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Comparative *In Vitro* Bioactivity of Traditional Aqueous and Alcoholic Preparations of Arnica (*Chiliadenus glutinosus*), Effects on Marine Fish Pathogens, PLHC1 Cells and Gilthead Seabream (*Sparus aurata*) Leucocytes

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Abstract

Arnica (*Chiliadenus glutinosus* (L.) Fourr.) is an endemic plant widely used in Spanish traditional medicine as infusions and alcoholic macerates for different ailments. Despite this use, information about the biological activity of these preparations in fish-related models is scarce. In the present study, aqueous, ethanolic, and methanolic extracts were tested at different doses (0, 0.001, 0.01, 0.125, 0.25, 0.5, and 1 mg mL^{-1}) to compare their antioxidant activity, effects on four marine fish pathogens, cytotoxicity on the PLHC1 tumour cell line, and their impact on immunological parameters in head-kidney leucocytes (HKLs) of gilthead seabream (*Sparus aurata*). All extracts showed dose-dependent antioxidant activity, while bactericidal effects depended on the solvent and were mainly observed at the highest concentrations. Ethanolic and methanolic extracts displayed clear cytotoxicity, whereas the aqueous extract showed lower toxicity and was therefore selected for further evaluation. In a second assay, selected concentrations (0, 0.25, and 0.5 mg mL^{-1}) of the aqueous extract were tested in leucocytes stimulated with λ-carrageenan (0 and 1,000 μg mL^{-1}), and respiratory burst and phagocytic activity, cell morphology, and gene expression were analysed. The aqueous extract reduced respiratory burst and phagocytic capacity in activated leucocytes and was associated with morphological signs of cell activation. It also down-regulated *crel* and *casp9* expression. These results provide a comparative view of the *in vitro* bioactivity of different traditional preparations of arnica and show that their biological effects strongly depend on the solvent used and the concentration tested, providing initial experimental information on their cellular effects in fish.

Keywords: arnica (*Chiliadenus glutinosus*); plant extracts; herbal preparations; carrageenan; innate immune system; gilthead seabream (*Sparus aurata*)

Key Contribution: The present study provides the first comparative assessment of the biological activity of traditional arnica extracts in fish-related models, with direct effects on marine fish pathogens and gilthead seabream immune cells.

1. Introduction

Since ancient times, plants and their derived preparations have been widely employed in traditional medicine to treat a broad range of human conditions, even when scientific evidence supporting their use has been limited [1–3]. Many of these remedies are still employed today as infusions, macerates, or topical applications, and their continued use has encouraged experimental studies aimed at documenting their biological activity and evaluating their potential benefits and

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risks. In recent years, plant-based products have also attracted attention in aquaculture research, where natural substances are being explored as alternative sources of bioactive compounds with antioxidant, antimicrobial, or immune-related effects in fish species [4]. Due to these properties, plant extracts have been proposed as potential alternatives to antibiotics and vaccines in the treatment or prevention of aquaculture diseases [4–7].

In this context, *Chiliadenus glutinosus* (L.) Fourr. (=*Jasonia glutinosa* (L.) DC.) is a native plant species of the Iberian Peninsula belonging to the Asteraceae family, traditionally known in Spain as "*arnica*", "*té de roca*" (rock tea) or "*té de montaña*" (mountain tea) and which should not be confused with *Arnica montana* [8–11]. Its characteristic lanceolate leaves and yellow flowers have been used for generations in the preparation of infusions, mainly as remedies for digestive discomfort, respiratory problems such as colds, sore throats, and asthma and also for regulating blood pressure [12]. In addition, alcoholic macerates have been applied topically on the skin to disinfect minor wounds and as painkillers [12]. In popular medicine, these preparations have also been associated with anti-inflammatory and antiseptic properties [12]. Despite this long history of use, most available information has focused on its phytochemical composition or on biological effects observed in mammalian models [13–15]. However, its activity in fish remains largely unexplored. Specifically, only one previous study evaluated the dietary inclusion of arnica at 0 (control), 10 and 30% in gilthead seabream specimens for 15 and 30 days and reported changes in antioxidant status and immune-related parameters in skin mucus, serum, head kidney leucocytes (HKLs), liver and gut after 15 days of feeding [16]. Therefore, little is known about the direct effects of different types of traditional preparations of this plant on fish cells or on aquatic pathogens. From an ethnopharmacological perspective, comparing aqueous and alcoholic extracts that resemble common traditional uses may help to better understand how the method of preparation influences biological activity.

Based on this context, the present study compared the *in vitro* bioactivity of aqueous, ethanolic, and methanolic extracts of arnica. To the best of our knowledge, this is the first study that evaluates the antioxidant activity of these extracts, effects on the viability of four marine fish pathogenic bacteria, and cytotoxicity on the PLHC1 tumour cell line, together with their impact on immune cellular parameters of HKLs from gilthead seabream, one of the most important fish species in the Mediterranean aquaculture. In addition, selected concentrations of the aqueous extract were tested in leucocytes stimulated with λ-carrageenan to explore its influence on cellular responses, ultrastructural morphology, and the expression of genes related to inflammation and apoptosis. Overall, this work provides a comparative assessment of the biological activity of different traditional preparations of arnica in fish-related *in vitro* models.

2. Materials and Methods

2.1. Collection and Preparation of Plant Extracts

Whole arnica plants were harvested in August 2019 (Lorca, Murcia, Spain, Latitude 37°49′27.8461″N and longitude 1°40′50.5888″W). A voucher specimen was deposited in the herbarium of the University of Murcia (Accession number: MUB 71209). The collected material was air dried at room temperature (RT) for 15 days. Subsequently, leaves and flowers were milled into a fine powder and kept in dark conditions at 4ºC until further use. This powder material was employed for the preparation of three different extracts by maceration using water, ethanol, and methanol as solvents. The aqueous extract was prepared to resemble traditional infusion practices, while ethanolic and methanolic extracts reflect the use of alcoholic macerates traditionally applied for the treatment of wounds and external ailments. For the aqueous extract, 1 g of dried plant powder was mixed with 40 mL of boiling distilled water and shaken at RT for 4 h. The suspension was filtered twice through a 100 µm nylon mesh filter and lyophilized (CHRIST, Model Alpha 1-2 LD, 101021). The dried aqueous extract was weighed and resuspended in Phosphate-Saline Buffer (PBS; 11.9 mM Phosphates, 137 mM NaCl, and 2.7 mM KCl, pH 7.4) (Fisher Bioreagents) to obtain the stock solution, which was stored in darkness at -20 °C [17]. For the ethanolic and methanolic extracts, 1 g of dry plant

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powder was incubated with 40 mL of absolute ethanol or methanol, respectively, and shaken for 48 h at RT. These extracts were also filtered twice as described above, lyophilized and stored in darkness at -20 °C. After weighing, both dried extracts were dissolved in dimethyl sulfoxide (DMSO, Sigma) to prepare stock solutions at 10 mg mL^{-1} [18]. Then, both extracts were filtered and stored at -20 °C until use. For all experiments, working solutions from the three arnica extracts were prepared immediately before use by diluting the corresponding stock solution in the appropriate medium used for each experiment, as detailed below. Then, final solutions of 0 (Control; PBS for the aqueous extract and DMSO for the ethanolic and methanolic extracts), 0.001, 0.01, 0.125, 0.25, 0.5, and 1 mg mL^{-1} were obtained and assayed.

2.2. Total Antioxidant Activity

The antioxidant capacity of the plant extracts prepared as described above was evaluated using the 2,2′-azino-bis-3-(ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay [19]. The method relies on the capacity of antioxidant compounds present in the sample to reduce the ABTS radical cation, which is assessed through the decolourization of $ABTS^{+}$ and the corresponding decrease in absorbance. Antioxidant activity was determined by comparing the values obtained for each sample with an ascorbic acid standard curve, and the results were expressed as ascorbic acid equivalents (mM). Briefly, 50 µL of aqueous, ethanolic or methanolic extracts, diluted in PBS to the final concentrations (as explained above) were mixed with 950 µL of $ABTS^{+}$ solution. Absorbance quenching was then measured at 730 nm using a spectrophotometer (BOECO S-22 UV/Vis) with the reaction PBS used as the blank. All samples were analysed in triplicate.

2.3. Bactericidal Activity

The bactericidal activity of the three arnica extracts against four Gram-negative marine bacteria (*V. harveyi* strain Lg 16/00, *V. anguillarum* strain CECT4344, *P. damselae* subsp. *piscicida* strain PP3 and *Tenacibaculum maritimum*) was assessed following the protocol described by Guardiola et al. with minor modifications [20]. Before each assay, a single colony of *V. harveyi*, *V. anguillarum,* and *P. damselae* was inoculated into Tryptic Soy Broth medium (TSB, Difco Laboratories) supplemented with 1.5 % NaCl, whereas a single colony of *T. maritimum* was cultured in *Flexibacter maritimus* medium (FMM, Difco Laboratories) using flasks filled to 10% of their volume. All flasks were incubated overnight at 25 °C with continuous agitation at 100 rpm. Bacteria in the exponential growth phase were then resuspended in sterile PBS and adjusted to a concentration of 1 x 10^{8} colony forming units (CFU) mL^{-1}. To evaluate bactericidal activity, 20 µL of aqueous, ethanolic or methanolic extracts, previously diluted in PBS to final concentration described above**,** were dispensed into triplicate wells of a 96-well U-shaped plate. Culture medium alone and PBS were included as negative and positive controls, respectively. Subsequently, 20 µL of each bacterial suspension were added to the corresponding wells, and the plates were incubated for 5 h at 25 °C. Bacterial viability was then determined by means of an MTT, which is based on the reduction of the soluble yellow tetrazolium salt (3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide) (MTT) into an insoluble blue formazan product by succinate dehydrogenase activity [21,22]. Thus, 25 µL of MTT (1 mg mL^{-1}, Sigma) were added to each well, and the plates were incubated for 10 min to allow formazan formation. The plates were then centrifuged (2000 *x g*, 10 min) and the resulting precipitate was dissolved in 200 µL of DMSO. Absorbance of the solubilized formazan was recorded at 570 nm and 690 nm using a microplate reader (BMG, SpectroStarnano). Bactericidal activity was expressed as the percentage of surviving bacteria relative to the number of bacteria in the positive controls control group, which was set at 100%.

