Bacterial culture and bactericidal activity

Vibrio anguillarum was obtained from Heriot Watt University (Edinburgh, UK) and cultured in TSA supplemented with 1% NaCl (24 h, 24 °C). The culture was centrifuged (3000 × *g*, 10 min, 4 °C), before the supernatants were discarded, and the pellets resuspended in 0.05 M phosphate buffer saline (PBS, pH 6.2). The bacterial suspensions were counted by Z2 Coulter Particle Counter and adjusted to 10^7 cells ml^{-1}. Serum bactericidal activity was done following the procedure of Kajita et al. [30]. Briefly, an equal volume (100 μl) of serum and bacterial suspension were mixed and incubated for 1 h at 25 °C. A blank control was also prepared by replacing serum with sterile PBS. The mixture was then diluted with sterile 0.05 M PBS at a ratio of 1:10. The mixture (50 μl) was plated onto nutrient agar plates and incubated for 24 h at 24 °C before the number of colonies was counted.

2.5. Statistical analyses

Data were analysed by one-way analysis of variance (ANOVA). The differences among treatments were compared by Tukey's test using Minitab statistical software (Minitab, Coventry, UK). Differences were considered significant at $p < 0.05$.

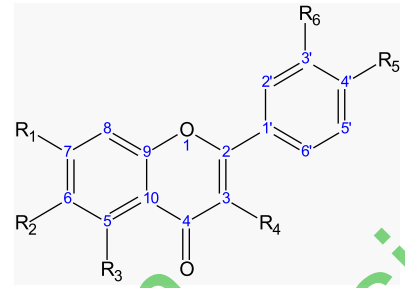
3. Results

3.1. Plant extraction and isolation

Phytochemical investigation of alcoholic extracts of aerial parts of *C. deodara* demonstrated the isolation of one compound. Its formula was structurally elucidated using UV spectrometry, electrospray ionization mass spectrometry (ESIMS) ^1H and ^{13} CNMR spectrometry (Fig. 1) and by comparing with published data [31] it was identified as dihydroquercetin (Fig. 1).

3.2. Cellular immune parameters

Similar responses were obtained for phagocytosis and respiratory burst activity from HK leucocytes isolated from fish fed enriched diets. Both activities were always increased respect to the values obtained for leucocytes from fish in control group. The highest phagocytic activity was recorded for HK leucocytes isolated



E_1: , R_1 =OH, R_2 = H, R_3 = OH, R_4= OH , R_5 = OH, R_6= OH

E_2: , R_1 =OH, R_2 = H, R_3 = OH, R_4= H , R_5 = OH, R_6=O H

Fig. 1. The isolated compounds from *Cedrus deodara* (Roxb.).

from fish fed 0.1% diet followed by those fed 1% and 0.5% doses, respectively (Figs. 2 and 4).

Regarding the *in vitro* study, the activities of HK leucocytes after being incubated with different concentrations of dihydroquercetin vary depending on the used dosage (Fig. 3). Interestingly, the lowest doses (0.025% for phagocytosis and 0.0125% for respiratory burst) revealed the highest activities ($p < 0.05$). On the other hand, the phagocytic activity of HK leucocytes incubated with doses of dihydroquercetin from 0.1% till 1% were always lower than those found for control samples. Respiratory burst of HK leucocytes incubated with 0.025% and 0.05% doses was higher (although not statistically significant) than the activity recorded for control leucocytes (non incubated with dihydroquercetin) (Fig. 5).

3.3. Humoral immune parameters

All the humoral immune assays tested (complement and antiprotease activities, total protein content, peroxidase level, bactericidal activity and IgM) (Figs. 6–11) were higher in fish fed dihydroquercetin supplemented diets compared to those found in control fish (fed commercial diet); particularly, fish fed 0.1% doses recorded the highest humoral immune activities ($p < 0.05$) in all assays compared to control.

