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In vitro effects of *Origanum vulgare* leaf extracts on gilthead seabream (*Sparus aurata* L.) leucocytes, cytotoxic, bactericidal and antioxidant activities

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ABSTRACT

Origanum vulgare is a well-known medicinal plant that has been used since ancient times as an additive in foods and cosmetic preparations. The possible application of *O. vulgare* extracts in fish was assessed by using gilthead seabream (*Sparus aurata*) as a marine fish model due to its importance in aquaculture. The *in vitro* effects of aqueous and ethanolic leaf extracts of *O. vulgare* were tested in order to observe any immunostimulant, cytotoxic, bactericidal or antioxidant properties. The results showed that medium or high concentration of aqueous extracts and low concentrations of ethanolic extract, increased head kidney leucocyte activities as well as the number of SAF-1 cells. However, moderate to high concentrations of ethanolic extracts decreased both leucocyte activities and the number of viable SAF-1 cells, suggesting some possible toxic effect towards them. Only the highest concentration of the aqueous extract and medium to high concentrations of the ethanolic extracts showed cytotoxic activity against the tumor PLHC-1 cell line. Bactericidal activity was only detected against *Vibrio harveyi, V. anguillarum* and *Photobacterium damselae* when using the highest concentration of aqueous extract and moderate to high concentrations of ethanolic extract. Finally, both plant extracts presented antioxidant activity particularly the aqueous extract. Overall, the results suggest that both extracts (when used at the appropriate concentration) have immunostimulant, cytotoxic, bactericidal and antioxidant properties, making *O. vulgare* an interesting candidate for incorporation as additive in functional diets for farmed fish.

1. Introduction

Global fish consumption and aquaculture activity have increased in recent decades, and this industry is one of the fastest-growing animal food producing sectors [1], with Spain being the biggest producer in the European Union [2]. However, the super-intensive practices developed in fish farms have led to problems, including environmental harm (e.g. bad water quality), increased number of opportunistic microorganisms and stress conditions. Such negative situations may compromise fish growth and health and make animals more susceptible to infections and diseases, resulting in substantial economic loss [3]. Furthermore, in recent years the use of antibiotics to treat and control fish diseases has been banned in the EU because they can accumulate in fish tissues and give rise to resistant bacteria. However, disease prevention is important in order to preserve a sustainable aquaculture, both environmentally and economically. Prophylactic methods based on stimulation of the fish immune system have been successfully used for this purpose and have become an integrated part of the management of modern aquaculture processes [4]. At present, the main prophylactic measures available for farmed fish include vaccination, probiotics and immunostimulation [5].

Oregano or Pot Marjoram (*Origanum vulgare*) is a well-known plant used worldwide since ancient times in traditional and folk medicine [6]. Oregano is the most important and variable species of this genus and is widespread throughout the world and is particularly abundant in the Mediterranean area [7–9], Eurasia and the North of Africa [10]. Many studies have demonstrated that the plant presents a wide variety of secondary metabolites, most of them phenolic compounds such as flavonoids, terpenoids, phenolic acids and alkaloids, and fatty acids among others [10–13], which are the principal components responsible for its activities and allows its use not only in traditional medicine, but also in foods and cosmetic preparations [13,14]. Among the

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pharmacological properties demonstrated for the compounds present in *O. vulgare* are antibacterial, antivirus, anti-inflammation and antioxidant activities [6,15]. For all these reasons, *O. vulgare* could be considered *a priori* as a good source of new natural compounds to treat, prevent and/or control fish diseases in aquaculture.

At present, there is intense and active research into natural products with immunostimulant or biocidal activities for fish [16]. In this sense, medicinal plants are a promising alternative to antibiotics, for several reasons, including all the beneficial properties that their biological compounds present in other animals or even in human beings, the low negative impacts on fish, the environment and the human health, low cost and eco-friendly origin [3,17–19]. In addition to the immunostimulant properties, it has also been demonstrated that many medicinal plants are also able to have other positive effects on fish, such as the stimulation of fish growth, weight gain and early maturation of cultured species [20].

There are many *in vivo* studies in which plants, their extracts or their essential oils have been used as additives in animal feed, particularly fish [13,17–19]. However, to the best of our knowledge, there are very few studies about the *in vitro* effect of these plants, extracts or essential oils on fish. The present study was undertaken taking into account all these considerations (including the prohibition of antibiotics, their possible replacement by medicinal plants, the abundance of *O. vulgare* and its bioactive compounds). The aim was to evaluate the *in vitro* effects of leaf extracts (both aqueous and ethanolic) obtained from *O. vulgare* on gilthead seabream (*S. aurata* L.) head kidney leucocyte activities (viability, phagocytosis, respiratory burst and peroxidase). Gilthead seabream was selected as a representative species of marine aquaculture. Furthermore, the possible cytotoxic activity of such extracts on SAF-1 cells (cell line obtained from *S. aurata* fibroblasts) and PLHC-1 cell line (hepato-carcinoma obtained from *Poeciliopsis lucida*, topminnow) and the bactericidal activity against three bacterial pathogens for fish (*Vibrio harveyi, V. anguillarum* and *P. damselae*) were also evaluated. Finally, the antioxidant activity of the extracts was determined. The results of the selected properties of the oregano extracts (immunostimulant, cytotoxic, bactericidal and antioxidant) are discussed and suggest that this plant may be considered an interesting candidate for incorporation as additive in functional diets for farmed fish.