2.4. Cytotoxic Activity Against PLHC1 Tumour Cell Line

The PLHC1 cell line (ATCC® CRL2406™), derived from *Poeciliopsis lucida* hepatocellular carcinoma, was seeded in plastic culture flasks of 25 cm^{2} containing Eagle's Minimum Essential

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Medium (EMEM) supplemented with 5 % foetal bovine serum (FBS, Life Technologies), 2 mM L-glutamine, 100 i.u. mL^{-1} penicillin/streptomycin, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate. Cells were cultured at 30 °C in a humidified incubator with85 % humidity and 5 % CO_2. Cells in exponentially growth phase were detached from the flasks by brief treatment with trypsin (0.05 % in PBS, pH 7.2-7.4), following standard trypsinization procedures. The detached cells were recovered by centrifugation (200 *x g*, 5 min, 30 °C) and their viability was assessed by trypan blue exclusion. The cytotoxicity assay was carried out in triplicate for each concentration tested. Once the cultures reached approximately 80 % confluence, the cells were detached with trypsin and 100 µL aliquots containing 30,000 cells per well were seeded into 96-well tissue culture plates and incubated (24 h, 30 ºC). This seeding density was previously evaluated to provide suitable absorbance values in the cytotoxic assay while preventing cell overgrowth. After this incubation period, the culture medium was replaced with 200 µL per well of EMEM supplemented culture medium containing aqueous, ethanolic or methanolic extract of arnica adjusted to the final concentration (see the section 2.1.) and the cells were incubated for a further 24 h at 30 °C. Culture medium alone was included as negative control, whereas cells maintained in the same culture medium served as positive control [23]. At the end of the exposure period, the medium was removed and 200 µL per well of MTT solution (1 mg mL^{-1} in EMEM supplemented without FBS) were added to determine cell viability. Following 4 h of incubation at 22 ºC, the MTT solution was discarded, and the resulting formazan crystals were dissolved with 100 µL per well of DMSO. The plates were then shaken (5 min, 100 rpm) in darkness and absorbance was measured at 570 nm and 690 nm using a microplate reader.

2.5. Head-Kidney Leucocytes (HKLs) Isolation, Viability, and Immune Parameters

2.5.1. Animals

Twelve gilthead seabream (*S. aurata* L.) specimens obtained from a local farm (Mazarrón, Spain), with a mean body weight of 128.49 ± 3.84 g and a mean total length of 18.11 ± 0.25 cm, were acclimatized for one month in the Marine Fish Facilities of the University of Murcia (Spain). Fish were maintained in a recirculation system connected to individual 450 L tanks equipped with aeration, as well as biological and mechanical filtration. Water temperature was kept at 20 ± 2 °C, and salinity at 28‰ Water quality parameters including ammonia, nitrate and nitrite, were monitored weekly. The photoperiod was adjusted to a 12 h light and 12 h dark cycle. Fish were fed a commercial diet (Skretting, Spain) at a daily ration corresponding to 2% of the total biomass. All experimental procedures were approved by the Ethical Committee of the University of Murcia under permit number A13160416.

2.5.2. HKLs Isolation and Incubation

Fish were anesthetized with clove oil (20 mg L-1, Guinama®) and bled from the caudal vein. Excised head-kidney was cut into small pieces and leucocytes were isolated Esteban et al. [24]. All extracts prepared, as was mentioned in the section 2.1., were measured in an osmometer (Wescor), resuspended in sRPMI and dilutions for each concentration were prepared. λ-Carrageenan (Sigma, CAS Number: 9064-57-7) was similarly diluted into sterile PBS to prepare a stock solution of 10 mg mL^{-1}, and resuspended in sRPMI to obtain the final doses. For each fish and assay, samples of 500 µL of seabream HKLs (previously adjusted to 2 x 10^7 cells mL^{-1}) were dispensed into 24-well plates (Nunc). Two consecutive experiments were then conducted. In the first experiment, HKLs were exposed to 500 µL of each arnica extract solution to obtain final concentrations of 0, 0.001, 0.01, 0.125, 0.25, 0.5, and 1 mg mL^{-1}. Cells were incubated with each extract for 24 h at 25 °C, 85 % humidity and 5% CO_2. We selected the aqueous extract of arnica in order to develop a second experiment. In this case, HKLs were incubated with 250 µL of λ-carrageenan at a final concentration of 0 (PBS diluted in sRPMI; used as control) and 1,000 µg mL^{-1}, together with250 µL aqueous extract of arnica [at a final concentration of 0 (PBS diluted in sRPMI; control), 0.25 and 0.5 mg mL^{-1}], under the same conditions described for the first experiment. The concentrations of λ-carrageenan were selected by taking into

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account its leucocyte-activating capacity, previously tested in *in vitro* assays [25–27]. In both assays, HKLs obtained from 6 independent fish specimens were used separately, without pooling.

2.5.3. HKLs Viability

Viability of HKLs in both experiments was assessed by flow cytometry using propidium iodide (PI) to determine the abundance of viable leucocytes, according to Cuesta et al. [28]. Positive controls consisted of cells lysed with 50 µL of 0.02% cetyltrimethyl ammonium bromide (CTAB, Sigma), shaken (10 min, 60 rpm, in the dark), and then mixed with 50 µL of PI. Dead cells were quantified as the percentage of PI positive cells. For each sample, both the total leucocyte viability and the specific viability of acidophilic granulocytes (AGs, R1), monocytes/macrophages (MM, R2) and lymphocytes (R3) were determined, these populations being represented in dot plots according to SSC and FSC.

2.5.4. Immune Cellular Parameters

The peroxidase activity in HKLs was measured by using the method described by Quade & Roth [29] andstandard samples without HKLswere used as blanks. The respiratory burst activity of HKLs was assayed by chemiluminescence [30] and the kinetic of the reaction was assessed by calculating the slope min^{-1} of each curve. The phagocytic activity of HKLs was studied by flow cytometry using heat-killed lyophilized *Saccharomyces cerevisiae*, (strain S288C), labelled with fluorescein isothiocyanate (FITC, Sigma), according to Rodríguez et al. [31]. Standard samples of FITC-labelled *S. cerevisiae* or HKLs were included in each phagocytosis assay. Phagocytic ability was expressed as the percentage of cells with one or more ingested yeast (green-FITC fluorescent cells) within the phagocytic cell population, whilst the phagocytic capacity was the mean fluorescence intensity.

2.5.5. HKLs Ultrastructure

To determine possible morphological alterations in HKLs following incubation with λ-carrageenan and the aqueous arnica extract, samples were processed for transmission electron microscopy (TEM), according to Reynolds [33]. After 24 h of exposure, HKLs were centrifuged (400 *x g*, 5 min, 22 °C), washed in 250 µL of sRPMI and fixed with 200 µL of 2.5% glutaraldehyde prepared in 0.1 M cacodylate buffer (pH 7.2–7.4, 5-10 min, 4°C). Samples were then post-fixed for 2 h in 1% OsO4 and embedded in Epon. Semithin and ultrathin sections were obtained using a Reichert-Jung Ultracut.Semithin sections were stained with toluidine blue whereas ultrathin sections were contrasted with uranyl acetate and lead citrate. Ultrathin sections were examined using a transmission electron microscope (TEM, Zeiss EM 10C).

2.5.6. HKLs Gene Expression by Real-Time PCR

The sequences of the selected genes were retrieved from a gilthead seabream database [34]. The open reading frames (ORF) were identified using the ExPASy translation software (SIB Bioinformatics Resource Portal) and were further verified by sequence alignment analysis with NCBI BLAST (NIH). The primers (Table 1) were designed with the Thermo Fisher OligoPerfectTM tool, according to the following criteria: *i)* each individual oligonucleotide was composed of 20 nucleotides, *ii)* the size of the amplicon was between 100 and 120 nucleotides, *iii)* with a guanine-cytosine (GC)% between 55 % and 60 %, *iv)* an annealing temperature (Melting temperature) as close as possible to 60 °C, and *v)* the selection of primers that self-inhibit forming hairpins was avoided as far as possible, in order not to hinder the amplification reaction. After 24 h incubation of HKLs with λ-carrageenan and aqueous arnica extract, cells were collected by centrifugation (400 *x g*, 10 min, 22° C), and stored at -80°C until analysis. Total RNA was extracted using the Pure Link® RNA Mini Kit (Life Technologies) following the manufacturer 's instructions. RNA quantity and purity were evaluated with a Nanodrop® spectrophotometer, and the 260:280 ratios ranged from1.8 to 2.0. RNA samples were subsequently treated with DNase I (Promega) to eliminate genomic DNA contamination. Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using the

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SuperScript IV reverse transcriptase (Life Technologies) with an oligo-dT18 primer. In the present study, the expression of the selected genes, shown in Table 1, was analysed by real-time qPCR with QuantStudio™ Real-Time PCR System Fast (Life Technologies). Each reaction mixture contained 5 µL of SYBR Green supermix, 2.5 µL of primers (0.6 µM each) and 2.5 µL of cDNA template. Amplification conditions consisted of an initial incubation for 10 min at 95 ºC, followed by 40 cycles of 15 s at 95 ºC, 1 min at 60 ºC, and a final step of15 s at 95 ºC, 1 min at 60 ºC and 15 s at 95 ºC. Gene expression was calculated using the 2−ΔCt method with some modifications [35,36], . Reaction without cDNA were included as negative controls, and all samples were analysed in triplicate. For each mRNA, expression levels were normalized using the geometric mean of ribosomal protein (*18s*) and elongation factor 1-alfa (*ef1a*) RNA levels in each sample. Gene names followed the accepted zebrafish nomenclature (http://zfin.org/).

2.6. Statistical Analysis

Results were expressed as mean ± standard error of the mean (SEM). n the first experiment, data were analysed by One-way ANOVA followed by Tukey's post hoc test to identify differences between experimental groups. i. In the second experiment, data were analysed by Two-way ANOVA followed by Tukey 's post hoc test to evaluate the effects of *λ*-carrageenan and increasing concentrations of arnica. Data normality was previously assessed using the Shapiro-Wilk test, and homogeneity of variances was verified using by means of Levene 's test. All statistical analyses were carried out using SPSS software (25.0v; SPSS Inc., Chicago, IL, USA) for Windows. Statistical significance was established at $p < 0.05$ in all analyses.

Table 1. Primers used for real-time qPCR.