4. Discussion

The present study revealed that dietary administration of dihydroquercetin significantly increased the innate humoral and cellular immune parameters of gilthead seabream. The phagocytes plays a vital role in defence mechanisms by engulf invading pathogen and

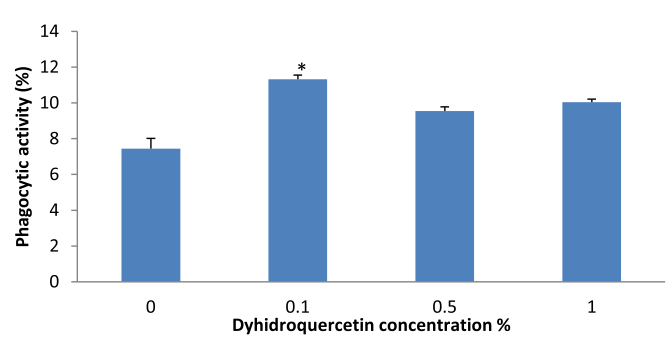


Fig. 2. Phagocytosis of FITC-labelled *Saccharomyces cerevisiae* by gilthead seabream head-kidney leucocytes. Dietary dihydroquercetin supplemented doses: 0% (control), 0.1%, 0.5% and 1%. Data are presented as mean ± S.E. Asterisk represents significant difference from control $p \leq 0.05$. Bars = mean ± S.E.

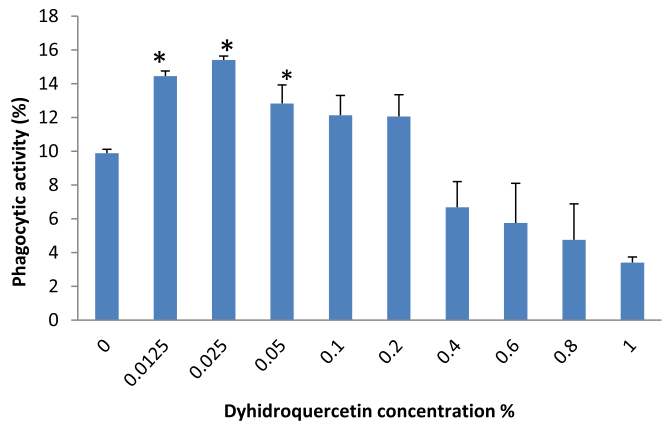


Fig. 3. Phagocytic activity (%) of head-kidney gilthead seabream leucocytes incubated *in vitro* with different concentrations of dihydroquercetin. Data represent mean ± S.E. Asterisks denote statistically significant differences ($p < 0.05$) between control (0%) and dihydroquercetin incubated groups.

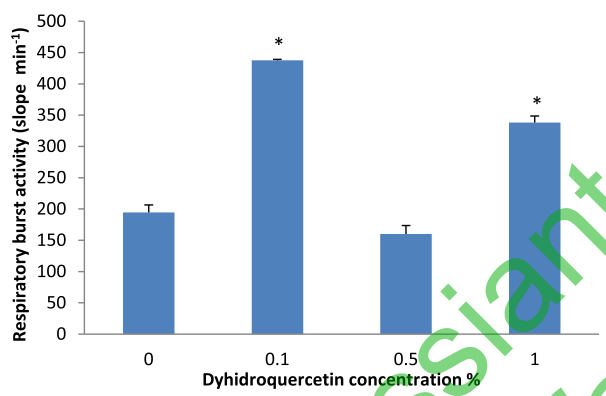


Fig. 4. Respiratory burst activity by gilthead seabream head-kidney leucocytes. Dietary dihydroquercetin supplemented doses: 0% (control), 0.1%, 0.5% and 1%. Data are presented as mean ± S.E. Asterisks represent significant difference from control $p \leq 0.05$. Bars = mean ± S.E.

then digest the cellular debris as well as stimulating lymphocytes and other immune cells to respond to the pathogen by synthesis the antibody [32]. An increase in phagocytic activity of leucocytes by immunostimulants has been documented by many authors [12,33–35]. However, the ideal time and dose of immunostimulants

