Article

Antimicrobial and Toxic Activity of Citronella Essential Oil (*Cymbopogon nardus*), and Its Effect on the Growth and Metabolism of Gilthead Seabream (*Sparus aurata* L.)

Jorge García-Márquez [1,*], Andre Barany [2], Álvaro Broz Ruiz [2], Benjamín Costas [3,4], Salvador Arijo [1] and Juan Miguel Mancera [2]

[1] Departamento de Microbiología, Facultad de Ciencias, Instituto de Biotecnología y Desarrollo Azul (IBYDA), Campus de Excelencia Internacional del Mar (CEI·MAR), Universidad de Málaga, 29071 Málaga, Spain; sarijo@uma.es

[2] Department of Biology, Faculty of Marine and Environmental Sciences, Instituto Universitario de Investigación Marina (INMAR), Campus de Excelencia Internacional del Mar (CEI·MAR), University of Cádiz, 11519 Cádiz, Spain; andre.barany@uca.es (A.B.); alvarobroz@hotmail.com (Á.B.R.); juanmiguel.mancera@uca.es (J.M.M.)

[3] Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR), Universidade do Porto, Avenida General Norton de Matos, S/N, 4450-208 Matosinhos, Portugal; bcostas@ciimar.up.pt

[4] Instituto de Ciências Biomédicas Abel Salazar (ICBAS-UP), Universidade do Porto, Rua de Jorge Viterbo Ferreira n 228, 4050-313 Porto, Portugal

* Correspondence: j.garcia@uma.es

Citation: García-Márquez, J.; Barany, A.; Ruiz, Á.B.; Costas, B.; Arijo, S.; Mancera, J.M. Antimicrobial and Toxic Activity of Citronella Essential Oil (*Cymbopogon nardus*), and Its Effect on the Growth and Metabolism of Gilthead Seabream (*Sparus aurata* L.). *Fishes* **2021**, *6*, 61. https://doi.org/10.3390/fishes6040061

Academic Editor: Francisco J. Moyano

Received: 19 October 2021
Accepted: 10 November 2021
Published: 12 November 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

Abstract: Aquaculture procedures usually induce stress that affects the physiological status of fish. For this reason, the inclusion of additives in fish feeds to palliate stress might be a good alternative. This study aimed to assess the antimicrobial activity of citronella (*Cymbopogon nardus*) essential oil (CEO) against bacterial pathogens and to determine its dietary impact on the growth performance of *Sparus aurata*. In vitro tests confirmed that CEO possesses antimicrobial activity against several fish-specific pathogens. For the in vivo tests, three experimental groups were fed for 60 days with different concentrations of CEO: CTRL (0 mL kg^{-1} fish feed); CEO1 (1 mL kg^{-1} fish feed); and CEO2 (2 mL kg^{-1} fish feed). At the end of the experiment, the physiological status was characterized. Subsequently, the specimens of the CTRL and CEO2 groups were subjected to a challenge with an injection of Poly I:C for immune stimulation. Although *S. aurata* individuals tolerated CEO inclusion without compromising growth performance, it significantly reduced glycogen in the CEO2 group, concomitant to an increment of total peripheral leucocytes. Moreover, different hematological profiles' responsive patterns against an inflammatory stimulus were observed. In conclusion, our results suggest that the use of CEO as a fish feed additive can prevent bacterial outbreaks and improve potential in vivo disease resistance in *S. aurata* without negatively affecting growth.

Keywords: acute toxicity; lemongrass; Sparidae; *Aeromonas hydrophila*; *Photobacterium damselae* subsp. *piscicida*; *Vibrio fischeri*; *Vibrio harveyi*

1. Introduction

Crowding densities, transportation, and handling cause major stress, altering the physiology and behavior of fish and ultimately causing significant economic losses produced by pathogen outbreaks [1,2]. Stress has a wide range of effects on overall physiology, involving several clinical manifestations related to metabolism, hydromineral balance, and/or immune system alterations [3,4].

Although antibiotics have been used to prevent and treat bacterial diseases in farmed fish, some pathogens have acquired resistance to many of these, including kanamycin, sulfonamide, tetracycline, ampicillin, chloramphenicol, florfenicol, and erythromycin [5]. Alternatives, such as medicinal plants in fish feed, have been a major focus recently [6,7].

Extracts and essential oils (EOs) of medicinal plants contain phenolics, polyphenols, alkaloids, terpenoids, and polypeptide compounds, which are natural and effective alternatives to antibiotics, agrochemicals, and other synthetic compounds [8,9]. These substances, which have growth-promoting properties, antimicrobial ability, and antistress properties, act as immune system enhancers and appetite stimulants [10,11]. Moreover, its minimal side effects on animal welfare [12] make their use an important means to improve farmed fish's health and/or performance [11,13].