Gene name	Gene abbreviation	GenBank number	Primer sequences (5′→3′)
v-rel avian reticuloendotheliosis viral oncogene homolog A	*rela*	B030837	F: GAACCCCACCCTCATGAGTG R: GTTCTGGGCAGCAGTAGAGG
v-rel avian reticuloendotheliosis viral oncogene homolog B	*relb*	B012502	F: ACAGAGGAGGTGGAGGTCAG R: TATGGATCTGGGTTGTGCGG
v-rel avian reticuloendotheliosis viral oncogene homolog	*rel*	B018958	F: AAGCAAGAGCCCCAGATCAC R: TAGGGCGAGGAAGCAAGTTG
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	*nfkb1*	B005908	F: CCGACAGACGTTCACAGACA R: TCTTCAGCTGGACGAACACC
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2	*nfkb2*	B012900	F: ATCACAGCGCAGAGATCGAG R: TGCGGGATGTAGGTGAACTG
Interleukin 1	*il1b*	XM030416076.1	F: GCGAGCAGAGGCACTTAGTC R: GGTAGGTCGCCATGTTCAGT
Tumour necrosis factor alpha	*tnfa*	AJ413189	F: CTGTGGAGGGAAGAATCGAG

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			R: TCCACTCCACCTGGTCTTTC
Interleukin-10	*il10*	XM030420872	F: CTCACATGCAGTCCATCCAG R: TGTGATGTCAAACGGTTGCT
Transforming growth factor 1 beta	*tgfb*	AF424703	F: GCATGTGGCAGAGATGAAGA R: TTCAGCATGATACGGCAGAG
Caspase 1	*casp1*	DQ198376	F: CCAGATCGTGGGTGTTTTCT R: TCTTCAAAGCGTTGCATGAC
Caspase 3	*casp3*	DQ345773	F: AATTCACCAGGCTTCAATGC R: CTACGGCAGAGACGACATCA
Caspase 8	*casp8*	FJ225665	F: ACACGTGTGAACAGGGAGGT R: TTGAGGACGAGCTTCTTGGT
Caspase 9	*casp9*	DQ345775	F: AACGAGTGGGGTTGTTTCAG R: ATGGGTCCAAGTCTCTCACG
Ribosomal protein 18S	*rps18*	AM490061	F: CGAAAGCATTTGCCAAGAAT R: AGTTGGCACCGTTTATGGTC
Elongation factor-1 alfa	*ef1a*	AF184170	F: TGTCATCAAGGCTGTTGAGC R: GCACACTTCTTGTTGCTGGA

3. Results

3.1. First Experiment

3.1.1. Total Antioxidant Activity

The aqueous, ethanolic and methanolic extracts of arnica showed dose-dependent antioxidant activity. However, the two highest concentrations of the aqueous extract (0.5 and 1 mg mL^{-1}) presented higher antioxidant activity compared to the other concentrations tested (Fig. 1A). The dose of 0.25 mg mL^{-1} of the same aqueous extract showed higher activity compared to the lower ones (0, 0.001 and 0.01 mg mL^{-1}). In the case of the ethanolic extract, only the highest concentration showed higher antioxidant activity than the rest of lower concentrations (Fig. 1B). Moreover, the next two doses of ethanolic extract (0.25 and 0.5 mg mL^{-1}) resulted in higher antioxidant activity compared to the lowest doses of the same extract. The three higher concentrations of methanolic extract, on the other hand, resulted in higher antioxidant activity compared to the doses of 0, 0.001 and 0.01 mg mL^{-1} (Fig. 1C).

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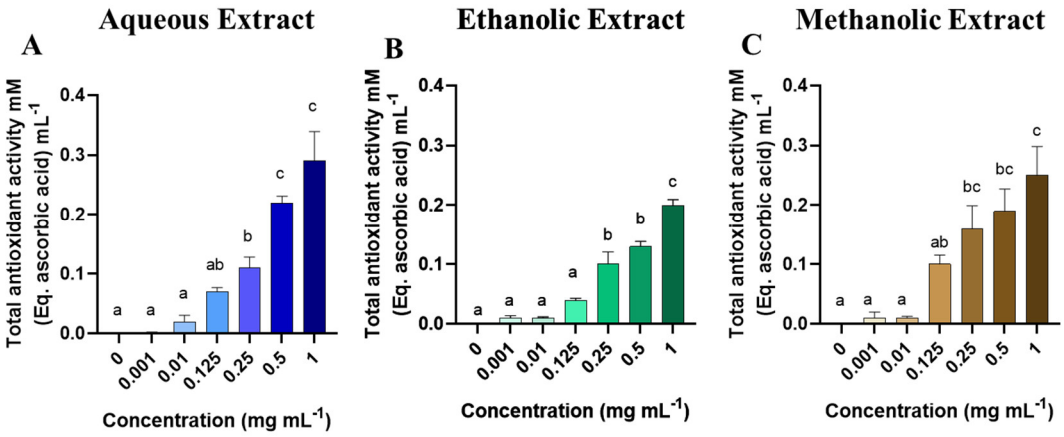

Figure 1. Total antioxidant activity (ascorbic acid equivalents) of different concentrations (0, 0.001, 0.01, 0.125, 0.0.25, 0.5, and 1 mg mL^{-1}) of (A) aqueous, (B) ethanolic and (C) methanolic extract obtained from arnica. Error bars represent the standard error of the means (n = 6). Different letters denote significant differences between control (0 mg mL^{-1}; PBS/DMSO diluted in PBS) and experimental concentrations (ANOVA; $p < 0.05$).

3.1.2. Bactericidal Activity

Regarding bactericidal activity, although no statistical differences were observed in the bactericidal activity against *V. harveyi* and *V. anguillarum* when incubated with the aqueous extract, the highest concentration (1 mg mL^{-1}) of this extract increased bactericidal activity against *P. damselae* compared to the other concentrations, except for the concentration of 0.5 mg mL^{-1} which showed no statistical differences (Fig. 2A). On the other hand, the same concentration (1 mg mL^{-1}) of the ethanolic and methanolic extracts showed an increase in bactericidal activity against *V. harveyi* compared to all other concentrations except the 0.5 mg mL^{-1} concentration. Moreover, a dose-dependent increase in bactericidal activity against *V. anguillarum* was observed when incubated with the three highest doses (0.25, 0.5 and 1 mg mL^{-1}) of ethanolic or methanolic extracts. In contrast, a decrease in bactericidal activity against *P. damselae* was found after incubation with 0.5 and 1 mg mL^{-1} of the ethanolic extract compared to the control and all other experimental doses tested. The highest dose of the methanolic extract also showed a high decrease in bactericidal activity against this pathogenic bacterium. No variations in the values of bactericidal activity against *T. maritimum* were detected after incubation with any dose of any of the extracts used in this study.

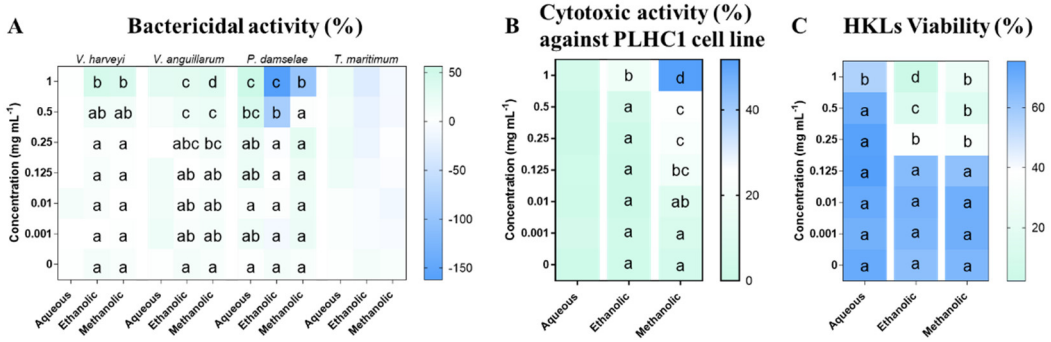

Figure 2. Heat map of (A) bactericidal activity against *Vibrio harveyi*, *V. anguillarum*, *Photobacterium damselae* and *Tenacibaculum maritimum*; (B) cytotoxic activity against PLHC1 tumour cell line; all incubated with different concentrations ; and (C) viability (%) of gilthead seabream head-kidney leucocytes; all incubated with different concentrations (0, 0.001, 0.01, 0.125, 0.0.25, 0.5 and 1 mg mL^{-1}) of aqueous, ethanolic and methanolic extracts of arnica. The colour scale on the right of the heat map represents the toxicity level, while green, white and blue colours indicate low, medium and high toxicity of the extract on the tested bacteria/cells, respectively.

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3.1.3. Cytotoxic Activity

The results of the cytotoxic assay revealed that the aqueous extract did not affect the viability of the PLHC1 cell line (Fig. 2B). Instead, an increase in cytotoxic activity was detected with the highest dose of the ethanolic extract compared to the other concentrations. Furthermore, the four highest doses of the methanolic extract (0.125, 0.25, 0.5 and 1 mg mL-*1*) showed a dose-dependent cytotoxic activity in the PLHC1 cell line compared to cells incubated with lower concentrations of this extract.

3.1.4. Total HKLs Viability

Our results revealed a decrease in the total viability of HKLs following 24 h of incubation with 1 mg mL^{-1} of aqueous extract compared to the values found in HKLs incubated with the other experimental doses (0, 0.001, 0.01, 0.125, 0.25 and 0.5 mg mL^{-1}) (Fig. 2C). Furthermore, HKLs incubated with the higher concentrations of ethanolic or methanolic extract (0.25, 0.5 and 1 mg mL^{-1}) showed a dose-dependent decrease in viability. On the other hand, although no significant differences were detected in the viability of AGs and lymphocytes incubated with any concentration of aqueous extract, the viability of MM decreased after incubation with 1 mg mL^{-1} of the same extract compared to the rest of the doses tested (Table 2). Interestingly, the viability of all cell types decreased dramatically after incubation with 1 mg mL^{-1} of both ethanolic and methanolic extracts. Moreover, this reduction in viability was dose-dependent in the case of MM and lymphocytes.

3.1.5. Immune Cellular Parameters

The peroxidase activity of HKLs decreased after 24 h of incubation with 1 mg mL^{-1} of aqueous, ethanolic and methanolic extracts compared to HKLs incubated with 0 (control) 0.001 and 0.01 mg mL^{-1} of the corresponding extract (Fig. 3A-3C). Moreover, this decrease was also statistically significant in HKLs incubated with 0.125, 0.25 and 0.5 mg mL^{-1} of methanolic extract, but not in HKLs incubated with the same concentrations of aqueous and ethanolic extracts. The respiratory burst activity of HKLs decreased in a dose-dependent manner, being totally inhibited when HKLs were incubated with 0.5 and 1 mg mL^{-1} of aqueous extract, and 0.125, 0.25, 0.5 and 1 mg mL^{-1} of ethanolic and methanolic extract, compared with the lowest doses tested and with the control (0 mg mL^{-1}) (Fig. 3D-3F).

Table 2. Viability (%) of gilthead seabream head-kidney leucocytes (acidophilic granulocytes, monocytes/macrophages and lymphocytes) after 24 h of incubation with different concentrations (0, 0.001, 0.01, 0.125, 0.0.25, 0.5, and 1 mg mL^{-1}) of aqueous, ethanolic or methanolic extracts obtained from arnica. Data represent the mean ± standard error (n = 6). Different letters denote significant differences between control (0 mg mL^{-1}; PBS diluted in RPMI medium) and experimental concentrations (ANOVA; $p < 0.05$).