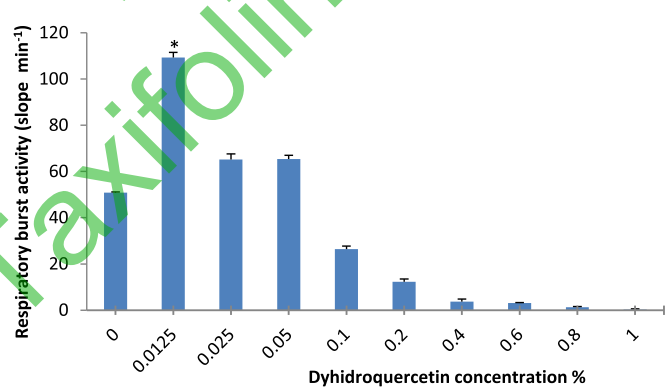


Fig. 5. Respiratory burst activity of head-kidney gilthead seabream leucocytes incubated *in vitro* with different concentrations of dihydroquercetin. Data represent mean ± S.E. Asterisk denotes statistically significant differences ($p < 0.05$) between control (0%) and dihydroquercetin incubated groups.

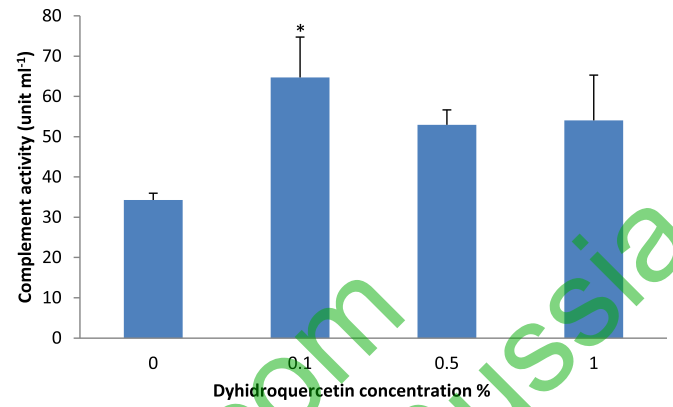


Fig. 6. Complement activity of gilthead seabream serum. Dietary dihydroquercetin supplemented doses: 0% (control), 0.1%, 0.5% and 1%. Data are presented as mean ± S.E. Asterisc represents significant difference from control $p \leq 0.05$. Bars = mean ± S.E.

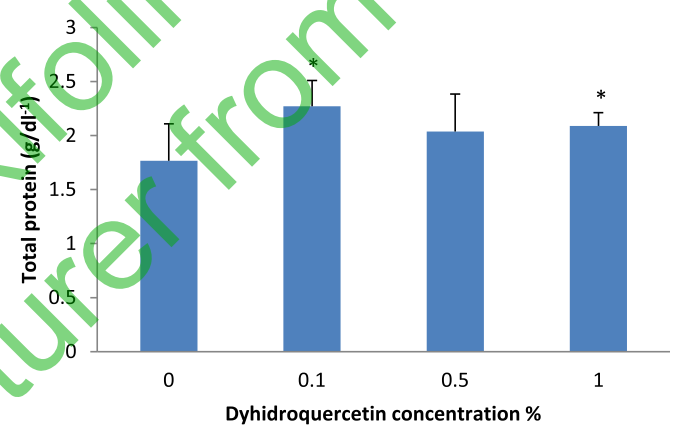


Fig. 7. Total protein of gilthead seabream serum. Dietary dihydroquercetin supplemented doses: 0% (control), 0.1%, 0.5% and 1%. Data are presented as mean ± S.E. Asterisks represent significant difference from control $p \leq 0.05$. Bars = mean ± S.E.

for enhancement of immunity is variable among many factors including immunostimulant tested and fish species. For example, Tilapia fed with 0.1% and 0.5% *Astragalus radix* extract for 3 week showed enhancement in phagocytosis. On the other hand, inhibition of phagocytosis and respiratory burst activity was found when fish were fed 0.5 and 1% doses of *Scutellaria radix* extract [34]. In common carp, phagocytic activity increased after feeding with 1% of herbal