2. Material and methods

2.1. Plant extracts

Dried leaves of *O. vulgare* were bought in a local market (Murcia, Spain). Leaves were crashed until to be powder. One g of powder and 40 mL of water or absolute ethanol were used for extract's preparations [21]. To prepare the aqueous extracts, leaves were macerated and shaken with boiling water for 4 h at 25 °C. The mixture was filtered twice using a nylon net filter with a 100 mm pore size, and evaporated in a rotary evaporator (Buchi Rotavapor R-215) until dryness. Prior to use in the assays, the extracts were filtered using sterile filters of 0.22 mm diameter. For the preparation of ethanolic extracts, dry leaves were macerated and shaken with pure ethanol (1:40, 48 h, and 25 °C). The resulting mixture was then filtered twice as described above, and concentrated by vaporizing using a rotary evaporator.

2.2. Animals

Five specimens (52.75 ± 3.62 g weight) of the seawater teleost gilthead seabream (*S. aurata* L.), obtained from a local farm (Murcia, Spain), were kept in re-circulating seawater aquaria (250 L) in the Marine Fish Facilities at the University of Murcia. The water temperature was maintained at 20 ± 2 °C with a flow rate of 900 L h^{-1} and 28‰ salinity. The photoperiod was 12 h light:12 h dark. Fish were allowed to acclimatize for 15 days before the start of the trial, where they were fed with a commercial pellet diet (Skretting, Spain) at a rate of 2% body weight day^{-1}. The fish were killed after starving for 24 h by using an overdose of MS-222 (Sandoz, 100 mg mL^{-1} water). All experimental protocols were approved by the Ethical Committee of the University of Murcia.

2.3. Head-kidney leucocyte isolation and incubation with extracts

Before the dissection of the head-kidney (HK), the specimens were bled. Blood was collected from the caudal vein and afterwards fish were dissected to obtain HK fragments, isolating the leucocytes according to Esteban et al. [22]. Briefly, HK were cut into small fragments and transferred to 12 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), 3% foetal calf serum (FCS, Gibco), 100 i.u. mL^{-1} penicillin (Flow) and 100 mg mL^{-1} streptomycin (Flow)]. HK leucocytes were obtained by forcing fragments of the organ through a nylon mesh (mesh size 100 mm), washed twice (400 g, 10 min), counted in an automatic counting chamber (BioRad) and adjusted to 2×10^7 cells mL^{-1} in sRPMI. Cell viability was determined by the trypan blue exclusion test.

To study the possible effects of aqueous and ethanolic extracts on HK leucocyte activities, aliquots of 50 µL of the HK leucocyte suspension containing 2×10^7 cells mL^{-1} were dispensed into glass tubes (Falcon, Becton Dickinson) to ascertain viability and phagocytic activity, 50 µL into a flat-bottomed 96-well plates to assess respiratory burst activity and 5 µL into a flat-bottomed 96-well plates for peroxidase activities. Afterwards, aliquots of 50 µL of aqueous or ethanolic extracts (0.002, 0.2, 1 and 2 mg mL^{-1} prepared in sRPMI) were added to each glass tubes for viability and phagocytic activity assays. Aliquots of 50 µL of the extracts were added to each well of flat-bottomed 96-well plates to check respiratory burst activity and aliquots of 5 µL of the extracts were added to each well of flat-bottomed 96-well plates for peroxidase activity. The extract aliquots were replaced by sRPMI on control samples for those assays developed with aqueous extracts. On the other hand, extracts were replaced by 1% dimethyl sulfoxide (DMSO, Sigma) in sRPMI in the case of control samples for assays carried out with ethanolic extracts. Cells were incubated in the presence of the extracts for 24 h at 21 °C in an incubator with 5% CO_2 and 85% humidity. After incubation, HK leucocyte viability, phagocytic, respiratory burst and peroxidase activities were determined as described below.

2.4. Leucocyte viability

Leucocyte viability was studied adding 50 µL of propidium iodide (PI) (400 mg mL^{-1}, Sigma) to each 100 µL aliquot of HK leucocytes (previously incubated with the extracts, as described above). The tubes were gently mixed before analysis in a FACScan (Becton Dickinson, Madrid, Spain) flow cytometer with an argon-ionlaser adjusted to 488 nm. Analyses were performed on 5000 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 computerized system. Dead cells were estimated as the percentage of cells with propidium iodide (red-PI fluorescent cells). A quantitative study of the flow cytometric results was made using the statistical option of the Lysis Software Package (Becton Dickinson).

2.5. Phagocytic activity

The phagocytic activity of gilthead seabream HK leucocytes was studied by flow cytometry according to Esteban et al. [23]. Heat killed (30 min, 60 °C) lyophilized *S. cerevisiae*, strain S288C, were washed twice, counted and adjusted to 10^8 yeast cells mL^{-1} in sRPMI-1640. To