Head-kidney leucocytes	**Aqueous extract concentration (mg mL^{-1})**						
	0	**0.001**	**0.01**	**0.125**	**0.25**	**0.5**	**1**
Acidophilic granulocytes	65.08 ± 3.15	61.42 ± 0.67	63.86 ± 4.52	59.31 ± 7.56	51.60 ± 9.44	54.39 ± 6.80	43.04 ± 5.14
Monocytes/macrophages	86.85 ± 1.38 a	84.10 ± 2.00 a	86.04 ± 1.29 a	86.24 ± 0.79 a	84.94 ± 1.96 a	77.75 ± 2.70 a	63.72 ± 2.99 b
Lymphocytes	88.78 ± 1.15	88.90 ± 0.77	89.41 ± 0.16	89.36 ± 0.85	90.21 ± 1.01	92.09 ± 0.13	92.17 ± 0.75
Head-kidney leucocytes	**Ethanolic extract concentration (mg mL^{-1})**						
	0	**0.001**	**0.01**	**0.125**	**0.25**	**0.5**	**1**
Acidophilic granulocytes	54.93 ± 3.93 a	53.47 ± 5.06 a	56.43 ± 2.13 a	64.37 ± 3.18 a	66.74 ± 4.37 a	68.15 ± 1.54 a	0.00 ± 0.00 b
Monocytes/macrophages	79.85 ± 2.89 ab	81.70 ± 2.64 ab	82.38 ± 2.86 ab	84.53 ± 1.26 a	71.15 ± 4.43 bc	62.95 ± 2.57 c	0.47 ± 0.12 d

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Lymphocytes	83.46 ± 1.61 ab	87.86 ± 1.14 a	86.10 ± 1.79 ab	85.60 ± 2.61 ab	75.62 ± 1.92 bc	71.44 ± 3.92 c	0.00 ± 0.00 d

Head-kidney leucocytes	**Methanolic extract concentration (mg mL^{-1})**						
	0	**0.001**	**0.01**	**0.125**	**0.25**	**0.5**	**1**
Acidophilic granulocytes	56.99 ± 2.16 a	60.45 ± 1.74 a	51.58 ± 3.98 a	55.44 ± 5.60 a	62.78 ± 0.60 a	66.9 ± 3.41 a	0.75 ± 0.35 b
Monocytes/macrophages	82.14 ± 1.63 ab	86.92 ± 1.00 a	83.14 ± 1.06 a	83.71 ± 1.40 a	75.14 ± 2.03 b	58.48 ± 2.86 c	2.16 ± 0.93 d
Lymphocytes	85.69 ± 1.03 a	87.44 ± 1.28 a	88.15 ± 0.03 a	87.83 ± 0.56 a	79.87 ± 1.19 a	75.64 ± 2.77 a	24.11 ± 9.24 b

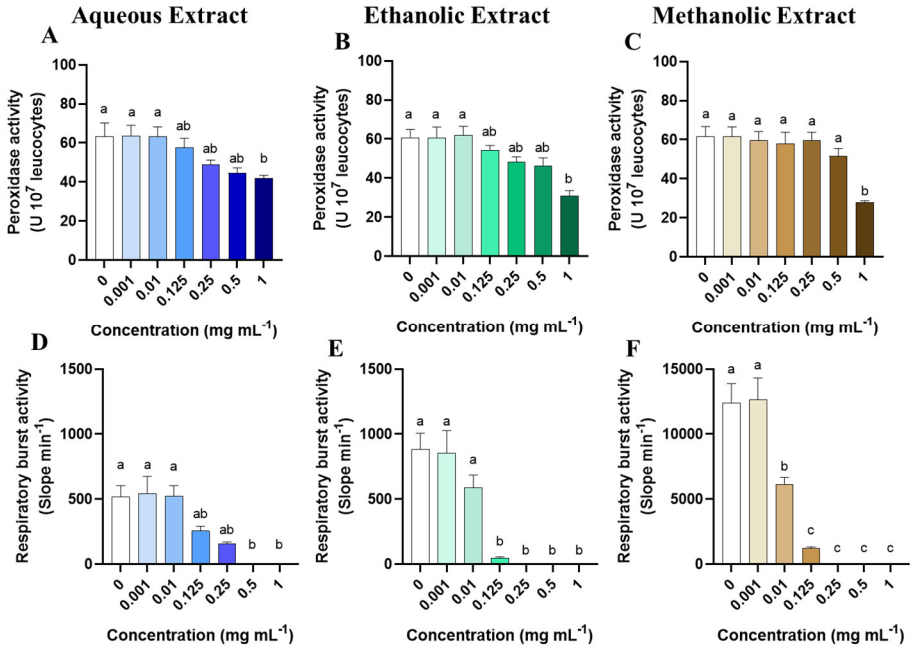

Figure 3. Peroxidase activity (U 10^7 leucocytes) and respiratory burst activity (Slope/min. a.u. luminiscence) of gilthead seabream head-kidney leucocytes after 24 h of incubation with different concentrations (0, 0.001, 0.01, 0.125, 0.0.25, 0.5, and 1 mg mL^{-1}) of (A, D) aqueous, (B, E) ethanolic and (C, F) methanolic extract obtained from arnica. Error bars represent the standard error of the means (n = 6). Different letters denote significant differences between control (0 mg mL^{-1}; PBS/DMSO diluted in PBS) and experimental concentrations (ANOVA; $p < 0.05$).

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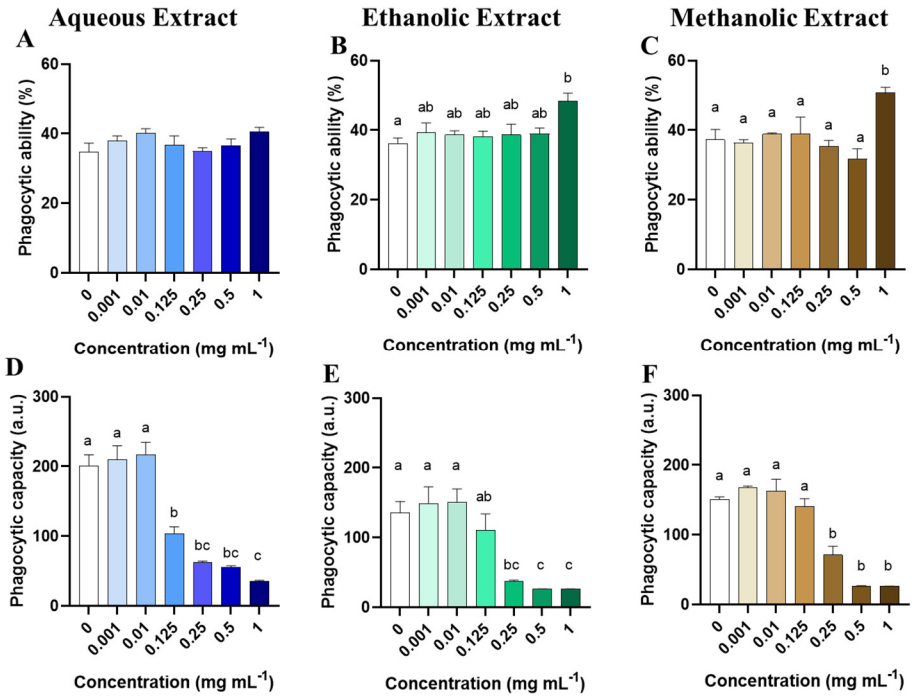

Figure 4. Phagocytic ability (%) and capacity (arbitrary units, a.u.) of gilthead seabream head-kidney leucocytes after 24 h of incubation with different concentrations (0, 0.001, 0.01, 0.125, 0.0.25, 0.5, and 1 mg mL^{-1}) of (A, D) aqueous, (B, E) ethanolic and (C, F) methanolic extract obtained from arnica. Error bars represent the standard error of the means (n = 6). Different letters denote significant differences between control (0 mg mL^{-1}; PBS/DMSO diluted in PBS) and experimental concentrations (ANOVA; $p < 0.05$).

Although no variations were recorded in the phagocytic ability of HKLs incubated with the aqueous extract, increased phagocytic ability was observed at 1 mg mL^{-1} for the ethanolic or methanolic extract, compared to control HKLs samples. Moreover, in the case of the methanolic extract, this increase was also statistically significant with the concentrations (0.001, 0.01 and 0.125 mg mL^{-1}) (Fig. 4A-4C). In contrast, HKLs incubated with any of the three extracts followed a dose-dependent inhibition of phagocytic capacity when incubated with 0.25, 0.5 and 1 mg mL^{-1} compared to the lowest doses tested. In addition, this activity also decreased in HKLs incubated with 0.125 mg mL^{-1} of the aqueous extract in comparison with the lowest doses (Fig. 4D-4F).

3.2. Second Experiment

3.2.1. Leucocyte Viability

Results of total viability did not show significant differences in HKLs incubated with any doses of λ-carrageenan and aqueous extract of arnica tested (data not shown). Regarding the viability of leucocyte subpopulations, the viability of AGs decreased in a dose-dependent manner when incubating with arnica (0.25 and 0.5 mg mL^{-1}) and in the absence of λ-carrageenan (Table 3). However, the viability of AGs incubated with λ-carrageenan decreased with highest doses of arnica (0.5 mg mL^{-1}). The viability of MMs decreased by arnica (0.5 mg mL^{-1}) compared to control. In contrast, lymphocyte viability increased slightly with the aqueous extract of arnica (0.25 and 0.5 mg mL^{-1}) independently of the presence/absence of λ-carrageenan.

Table 3. Viability (%) of head-kidney leucocytes of gilthead seabream (acidophilic granulocytes, monocytes/macrophages and lymphocytes) after 24 hours of incubation with different concentrations of aqueous extract of arnica (0, 0.25 and 0.5 mg mL^{-1}) and λ-carrageenan (0 and 1000 μg mL^{-1}). Data represent the mean ± standard error (n = 6). Capital letters denote significant differences between the control of the aqueous extract of arnica (0 mg mL^{-1}; PBS diluted in RPMI medium) and the experimental concentrations, while lower case denotes

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significant differences between the control of λ-carrageenan (0 mg mL^{-1}; PBS diluted in RPMI medium) and the experimental concentrations (Two-way ANOVA; $p < 0.05$).

Head-kidney leucocytes	Arnica concentration (mg mL^{-1})			λ-carrageenan concentration (μg mL^{-1})
	0	0.25	0.5	
Acidophilic granulocytes	84.56 ± 0.39a	57.49 ± 1.33b	34.32 ± 2.70c	**0**
	66.24 ± 4.18a	59.03 ± 4.47a	38.27 ± 5.73b	**1,000**
Monocytes/Macrophages	83.45 ± 0.59a	70.86 ± 4.96ab	57.58 ± 2.87b	**0**
	71.15 ± 4.62	69.09 ± 4.59	53.96 ± 5.03	**1,000**
Lymphocytes	90.77 ± 0.72a	96.32 ± 0.43b	96.49 ± 0.49b	**0**
	92.88 ± 0.40a	95.89 ± 0.30b	96.83 ± 0.33b	**1,000**

3.2.2. Cellular Immune Parameters

Peroxidase activity of HKLs did not show variations by the incubation with any of the combinations of λ-carrageenan and arnica (Fig. 5A). Respiratory burst activity increased in the HKLs incubated with only λ-carrageenan compared to control (0 μg mL^{-1} of λ-carrageenan and 0 mg mL^{-1} of arnica) (Fig. 5B). However, this activity decreased in a dose-dependent manner with increasing doses of arnica and 1,000 μg mL^{-1} of λ-carrageenan. Regarding phagocytic activities, the phagocytic ability of HKLs was not affected by λ-carrageenan and arnica (Fig. 5C). Otherwise, the phagocytic capacity decreased in HKLs by λ-carrageenan and without arnica (0 mg mL^{-1}) compared to control cells (Fig. 5D). In addition, this activity decreased in a dose-dependent manner with increasing doses of arnica and without λ-carrageenan (0 μg mL^{-1}). However, the mixture of both substances only decreased the phagocytic capacity of HKLs incubated with the highest concentration of arnica (0.5 mg mL^{-1}) and λ-carrageenan.