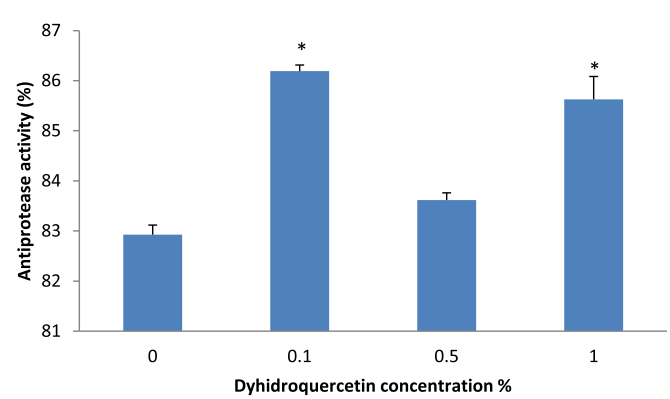


Fig. 8. Antiprotease activity of gilthead seabream serum. Dietary dihydroquercetin supplemented doses: 0% (control), 0.1%, 0.5% and 1%. Data are presented as mean ± S.E. Asterisk represents significant difference from control $p \leq 0.05$. Bars = mean ± S.E.

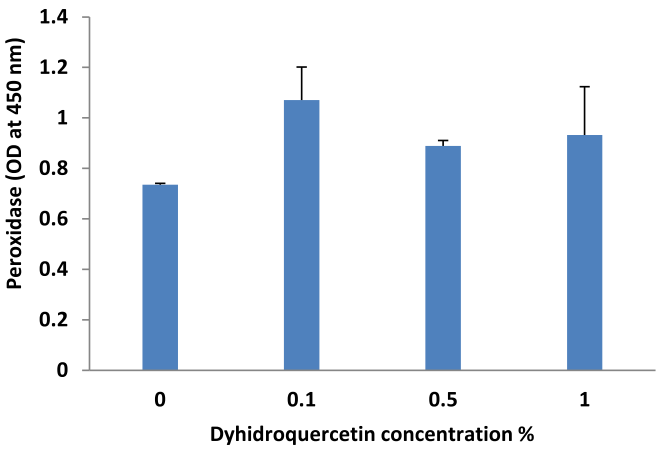


Fig. 9. Peroxidase activity of gilthead seabream head-kidney leucocytes. Dietary dihydroquercetin supplemented doses: 0% (control), 0.1%, 0.5% and 1%. Data are presented as mean ± S.E. Bars = mean ± S.E.

mixture extracts for 4 weeks, more than with 0.5% dose [36]. Interestingly, the present results demonstrated significant increase in phagocytic activity of fish fed with 0.1% of dihydroquercetin. Similarly, incubation of seabream leucocytes with 0.0125%, 0.025% or 0.05% dihydroquercetin also resulted in increased phagocytic activity. This could be attributed to the efficiency of this compound as immunomodulatory agent to persuade the leucocyte to attack the pathogens. Similar observations were recorded in a previous study, where the dihydroquercetin play vital role as anti-inflammatory factors in rat peritoneal macrophages [18].

Several forms of reactive oxygen intermediates are produced during phagocytosis, which are considered as toxic for bacterial fish pathogens [37,38]. Superoxide anion is one of the first forms that released from the respiratory burst; therefore the measurement of O_2^- has been accepted as an accurate method of measuring this activity [39]. The study recorded significantly higher in respiratory burst activity in groups fed with 0.1% and 1% doses of dihydroquercetin compared to control, whilst 0.5% dose showed reduction in respiratory bust activity. This could be attributed to dose-dependent of immunostimulant which recorded by many researcher and its role for enhance the immune response in fish. For example, grouper (*Epinephelus coioides*) fed for 2 weeks with 1%, 2.5% or 5% and Katuk extract, (*Sauropus androgynus*), showed increased respiratory burst and phagocytosis activities [40]. Also, respiratory burst activity was significantly higher in carp fish fed