Citronella (*Cymbopogon nardus*) is a plant used to obtain an essential oil mainly composed of citronellal, citronellol, and geraniol [14]. These substances have been described as insect repellent, antiseptic, and bactericidal [15–17]. The toxicity of a substance can be measured via in vitro tests without the need for the use of experimental animals. One of the most commonly used in vitro toxicity tests is the Microtox test [18–20], where the decrease in the bioluminescence of the bacterium *Vibrio fischeri* is used as an indicator of acute toxicity. However, data regarding the dietary effects of citronella essential oil (CEO) on fish metabolism and its potential toxicity are scarce. CEO's effects have been shown to depress the central nervous system in rodents and dogs. In fish, it has also been demonstrated that CEO promotes muscle relaxation and significantly modulates swimming behavior and cardioventilatory responses [21]. Remarkably, CEO may also induce lower cortisol levels in treated *Cyprinus carpio* than other EOs [22]. Furthermore, previous studies on the high inclusion of citronella (400 mg kg^{-1} fish feed) in a diet showed that the fish had lower intestinal total bacteria while enhancing growth performance, feed utilization, and disease resistance [23]. Lastly, based on the available ecological data, exposure information, and the oil of citronella's non-toxic mode of action, the uses of citronella oil pose minimal risk to nontarget wildlife and fish, according to the U.S. Environmental Protection Agency (EPA).

Gilthead seabream (*Sparus aurata* L.) is one of the most important Mediterranean aquaculture species [24]. However, intensive rearing conditions cause stress to fish, making them vulnerable to infectious diseases [3]. Some bacterial pathogens that affect gilthead seabream farming are *Vibrio harveyi*, *Aeromonas hydrophila,* and *Photobacterium damselae* subsp. *piscicida* [25]. Under this framework, CEO supplementation in fish feeds could be of interest as a preventive measure or as an alternative to antibiotic use in order to improve the immune status while not altering the growth and health of the gilthead seabream.

Thus, this work aimed (1) to determine the capacity of CEO to inhibit bacterial pathogens in *S. aurata*; (2) to assess the in vitro acute toxicity of CEO; (3) to evaluate the influence of dietary CEO on *S. aurata* growth and metabolism; and (4) to assess immune stimulation after challenging fish fed with CTRL and CEO2 to a subsequent viral infection-like treatment (i.e., Poly I:C).

2. Materials and Methods

2.1. In Vitro Antimicrobial Activity

According to Wei and Wee [16], citronella essential oil (CEO) was obtained from *Cymbopogon nardus* by steam distillation. Specifically, 600 mL of CEO was obtained from every 100 kg of green biomass from the plant. Its density at 20 °C was in the range of 0.87–0.89 g mL^{-1}. See Supplementary Materials for further information.

For the antimicrobial test, three fish pathogenic bacterial strains—*Vibrio harveyi* Lg16/00, *Photobacterium damselae* subsp. *piscicida* Lg41/01, and *Aeromonas hydrophila* Lg28/4—were used [26,27]. All of them were previously isolated from *Solea senegalensis*. The non-virulent bacterium *Vibrio fischeri* (isolated from seawater) was also used. Strains were cultivated in trypticase soy agar (TSA; Oxoid Ltd., Basingstoke, Hampshire, UK) with 2% NaCl (TSAs) for 24–48 h at 22 °C.

Aqueous and ethanolic dilutions of CEO were prepared using a saline solution and ethanol, respectively. The aqueous extracts were tested using the diffusion method, as Rattanachuay et al. [28] described. Bacterial colonies were suspended in a saline solution (2% of NaCl), matched to a turbidity of 0.5 McFarland standard (approximately 10^8 CFU mL^{-1}). The bacterial suspension was spread over the surface of the TSA plates (with a 5 mm media

thickness) to obtain an even inoculum. Wells with a 6 mm diameter were cut into the agar and then filled with 60 µL of different CEO concentrations. Saline solution was added as a negative control. A sterilized filter paper disc with a 6 mm diameter (Whatman no. 1 filter paper) was used for the ethanolic extracts. An inoculum of 10 µL for each tested concentration was transferred to each disc. As a negative control, 10 µL of ethanol was applied to the filter paper disc. The disks were dried at 37 °C for 15 min to evaporate the ethanol. The suspensions of microorganisms were inoculated into TSA plates as previously described, and then the disks were placed over the plates. The plates were incubated at 22 °C for 24–48 h. The antimicrobial activity was determined by the presence/absence of an inhibition area around each well or disc. The lowest CEO concentration that inhibited the growth of microorganisms was designated as the minimum inhibitory concentration (MIC).

2.2. Assessment of CEO Acute Toxicity

The acute toxicity test was performed by modifying the method described by Johnson [29] using the bioluminescent bacterium *V. fischeri* and the Microtox® M500 test (Microbics Corporation, Carlsbad, CA, USA). Briefly, the CEO was diluted in serial fold 1:2 in 2% saline solution. Cuvettes with 1 mL of each dilution and control (2% saline solution) were maintained at 15 °C. *V. fischeri*, grown for 24 h in TSAs, was suspended in 2% saline solution at 0.6 optical density (600 nm), and maintained at 5 °C before use. Then, all the cuvettes were inoculated with 20 µL of the *V. fischeri* suspension. The samples were incubated at 15 °C for 15 min. After incubation, the bioluminescence of *V. fischeri* in each cuvette was measured by Microtox luminometer and relativized with those of the control, obtaining a percent decrease in bioluminescence. The luminescence inhibition was correlated with the toxicity of the concentration of CEO [30]. The percent reduction in the bioluminescence of *V. fischeri* produced by the CEO was recorded as the median and maximal effective concentration (EC_{50} and EC_{100}) values.