3.2.3. Ultrastructure of leucocytes

The electron microscopic study did not reveal morphological variations in AGs, MMs and lymphocytes incubated with the control solutions of the two mixtures of λ-carrageenan, or arnica prepared (Fig. 6A-6C). Nonetheless, clear ultrastructural alterations were detected on AGs and MMs by λ-carrageenan (1,000 μg mL^{-1}) and arnica (0.5 mg mL^{-1}) (Fig. 6D, 6E). Both cells went from a more rounded shape to a more elongated irregular and larger one with large cytoplasmic prolongations, secondary lysosomes in the cytoplasm and even with the visualization of the phagocytosis of cellular debris. No morphological variations were detected on lymphocytes exposed to the same mixtures (Fig. 6F).

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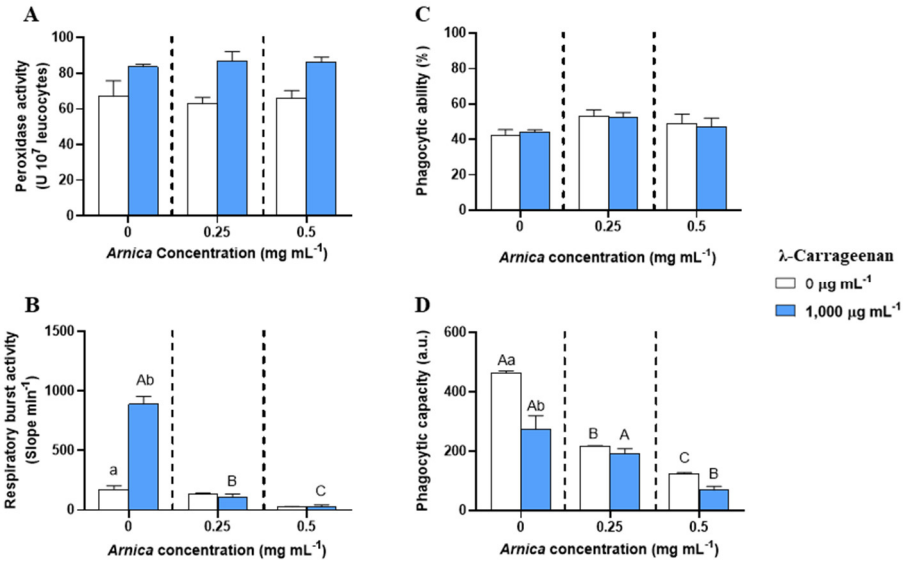

Figure 5. Peroxidase activity (U 107 leucocytes) (A), respiratory burst activity (slope min-1) (B), phagocytic ability (%) (C) and phagocytic capacity (a.u.) (D) of head-kidney leucocytes of gilthead seabream after 24 hours of incubation with different concentrations aqueous extract of arnica (0 and 0.5 mg mL-1) and λ-carrageenan (0 and 1,000 µg mL-1). Data represent the mean ± standard error (n = 6). Different letters denote significant differences between experimental concentrations of cantharidin (Two-way ANOVA; $p < 0.05$).

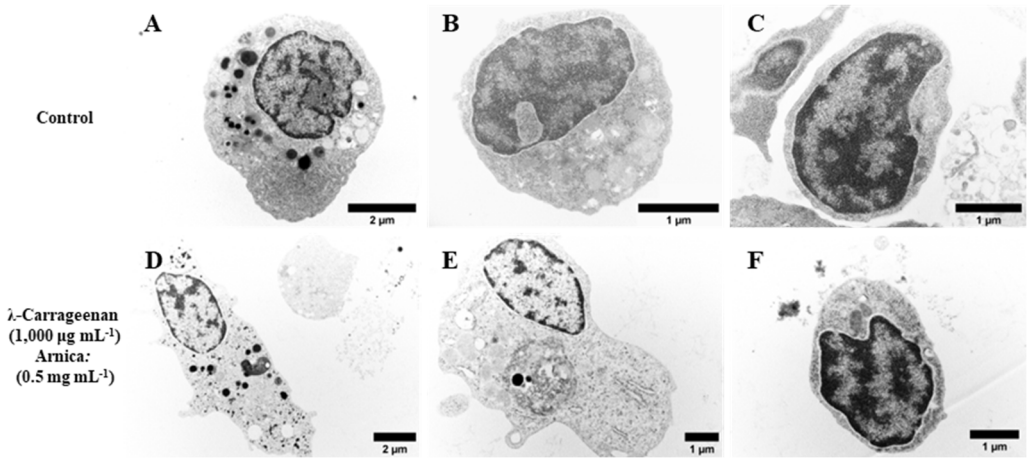

Figure 6. Transmission electron micrographs of head-kidney leucocytes of gilthead seabream after 24 hours of incubation with arnica (0.5 mg mL^{-1}) and λ-carrageenan (1,000 µg mL^{-1}). Acidophilic granulocyte (A), Scale bar = 2µm; monocyte/macrophage (B) Scale bar = 1µm; lymphocyte (C) Scale bar = 1µm.

3.2.4. Gene Expression Analysis

The gene expression profile of NF-κB subunits (*rela*, *relb*, *crel*, *nfkb1* and *nfkb2*), two proinflammatory cytokines (*il1b* and *tnfa*), two anti-inflammatory cytokines (*il10* and *tgfb*) and four caspases involved in apoptosis cell death (*casp1*, *casp3*, *casp8* and *casp9*) was analysed. The data are summarized in Fig. 7. First, the expression of *crel* and *casp9* was down-regulated in HKLs by λ-carrageenan (1,000 µg mL^{-1}) compared to control. Furthermore, the expression of these both genes was also down-regulated by arnica (0.5 mg mL^{-1}). In contrast, the expression of *il1b* gene was up-regulated by arnica (0.5 mg mL^{-1}). No statistical variations were detected in the values of the expression of *rela*, *relb*, *nfkb1*, *nfkb2*, *tnfa*, *il10*, *tgfb*, *casp1*, *casp3* and *casp8* genes in HKLs incubated with any of the mix tested.

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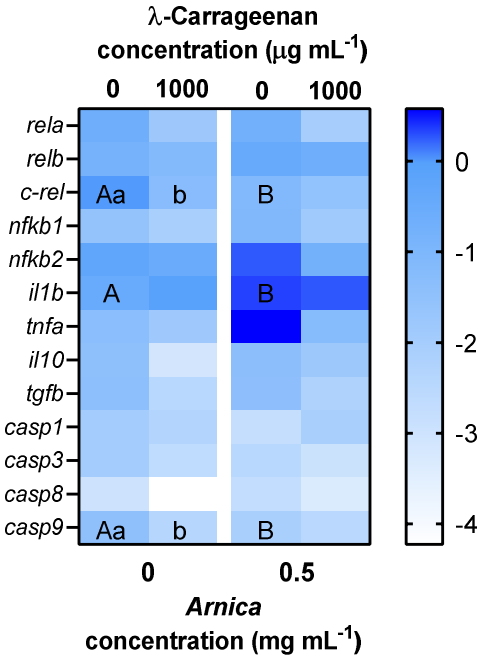

Figure 7. Heatmap of relative expression of proinflammatory (*rela*, *relb*, *crel*, *nfkb1*, *nfkb2*, *il1b*, *tnfa*), anti-inflammatory (*il10*, *tgfb*) and apoptotic (*casp1*, *casp3*, *casp8*, *casp9*) gene of head-kidney leucocytes of gilthead seabream after 24 hours of incubation with different concentrations of aqueous extract of arnica (0 and 0.5 mg mL^{-1}) and λ-carrageenan (0 and 1,000 μg mL^{-1}). Data represent the mean ± standard error (n = 6). Different letters denote significant differences between experimental concentrations of λ-carrageenan (Two-way ANOVA; $p < 0.05$).

All the results of the effects of arnica extracts analysed in the present work have been summarized in a schematic summary of the observed cellular effects (Fig. 8).

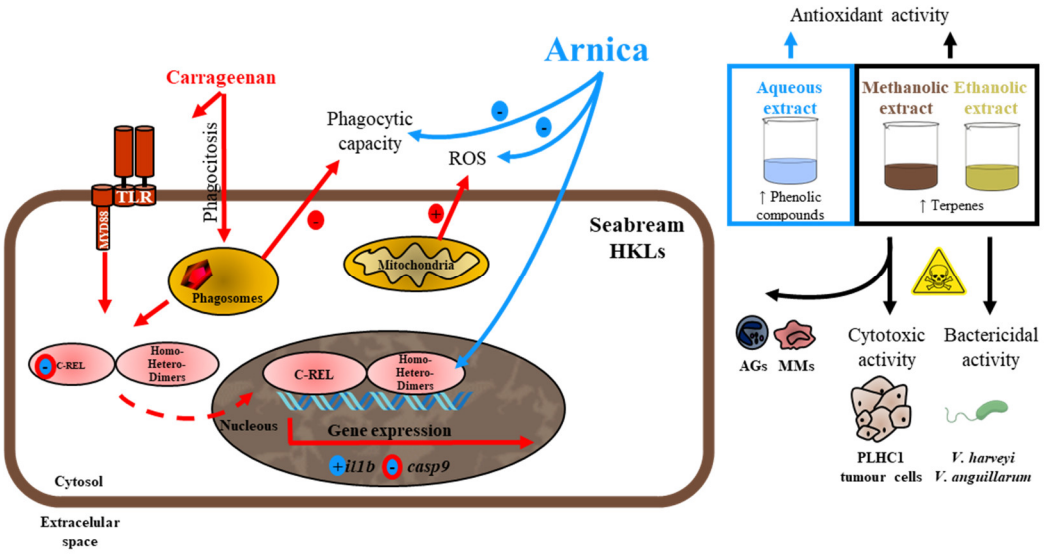

Figure 8. Proposed schematic model of carrageenan-transduction pathway, and effects of aqueous extract of arnica after 24 h of exposure with acidophilic granulocytes and monocytes/macrophages in the head kidney of gilthead seabream. Symbols + and – represent induction and repression interactions, while red and blue arrows point to λ-carrageenan and aqueous extract of arnica functions, respectively. Black arrow represents the cytotoxic and bactericidal activity of the methanolic and ethanolic extracts. HKLs: Head kidney leucocytes; AGs: Acidophilic granulocytes; MMs: Monocytes/Macrophages.