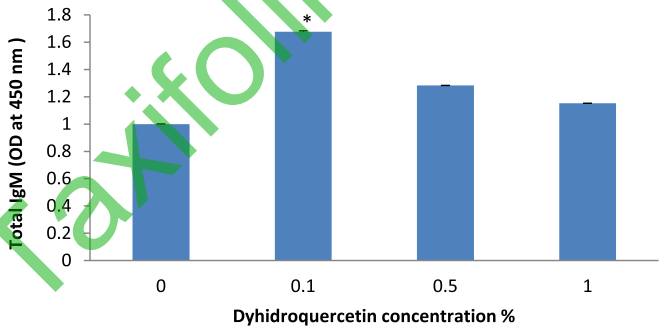


Fig. 10. Total IgM present in gilthead seabream serum. Dietary dihydroquercetin supplemented doses: 0% (control), 0.1%, 0.5% and 1%. Data are presented as the stimulation index (mean value ± S.E.) obtained by dividing each sample value by its control group value. The control value is 1 and values higher than 1 express an increase in the total serum content of IgM. Data are presented as mean ± S.E. Asterisk represents significant difference from control $p \leq 0.05$. Bars = mean ± S.E.

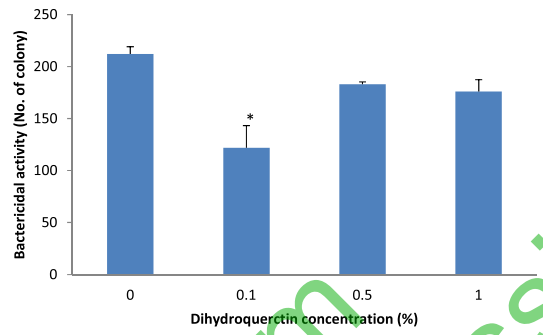


Fig. 11. Bactericidal activity of gilthead seabream serum. Dietary dihydroquercetin supplemented doses: 0% (control), 0.1%, 0.5% and 1%. Data are presented as mean ± S.E. Asterisk represents significant difference from control $p \leq 0.05$. Bars = mean ± S.E.

diets supplements with 0.5% and 1% of *Achyranthes aspera* seeds extract and immunized with heat-killed *Aeromonas hydrophila* compared to the control [10]. Moreover, using different doses of water-soluble fraction of *Tinospora Cordifolia* leaves caused significant increase in respiratory burst activity of *Oreochromis mossambicus* after 10 days [1]. In the present study, the direct interaction between leucocytes and dihydroquercetin was studied as a direct indicator of stimulation or toxicity of this antioxidant compound. *In vitro*, respiratory burst activity of gilthead seabream HK leucocytes was enhanced when incubated with lower concentrations of dihydroquercetin (0.0125%), while higher concentrations reduced it. The present results are in agreement with a previous *in vitro* study in which low concentrations of *Paecilomyces japonica* extracts (ranging from 31.3 to 250 ppm) significantly enhanced the respiratory burst of head-kidney leucocytes of juvenile olive flounder (*Pleuronectes platessa*). However, doses of 0.5% and 1% of *P. japonica* extract showed the highest respiratory burst *in vivo* [41].

Complement is a common protective system in vertebrates, containing ~35 soluble glycoproteins and membrane-bound proteins which interact sequentially with each other leading to lysis or protection against a variety of microorganisms [42]. The results of this study showed enhancement in complement activity in fish fed with all doses of dihydroquercetin especially in the group receiving 0.1% dose. Similarly, 0.1% of aqueous *Eclipta alba* leaf extract resulted in significantly increased complement activity in tilapia, *O. mossambicus* after 2 weeks [43]. It could be suggested that the increases observed in complement activity of gilthead seabream specimens fed dyhidroquercetin could contributed to the increase in the HK phagocytic activity, due to their role as an opsonin [44,45].