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4. Discussion

The present study compared the biological activity of different traditional preparations of arnica in fish *in vitro* assays, with the aim of determining whether extraction solvent influences responses in pathogens and host cells. Aqueous preparations were included to resemble infusions, whereas ethanolic and methanolic extracts were included to represent alcoholic macerates, since solvent and extraction conditions are known to influence the chemical profile of recovered compounds [37]. The concentration range (from 0.001 to 1 mg mL^{-1}) was selected based on previous *in vitro* work in gilthead seabream leucocytes and fish cell lines using medicinal plant extracts such as *Origanum vulgare* and *Lavandula sp*., where responses depend strongly on extract type and dose [23,38]. To date, most publications on arnica have focused on its phytochemical composition, which have confirmed and identified a high presence of monoterpenes (camphor, borneol and nerolidol mainly), sesquiterpenes (lucinone, glutinone and kudtriol), polyphenolic compounds such as flavonoids (quercetin and kaempferol), among the major classes described [9,39–41]. In this scenario, solvent comparisons in this and other species such as *Rosmarinus officinalis* L., *Thymus vulgaris* L. and *O. vulgare* L. indicate that aqueous extracts often recover a higher proportion of phenolic acids (mainly dicaffeoylquinic acids), whereas organic solvents such as ethanol and methanol tend to recover more lipophilic constituents such as terpenoid compounds [42,43]. Thus, this background may help to contextualise the differences found in this study between arnica preparations.

Among the biological activities commonly reported for medicinal plants, antioxidant capacity is frequently described and is linked to radical scavenging and metal chelation, thereby limiting damage caused by oxidative stress [44,45]. Within this context, in our study all three extracts showed antioxidant activity in a dose-dependent manner indicating that this property is retained across the traditional preparations tested under the present conditions. Similar dose-related patterns have been widely described for plant extracts evaluated in comparable *in vitro* assays, where the effect becomes more evident as concentration increases [23,37,43].

Another relevant aspect when studying medicinal plants is their potential activity against fish bacterial pathogens, since opportunistic infections represent one of the main constraints in marine aquaculture [46,47]. Previous *in vitro* studies have reported anti-protozoal activity of arnica ketone extracts against *Leishmania donovani* and *Entamoeba histolytica* [48]. Furthermore, arnica ethyl acetate and dichloromethane extracts showed *in vitro* antifungal activity against *Candida albicans*, and *Rhizopus stolonifera*, and antimicrobial activity against several human bacterial pathogens such as *Mycobacterium phlei*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* [49]. However, information about its effects on fish pathogens is still scarce. In the present study, the bactericidal activity of the three extracts was evaluated against four opportunistic marine bacteria (*V. harveyi*, *V. anguillarum*, *P. damselae* and *T. maritimum*), selected due to their relevance as causative agents of disease in farmed marine species and their associated economic impact [50,51]. In addition, it is also important to consider that all tested species were Gram-negative marine bacteria, which are generally less susceptible to plant extracts than Gram-positive bacteria, a factor that may partly explain the moderate activity observed in some cases [52,53]. Under the conditions tested in our assay, the aqueous extract showed a limited bactericidal activity, with a significant reduction only detected against *P. damselae* at the highest concentration. In contrast, ethanolic and methanolic extracts showed clearer activity at elevated doses, particularly against *V. harveyi* and *V. anguillarum*, where the highest concentrations were associated with a more marked reduction in bacterial viability. These observations are in agreement with previous similar *in vitro* studies carried out with other medicinal plants such as *O. vulgare* L. and *Ceratonia siliqua* L, in which organic solvent extracts often show stronger antibacterial effects than aqueous preparations and the response vary depending on the pathogen analysed [17,23]. Interestingly, a decrease in bactericidal activity against *P. damselae* was observed at the highest concentrations of the ethanolic and methanolic extracts, which contrasts with the response recorded for *V. harveyi* and *V. anguillarum*. In this context, it has been reported that *P. damselae* is able to degrade extracellular lipids and use them as a carbon and energy source, which can favour its growth under suitable conditions [54]. This metabolic capacity could partly explain the

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distinct behaviour observed in our assays at the highest concentrations, where the presence of additional organic material from the extracts may have supported bacterial persistence instead of limiting it. However, further studies would be needed to better understand the mechanisms underlying this response.

In addition to the aforementioned bioactivities, the evaluation of cytotoxicity is essential to define the concentration range in which cell-based responses can be interpreted. In the present study, ethanolic and methanolic extracts produced a strong reduction in viability at moderate and high concentrations, both in the PLHC1 tumour cell line and in HKLs, whereas the aqueous extract showed a comparatively lower impact. The marked response observed in the PLHC1 tumour cell line could suggest that the alcoholic extracts may contain bioactive components able to interfere with cellular survival, and this is consistent with previous studies reporting cytotoxic effects of ethanolic plant extracts evaluated in tumour cell lines, including *O. vulgare* extracts tested on human lung adenocarcinoma A549 cells and *Moringa stenopetala* ethanolic leaf and seed extracts tested on human hepatoblastoma HepG2 cells [18,43]. In this context, certain lipophilic compounds previously described in arnica, such as the monoterpene borneol, which are more efficiently extracted in alcoholic preparations than in water, have shown *in vitro* cytotoxic effects on human epithelial colorectal carcinoma cells [55,56], which is consistent with the stronger effects observed for alcoholic preparations compared to the aqueous one.

HKLs constitute the main effector population of the innate immune response in gilthead seabream, where AGs and monocyte-macrophages actively participate in phagocytosis and other defence mechanisms in response to external stimuli or foreign substances [24]. In addition, AGs can trigger respiratory burst activity, increasing oxygen consumption and the production of reactive oxygen species (ROS) through the activation of NADPH oxidase, and can also promote degranulation mechanisms with the release of peroxidase enzymes (with antimicrobial activity) into the extracellular space [57,58]. In the first experiment, our results showed an evident reduction in peroxidase activity, respiratory burst and phagocytic capacity at concentrations that also reduced viability, particularly for ethanolic and methanolic extracts. When focusing on concentrations compatible with viability, peroxidase activity remained largely unchanged for aqueous and ethanolic extracts, respiratory burst showed a clearer reduction for the methanolic extract at low-medium concentrations (0.01 and 0.125 mg mL^{-1}), and phagocytic capacity was reduced by the aqueous extract at intermediate concentrations (0.125, 0.25, 0.5 mg mL^{-1}), pointing to a possible inhibitory effect on these specific functions even in the absence of marked loss of viability. These results are in agreement with several *in vitro* studies that reported that not only direct application of plant extracts at high doses to HKLs did not have a stimulatory effect on the immune cells, but also decreased their activity [2,38]. In sharp contrast, dietary administration of arnica in this same species has been associated with immunostimulatory effects after 15 days, including increased phagocytic activity in HKLs and higher peroxidase levels in skin mucus [16], which highlights the differences between direct *in vitro* exposure and systemic responses *in vivo*.

Based on these observations, the aqueous extract was selected for a second assay in HKLs stimulated with λ-carrageenan, using concentrations that were compatible with viability (0, 0.25 and 0.5 mg mL^{-1}). The carrageenan doses (0 and 1,000 μg mL^{-1}) were selected based on their established capacity to activate HKLs including stimulation of phagocytosis and ROS related responses and the up-regulation of proinflammatory genes such as *il1b*, *tnfa*, and *il6* [25]. This ability comes from its atypical structure with sulphated moieties and unusual α-1,3-galactosidic linkages, characteristics that seem to be crucial in its action mechanisms to stimulate fish leucocytes *in vitro*, similar to what happens in mammals [25]. In agreement with this rationale, HKLs exposed to λ-carrageenan alone showed the expected activation profile including changes in phagocytosis and respiratory burst activity. Thus, when the aqueous extract of arnica was applied after this stimulation, respiratory burst and phagocytic activity tended to return towards values closer to controls levels, suggesting an immunomodulatory effect of arnica on previously activated leucocytes. In addition, ultrastructural changes in AGs and MMs evidenced by TEM after the exposure of high doses of λ-carrageenan and

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arnica showed morphological features associated with activation, including cytoplasmic extensions and the presence of secondary lysosomes compatible with active phagocytic processes, as previously evidenced in an *in vitro* assay developed by our research group (probably due to the effects of λ-carrageenan) [25]. This activation was also corroborated *in vivo*, where carrageenan was able to produce recruitment of acidophilic granulocytes and monocytes/macrophages as well as other cell types such as skin mucus-secreting cells and adipocytes shortly after intramuscular injection [59,60].

Regarding the gene expression analysis, we focused first on the expression of NF-κB subunits (RelA, RelB, C-Rel, NF-κB1, and NF-κB2) given their central role in coordinating transcriptional regulation of cytokines and proinflammatory molecules [61]. In mammals, RelA:NF-κB1 heterodimers are typically associated with canonical inflammation signalling pathway, whereas RelB:NF-κB2 combinations constitute a non-canonical or alternative activation pathway, with the c-REL subunit relegated to a secondary role in this process [62]. In our study, the gene expression of *crel* was down-regulated in HKLs incubated with λ-carrageenan or arnica in line with previous work from our group suggesting that c-REL may reflect a relevant regulatory node in fish leucocytes [63,64]. In addition, caspase-9, which is a responsible for activating downstream executioner caspases (caspase-3, -6 and -7), is a critical initiator, and therefore regulator, of the intrinsic pathway of apoptosis [65]. Then, the down-regulation of *casp9* by λ-carrageenan and arnica could be another regulatory mechanism avoiding the initiation of unnecessary apoptosis. Thus, although arnica has been related with an important anti-inflammatory agent, the up-regulation of *il1b* that we found in this study could be better interpreted as a marker of cellular activation or stress, or changes in the proportion of responsive subpopulations within the HKL pool, rather than a change in inflammatory status. This interpretation is consistent with *in vitro* studies showing that direct exposure of HKLs to plant extracts do not have to present the same effect as expected in *in vivo* experiments [2,17,38].

5. Conclusions

The present work provides an *in vitro* comparison of traditional aqueous and alcoholic preparations of arnica in fish-related *in vitro* assays, the three extracts tested displaying dose-dependent antioxidant capacity, while their effects on bacteria and cells differed clearly across preparations and concentrations, particularly when cell viability was taken into account. Ethanolic and methanolic extracts showed the strongest bactericidal activity at the highest concentrations and the most pronounced reduction in PLHC1 viability, while the aqueous extract showed its ability to reduce immune parameters in HKLs previously stimulated with λ-carrageenan. Overall, these results provide a first comparative basis to guide future work on arnica preparations in fish.

Author Contributions: J.C.C.S.: Methodology, Investigation, Writing—original draft preparation, Writing—review & editing, Visualization. F.A.G.: Methodology, Validation, Formal analysis, Investigation, Data curation, Writing—original draft preparation, Writing—review & editing, Visualization. M.Á.E.: Conceptualization, Validation, Investigation, Resources, Writing—review & editing, Visualization, Supervision, Project administration, Funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the EU Directive 2010/63/EU and was approved by the UMU Ethics Committee (Permit No. A13160416 and approval date: 24 September 2014).

Informed Consent Statement: Not applicable.

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Data Availability Statement: The data are available on the DIGITUM institutional repository from the University of Murcia: http://hdl.handle.net/10201/131623 (accessed on 30 May 2023).

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Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Adel, M.; Yeganeh, S.; Dadar, M.; Sakai, M.; Dawood, M.A.O. Effects of Dietary *Spirulina platensis* on Growth Performance, Humoral and Mucosal Immune Responses and Disease Resistance in Juvenile Great Sturgeon (*Huso huso* Linnaeus, 1754). Fish Shellfish Immunol. 2016, 56, 436–444, doi:10.1016/j.fsi.2016.08.003.
2. García Beltrán, J.M.; Espinosa, C.; Guardiola, F.A.; Esteban, M.Á. Dietary Dehydrated Lemon Peel Improves the Immune but Not the Antioxidant Status of Gilthead Seabream (*Sparus aurata* L.). Fish Shellfish Immunol. 2017, 64, 426–436, doi:10.1016/j.fsi.2017.03.042.
3. Van Hai, N. The Use of Medicinal Plants as Immunostimulants in Aquaculture: A Review. Aquaculture 2015, 446, 88–96, doi:10.1016/j.aquaculture.2015.03.014.
4. Reverter, M.; Bontemps, N.; Lecchini, D.; Banaigs, B.; Sasal, P. Use of Plant Extracts in Fish Aquaculture as an Alternative to Chemotherapy: Current Status and Future Perspectives. Aquaculture 2014, 433, 50–61, doi:10.1016/j.aquaculture.2014.05.048.
5. Harikrishnan, R.; Balasundaram, C.; Heo, M.S. Impact of Plant Products on Innate and Adaptive Immune System of Cultured Finfish and Shellfish. Aquaculture 2011, 317, 1–15, doi:10.1016/j.aquaculture.2011.03.039.
6. Oulahal, N.; Degraeve, P. Phenolic-Rich Plant Extracts with Antimicrobial Activity: An Alternative to Food Preservatives and Biocides? Front. Microbiol. 2022, 12, doi:10.3389/fmicb.2021.753518.
7. Su, H.; Yakovlev, I.A.; van Eerde, A.; Su, J.; Clarke, J.L. Plant-Produced Vaccines: Future Applications in Aquaculture. Front. Plant Sci. 2021, 12, doi:10.3389/fpls.2021.718775.
8. Muñoz-Centeno, L.; Rico, E. Chiliadenus Cass. In Flora Iberica. Plantas Vasculares de la Península Ibérica e Islas Baleares. Vol. XVI (III) Compositae (Partim), 1st ed.; Real Jardín Botánico, Consejo Superior de Investigaciones Científicas: Madrid, Spain, 2019; pp. 2092–2095.
9. Guillén, M.D.; Ibargoitia, M.L. Volatile Components Obtained from the Leaves of *Jasonia glutinosa*. Food Chem. 1996, 56, 155–158, doi:10.1016/0308-8146(95)00156-5.
10. Obón, C.; Rivera, D.; Verde, A.; Fajardo, J.; Valdés, A.; Alcaraz, F.; Carvalho, A.M. Árnica: A Multivariate Analysis of the Botany and Ethnopharmacology of a Medicinal Plant Complex in the Iberian Peninsula and the Balearic Islands. J. Ethnopharmacol. 2012, 144, 44–56, doi:10.1016/j.jep.2012.08.024.
11. Pardo de Santayana, M.; Morales, R. Consideraciones Sobre *El Género Jasonia* (Compositae, Inuleae). *Sistemática y Usos.* Acta Bot. Malacit. 2004, 29, 221–232, doi:10.24310/abm.v29i0.7232.
12. Las Heras Etayo, N.; Llamas, F.; Acedo, C. Ethnobotanical Research and Compilation of the Medicinal Uses in Spain and the Active Principles of *Chiliadenus glutinosus* (L.) Fourr. for the Scientific Validation of Its Therapeutic Properties. Plants 2021, 10, 1–21, doi:https://doi.org/10.3390/plants10030584.
13. Bermejo, B.P.; Abad, M.J.; Díaz, A.M.; Villaescusa, L.; González, M.A.; Silván, A.M. Sesquiterpenes from *Jasonia glutinosa*: *In vitro* Anti-Inflammatory Activity. Biol. Pharm. Bull. 2002, 25, 1–4, doi:10.1248/bpb.25.1.
14. Valero, M.S.; Oliván-Viguera, A.; Garrido, I.; Langa, E.; Berzosa, C.; López, V.; Gómez-Rincón, C.; Murillo, M.D.; Köhler, R. Rock Tea Extract (*Jasonia glutinosa*) Relaxes Rat Aortic Smooth Muscle by Inhibition of L-Type Ca^{2+} Channels. J. Physiol. Biochem. 2015, 71, 785–793, doi:10.1007/s13105-015-0442-8.
15. Castro, M.; Ramón-Giménez, M.; Les, F.; Trejo, L.; Plaza, M.Á.; López, V.; Murillo, M.D.; Valero Gracia, M.S. Spasmolytic Effect of *Jasonia glutinosa* on Rodent Intestine. Rev. Esp. Enfermedades Dig. 2016, 108, 785–789, doi:10.17235/reed.2016.4327/2016.

 doi:10.20944/preprints202603.2406.v1

16. Espinosa, C.; García Beltrán, J.M.; Messina, C.M.; Esteban, M.Á. Effect of *Jasonia glutinosa* on Immune and Oxidative Status of Gilthead Seabream (*Sparus aurata* L.). Fish Shellfish Immunol. 2020, 100, 58–69, doi:10.1016/j.fsi.2020.02.068.
17. Othmen, K. Ben; Elfalleh, W.; García Beltrán, J.M.; Esteban, M.Á.; Haddad, M. An *In vitro* Study of the Effect of Carob (*Ceratonia siliqua* L.) Leaf Extracts on Gilthead Seabream (*Sparus aurata* L.) Leucocyte Activities. Antioxidant, Cytotoxic and Bactericidal Properties. Fish Shellfish Immunol. 2020, 99, 35–43, doi:10.1016/j.fsi.2020.02.005.
18. Mekonnen, N.; Houghton, P.; Timbrell, J. The Toxicity of Extracts of Plant Parts of *Moringa Stenopetala* in HEPG2 Cells *In vitro*. Phyther. Res. 2005, 19, 870–875, doi:10.1002/ptr.1720.
19. Arnao, M.B.; Cano, A.; Acosta, M. Methods to Measure the Antioxidant Activity in Plant Material. A Comparative Discussion. Free Radic. Res. 1999, 31, doi:10.1080/10715769900301371.
20. Guardiola, F.A.; Cuartero, M.; del Mar Collado-González, M.; Díaz Baños, F.G.; Cuesta, A.; Moriñigo, M.Á.; Esteban, M.Á. Terminal Carbohydrates Abundance, Immune Related Enzymes, Bactericidal Activity and Physico-Chemical Parameters of the Senegalese Sole (*Solea senegalensis*, Kaup) Skin Mucus. Fish Shellfish Immunol. 2017, 60, 483–491, doi:10.1016/j.fsi.2016.11.025.
21. Berridge, M.; Tan, A. Characterization of the Cellular Reduction of 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT): Subcellular Localization, Substrate Dependence, and Involvement of Mitochondrial Electron Transport in MTT Reduction. Arch Biochem Biophys 1993, 303, 474–482, doi:10.1006/abbi.1993.1311.
22. Denizot, F.; Lang, R. Rapid Colorimetric Assay for Cell Growth and Survival. Modifications to the Tetrazolium Dye Procedure Giving Improved Sensitivity and Reliability. J. Immunol. Methods 1986, 89, 271–277, doi:10.1016/0022-1759(86)90368-6.
23. García Beltrán, J.M.; Espinosa, C.; Guardiola, F.A.; Esteban, M.Á. *In vitro* Effects of *Origanum vulgare* Leaf Extracts on Gilthead Seabream (*Sparus aurata* L.) Leucocytes, Cytotoxic, Bactericidal and Antioxidant Activities. Fish Shellfish Immunol. 2018, 79, 1–10, doi:10.1016/j.fsi.2018.05.005.
24. Esteban, M.A.; Mulero, V.; Muñoz, J.; Meseguer, J. Methodological Aspects of Assessing Phagocytosis of *Vibrio anguillarum* by Leucocytes of Gilthead Seabream (*Sparus aurata* L.) by Flow Cytometry and Electron Microscopy. Cell Tissue Res. 1998, 293, 133–141, doi:10.1007/s004410051105.
25. Campos-Sánchez, J.C.; Guardiola, F.A.; Esteban, M.Á. *In vitro* Effects of λ-Carrageenin in the Head-Kidney Leucocytes of Gilthead Seabream (*Sparus aurata*). Fish Shellfish Immunol. 2022, 127, 813–821, doi:10.1016/j.fsi.2022.07.030.
26. Campos-Sánchez, J.C.; Guardiola, F.A.; Esteban, M.Á. *In vitro* Effects of a Natural Marine Algae Polysaccharide (λ-Carrageenan) on Seabream Erythrocytes, Tumour Cell Lines and Marine Bacterial Pathogens. J. Appl. Phycol. 2023, doi:10.1007/s10811-023-03133-6.
27. Campos-Sánchez, J.C.; Serna, J.A.; Carmen, D.; Francisco, A.; Esteban, M.Á. Participation of Hepcidins in the Inflammatory Response Triggered by Λ-carrageenin in Gilthead Seabream (*Sparus aurata*). Mar. Biotechnol. 2024, doi:10.1007/s10126-024-10293-0.
28. Cuesta, A.; Esteban, M.A.; Meseguer, J. Natural Cytotoxic Activity of Gilthead Seabream (*Sparus aurata* L.) Leucocytes Assessment by Flow Cytometry and Microscopy. Vet. Immunol. Immunopathol. 1999, 71, 161–171, doi:10.1016/S0165-2427(99)00063-X.
29. Quade, M.J.; Roth, J.A. A Rapid, Direct Assay to Measure Degranulation of Bovine Neutrophil Primary Granules. Vet. Immunol. Immunopathol. 1997, 58, 239–248, doi:10.1016/S0165-2427(97)00048-2.
30. Bayne, C.J.; Levy, S. Modulation of the Oxidative Burst in Trout Myeloid Cells by Adrenocorticotropic Hormone and Catecholamines: Mechanisms of Action. J. Leukoc. Biol. 1991, 50, 554–560, doi:10.1002/jlb.50.6.554.
31. Rodríguez, A.; Esteban, M.Á.; Meseguer, J. Phagocytosis and Peroxidase Release by Seabream (*Sparus aurata* L.) Leucocytes in Response to Yeast Cells. Anat. Rec. - Part A Discov. Mol. Cell. Evol. Biol. 2003, 272, 415–423, doi:10.1002/ar.a.10048.
32. Rodríguez, A.; Esteban, M.A.; Meseguer, J. A Mannose-Receptor Is Possibly Involved in the Phagocytosis of *Saccharomyces cerevisiae* by Seabream (*Sparus aurata* L.) Leucocytes. Fish Shellfish Immunol. 2003, 14, 375–388, doi:10.1006/fsim.2002.0446.