Peroxidase is an important enzyme with microbicidal properties, which utilises one of the oxidative radicals (H_2O_2) to produce hypochlorous acid [46]. This process is important in killing the foreign microorganisms [47]. Present results demonstrated an increase in peroxidase content in all treated groups as compared to the control. In agreement with this study, peroxidase activity increased significantly in rainbow trout fed for 2 weeks with 0.5% and 1% dose of quercetin (fraction from Nettle extract) and 1%, 2% and 3% dose of *Nigella sativa* oil [2]. Furthermore, different doses of aqueous extract of *E. alba* leaf showed significantly increased in peroxidase activity of common tilapia after 1 week, whereas feeding for 2 or 3 weeks did not result in any significant increase [43]. New studies will give light to the possible way of action of these substances, although their antioxidant properties are probably involved.

Antiproteases play vital roles in inhibition the action of proteases either by binding to their active sites or by 'trapping' the protease to prevent protein hydrolysis [48] and, thus restrict the ability of bacteria to invade and to grow in fish [49]. All the assayed dihydroquercetin doses (but especially 0.1%) increased the

antiprotease activity present in seabream sera compared to the values found in control fish. In this sense, the present results seems to suggest that the dyhidroquercetin is more effective than quercetin because lower dosages of dyhidroquercetin are necessaries to increase fish antiprotease activity [2]. Similarly, Alexander et al. [1] recorded significantly enhanced in the serum antiprotease, lysozyme, and natural haemolytic complement activities of *O. mossambicus* treated with 6, 60 or 600 mg kg^{-1} body weight, of the water-soluble fraction of *Tinospora cordifolia* leaves and some previous works reported also an enhancement in antiprotease activity after using herbal extracts or fractions [43,50–52]. Further studies are needed to identify if dyhidroquercetin is present or not in such herbal extracts.

Many researchers attributed the high concentrations of total protein in fish fed with immunostimulants to the enhancement of the non-specific immune response. For example, the use of 0.1% and 1% ginger, mistletoe and stinging nettle extracts enhances the immune response, and concomitantly, increases in serum total protein of rainbow trout was observed [33]. The present results revealed highly significant increase in total protein level in seabream fed for 2 weeks with 0.1% and 1% doses compared to control. Present results are also in agreement with those previously obtained by Mohamad and Abasali [53] and Ardó [35].

Teleost IgM resembles mammalian IgM in structure, physiological characteristics, soluble forms and membrane-bound forms [54,55]. It plays an important role as an immune effect or molecule in the blood [56]. Previous studies have reported a considerable individual variation in serum IgM levels among fish related to size and/or age [57,58], environmental conditions [59,60], disease status [61,62] or immunostimulant types [2,63,64]. Moreover, dosage and time of administration created other variation factors. The use of 5% dose of *Ficus benghalensis* root extract on Indian freshwater murrel, *Channa punctatus* diet resulted in increasing in seric IgM level and total protein [65]. Our results showed increasing in serum IgM levels in all treatments groups compared to the control especially in the group fed with the lowest dose of dihydroquercetin (0.1%). Curiously, similar results were recorded in rainbow trout fed for 2 weeks with quercetin (0.1%) [2]. Finally, serum bactericidal activity is a mechanism noted for the killing and clearing of pathogenic organisms in fish [49]. *V. anguillarum* was used as a model to examine the effectiveness of dihydroquercetin to kill the bacterial infection. Therefore, the lowest number of bacterial colonies grown on TSA media indicated the efficiency of immune cells in serum to kill this pathogen. Our results revealed higher serum bactericidal activity in treated groups, especially in the group fed with 0.1% dose. This is in agreement with previous works on rainbow trout fed different concentrations of quercetin [2] and Quit-A, a fraction from *Quillaja saponaria* Molina [66]. Perhaps, the increments observed in the complement of fish fed dihydroquercetin supplemented diets are involved in the observed increases in bactericidal activity present in serum in which also the complement is involved [49,67].

To conclude, the use of low doses of dihydroquercetin (fraction from deodar, *Cedrus deodara)* in gilthead seabream diet give enhancements in all the innate and adaptive immune parameters tested. Such findings suggest a promising role for dihydroquercetin as immunostimulant compound in fish food for resist and/or treat disease problems. Further investigations could be useful to elucidate its way of action although perhaps its antioxidant and anti-inflammatory properties are involved, as in other vertebrates.

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