 doi:10.20944/preprints202603.2406.v1

33. Reynolds, E.S. The Use of Lead Citrate at High PH as an Electron-Opaque Stain in Electron Microscopy. J. Cell Biol. 1963, 17, 208–212, doi:10.1083/jcb.17.1.208.
34. Pareek, C.S.; Smoczynski, R.; Tretyn, A. Sequencing Technologies and Genome Sequencing. J. Appl. Genet. 2011, 52, 413–435, doi:10.1007/s13353-011-0057-x.
35. Livak, K.J.; Schmittgen, T.D. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2-ΔΔCT Method. Methods 2001, 25, 402–408, doi:10.1006/meth.2001.1262.
36. Cordero, H.; Brinchmann, M.F.; Cuesta, A.; Meseguer, J.; Esteban, M.A. Skin Mucus Proteome Map of European Sea Bass (*Dicentrarchus labrax*). Proteomics 2015, 15, 4007–4020, doi:10.1002/pmic.201500120.
37. Koldaş, S.; Demirtas, I.; Ozen, T.; Demirci, M.A.; Behçet, L. Phytochemical Screening, Anticancer and Antioxidant Activities of *Origanum vulgare* L. Ssp. *Viride* (Boiss.) Hayek, a Plant of Traditional Usage. J. Sci. Food Agric. 2015, 95, 786–798, doi:10.1002/jsfa.6903.
38. Fazio, A.; Cerezuela, R.; Panuccio, M.R.; Cuesta, A.; Esteban, M.Á. *In vitro* Effects of Italian *Lavandula multifida* L. Leaf Extracts on Gilthead Seabream (*Sparus aurata*) Leucocytes and SAF-1 Cells. Fish Shellfish Immunol. 2017, 66, 334–344, doi:10.1016/j.fsi.2017.05.033.
39. Muñoz Centeno, L.M. Plantas Medicinales Españolas: *Jasonia glutinosa* (L.) DC. (Asteraceae) (Té de Roca). Acta Bot. Malacit. 2003, 28, 221–227, doi:10.24310/abm.v28i0.7288.
40. Villaescusa Castillo, L.; Lanza, A.M.D.; Faure, R.; Debrauwer, L.; Elias, R.; Balansard, G. Two Sesquiterpenoids, Lucinone and Glutinone, from *Jasonia glutinosa*. Phytochemistry 1995, 40, 1193–1195, doi:10.1016/0031-9422(95)00377-J.
41. Valero, M.S.; Berzosa, C.; Langa, E.; Gómez-Rincón, C.; López, V. *Jasonia glutinosa* D.C ("Rock Tea"): Botanical, Phytochemical and Pharmacological Aspects. Bol. Latinoam. y del Caribe Plantas Med. y Aromat. 2013, 12, 543–557.
42. Kozłowska, M.; Laudy, A.E.; Przybył, J.; Ziarno, M.; Majewska, E. Chemical Composition and Antibacterial Activity of Some Medicinal Plants from Lamiaceae Family. Acta Pol. Pharm. - Drug Res. 2015, 72, 757–767.
43. Coccimiglio, J.; Alipour, M.; Jiang, Z.H.; Gottardo, C.; Suntres, Z. Antioxidant, Antibacterial, and Cytotoxic Activities of the Ethanolic *Origanum vulgare* Extract and Its Major Constituents. Oxid. Med. Cell. Longev. 2016, 2016, doi:10.1155/2016/1404505.
44. Yanishlieva, N. V.; Marinova, E.M.; Gordon, M.H.; Raneva, V.G. Antioxidant Activity and Mechanism of Action of Thymol and Carvacrol in Two Lipid Systems. Food Chem. 1999, 64, 59–66, doi:10.1016/S0308-8146(98)00086-7.
45. Ruberto, G.; Baratta, M.T. Antioxidant Activity of Selected Essential Oil Components in Two Lipid Model Systems. Food Chem. 2000, 69, 167–174, doi:10.1016/S0308-8146(99)00247-2.
46. Direkbusarakom, S. Application of Medicinal Herbs to Aquaculture in Asia. Walailak J. Sci. Technol. 2011, 1, 7–14.
47. Turker, H.; Yildirim, A.B.; Karakaş, F.P. Sensitivity of Bacteria Isolated from Fish to Some Medicinal Plants. Turkish J. Fish. Aquat. Sci. 2009, 9, 181–186, doi:10.4194/trjfas.2009.0209.
48. Gothandam, K.M.; Aishwarya, R.; Karthikeyan, S. Preliminary Screening of Antiprotozoal Activity of *Jasonia glutinosa* Aerial Parts. Cultures 2010, 2, 1–6.
49. López, V.; Akerreta, S.; Casanova, E.; Garcia-Mina, J.M.; Cavero, R.Y.; Calvo, M.I. Screening of Spanish Medicinal Plants for Antioxidant and Antifungal Activities. Pharm. Biol. 2008, 46, 602–609, doi:10.1080/13880200802179634.
50. Rivas, A.J.; Lemos, M.L.; Osorio, C.R. *Photobacterium damselae* Subsp. *Damselae*, a Bacterium Pathogenic for Marine Animals and Humans. Front. Microbiol. 2013, 4, 1–6, doi:10.3389/fmicb.2013.00283.
51. Nguyen, A.; Jacq, A. Small RNAs in the Vibrionaceae: An Ocean Still to Be Explored. Wiley Interdiscip. Rev. RNA 2014, 5, 381–392, doi:10.1002/wrna.1218.
52. Dubber, D.; Harder, T. Extracts of *Ceramium rubrum*, *Mastocarpus stellatus* and *Laminaria digitata* Inhibit Growth of Marine and Fish Pathogenic Bacteria at Ecologically Realistic Concentrations. Aquaculture 2008, 274, 196–200, doi:10.1016/j.aquaculture.2007.11.029.
53. Suzuki, M.; Nakagawa, Y.; Shigeaki, H.; Yamamoto, S. Phylogenetic Analysis and Taxonomic Study of Marine Cytophaga-like Bacteria: Proposal for *Tenacibaculum* Gen. Nov. with *Tenacibaculum maritimum*

 doi:10.20944/preprints202603.2406.v1

Comb. Nov. and *Tenacibaculum ovolyticum* Comb. Nov., and Description of *Tenacibaculum mesophilum* Sp. Nov. and Ten. Int. J. Syst. Evol. Microbiol. 2001, 51, 1639–1652.

54. Matanza, X.M.; Osorio, C.R. Transcriptome Changes in Response to Temperature in the Fish Pathogen *Photobacterium damselae* Subsp. *Damselae*: Clues to Understand the Emergence of Disease Outbreaks at Increased Seawater Temperatures. PLoS One 2018, 13, 1–23, doi:10.1371/journal.pone.0210118.
55. Petrović, A.; Milutinović, M.M.; Petri, E.T.; Živanović, M.; Milivojević, N.; Puchta, R.; Scheurer, A.; Korzekwa, J.; Klisurić, O.R.; Bogojeski, J. Synthesis of Camphor-Derived Bis(Pyrazolylpyridine) Rhodium(III) Complexes: Structure-Reactivity Relationships and Biological Activity. Inorg. Chem. 2019, 58, 307–319, doi:10.1021/acs.inorgchem.8b02390.
56. Su, J.; Chen, J.; Liao, S.; Li, L.; Zhu, L.; Chen, L. Composition and Biological Activities of the Essential Oil Extracted from a Novel Plant of *Cinnamomum camphora* Chvar. Borneol. J. Med. Plants Res. 2012, 6, 3487–3494, doi:10.5897/jmpr12.157.
57. Kinkade, J.M.; Pember, S.O.; Barnes, K.C.; Shapira, R.; Spitznagel, J.K.; Martin, L.E. Differential Distribution of Distinct Forms of Myeloperoxidase in Different Azurophilic Granule Subpopulations from Human Neutrophils. Biochem. Biophys. Res. Commun. 1983, 114, 296–303, doi:10.1016/0006-291X(83)91627-3.
58. Klebanoff, S.J. Myeloperoxidase: Friend and Foe. J. Leukoc. Biol. 2005, 77, 598–625, doi:10.1189/jlb.1204697.
59. Campos-Sánchez, J.C.; Vitarelli, E.; Guardiola, F.A.; Ceballos-Francisco, D.; García Beltrán, J.M.; Ieni, A.; Esteban, M.Á. Implication of Mucus-secreting Cells, Acidophilic Granulocytes and Monocytes/Macrophages in the Resolution of Skin Inflammation Caused by Subcutaneous Injection of λ/K-carrageenin to Gilthead Seabream (*Sparus aurata*) Specimens. J. Fish Dis. 2021, 00, 1–15, doi:10.1111/jfd.13528.
60. Campos-Sanchez, J.C.; Gonzalez-silvera, D.; Gong, X.; Broughton, R.; Guardiola, F.A.; Betancor, M.B.; Esteban, M.A. Implication of Adipocytes from Subcutaneous Adipose Tissue and Fatty Acids in Skin Inflammation Caused by λ-Carrageenin in Gilthead Seabream (*Sparus aurata*). Fish Shellfish Immunol. 2022, 131, 160–171, doi:10.1016/j.fsi.2022.09.066.
61. Jiang, G.T.; Chen, X.; Li, D.; An, H.X.; Jiao, J.D. Ulinastatin Attenuates Renal Interstitial Inflammation and Inhibits Fibrosis Progression in Rats under Unilateral Ureteral Obstruction. Mol. Med. Rep. 2014, 10, 1501–1508, doi:10.3892/mmr.2014.2323.
62. Mulero, M.C.; Wang, V.Y.F.; Huxford, T.; Ghosh, G. Genome Reading by the NF-KB Transcription Factors. Nucleic Acids Res. 2019, 47, 9967–9989, doi:10.1093/nar/gkz739.
63. Campos-Sánchez, J.C.; Mayor-Lafuente, J.; González-Silvera, D.; Guardiola, F.A.; Esteban, M.Á. Acute Inflammatory Response in the Skin of Gilthead Seabream (*Sparus aurata*) Caused by Carrageenin. Fish Shellfish Immunol. 2021, 119, 623–634, doi:10.1016/j.fsi.2021.10.009.
64. Campos-Sánchez, J.C.; Mayor-Lafuente, J.; Guardiola, F.A.; Esteban, M.Á. *In silico* and Gene Expression Analysis of the Acute Inflammatory Response of Gilthead Seabream (*Sparus aurata*) after Subcutaneous Administration of Carrageenin. Fish Physiol. Biochem. 2021, doi:10.1007/s10695-021-00999-6.
65. Sakamaki, K.; Satou, Y. Caspases: Evolutionary Aspects of Their Functions in Vertebrates. J. Fish Biol. 2009, 74, 727–753, doi:10.1111/j.1095-8649.2009.02184.x.