2.3. Experimental Diets and Feeding Trial

A commercial isoproteic, isolipidic, and isoenergetic fish feed (57% crude protein, 18% crude fat, 10% ash, 1.6% phosphorus, and 19.5 mJ kg^{-1} digestible energy, Skretting, Burgos, Spain) was used as a basis to prepare the experimental diets. Three concentrations of CEO were added: (i) CTRL (0 mL CEO kg^{-1} fish feed), (ii) CEO1 (1 mL CEO kg^{-1} fish feed), and CEO2 (2 mL CEO kg^{-1} fish feed). All experimental feeds were elaborated by the Service of Experimental Diets from the University of Almería (Campus de Excelencia Internacional del Mar CEI·MAR, Almería, Spain). The addition of CEO to the feed was carried out by diluting this CEO in 100–150 mL of ethanol. Then, it was homogeneously sprayed onto the feed following a modified methodology described by Morgado et al. [31], i.e., 885 and 1770 mg of CEO kg^{-1} fish feed. The feed was dried at 40 °C on a mesh to allow for the evaporation of ethanol and stored at −20 °C until use. Note that control pellets were treated with equal amounts of vehicle (ethanol).

Juvenile individuals of *S. aurata* (n = 162, 17.7 ± 1.5 g body weight, 10.2 ± 0.3 cm length) were provided by *Servicios Centrales de Investigación en Cultivos Marinos* (SCI-CM, CASEM, University of Cádiz, Puerto Real, Cádiz, Spain; Operational Code REGA ES11028000312) and were randomly distributed in a flow-through setup of nine 80 L tanks (n = 18 per tank). The three experimental dietary groups (CTRL, CEO1, and CEO2) were then established in triplicates. The fish were maintained under natural photoperiod (May–July 2017), temperature (18–19 °C), and salinity (39 g L^{-1}). Supplemental aeration was provided to maintain dissolved oxygen at 6.8 ± 0.4 mg L^{-1}. Ammonia (<0.1 mg L^{-1}), nitrite (<0.2 mg L^{-1}), and nitrate (<50 mg L^{-1}) were determined once weekly. Fish were fed twice per day (9:00 a.m. and 1:00 p.m.) at a rate of 2% of their body weight over the 60-day trial.

All the experimental procedures complied with guidelines of the University of Cádiz (Spain) and the European Union Council (2010/63/EU) for the use of animals in research. The experimental procedures were previously approved by the Spanish Government's

Ethics and Animal Welfare Committee (RD53/2013) and endorsed by the Regional Government (Junta de Andalucía reference number 28-04-15-241).

2.4. Injection of Poly I:C for Immune-Stimulation after Feeding Trial

At the end of the experiment, a total of 64 individuals (previously fed with CTRL and CEO2 for 60 days; 44.7 ± 0.8 g body weight) were distributed in a flow-through setup of three 400 L fiberglass tanks (n = 16 in each). Fish were anesthetized with 2-phenoxyethanol (0.5 mL L^{-1} of 2-phenoxyethanol; Sigma P1126) before handling for injections. Fish were injected either intraperitoneally with 0.1 mL of Hank's Balanced Salt Solution (HBSS) and were regarded as sham groups, or with 0.1 mL of the inflammatory agent poly I:C (50 µg mL^{-1}; Merck Chemicals P1038), a synthetic analog of double-stranded RNA that mimics a viral agent.

2.5. Sampling Procedures

All the fish from each experimental tank were slightly anesthetized with 2-phenoxyethanol, weighed, and measured at 0, 15, 30, 45, and 60 days of the experiment, and then returned to their respective tanks. These sampling points were used to adjust the feed ratio to maintain the initial 2% of their body mass during the experiment and record the biometric parameters for each tank and treatment. For each sampling point, the fish were fasted for 24 h before sampling and anesthetized with 2-phenoxyethanol (0.5 mL L^{-1} of 2-phenoxyethanol; Sigma P1126) before handling.

At the end of the experiment (day 60), the fish were netted and euthanized with a lethal dose of 2-phenoxyethanol (1 mL L^{-1} in seawater). All fish were weighed and measured, and six fish per tank were sampled (n = 18 per experimental diet). Blood was collected from caudal vessels with ammonium-heparinized syringes (Sigma-Aldrich H6279; 25,000 units in 3 mL of saline 0.6% NaCl). Plasma was separated from cells by centrifugation of whole blood (3 min, 10,000× g, 4 °C) and stored at –80 °C for further analysis. The fish were subsequently beheaded and opened. The livers were removed and weighted to determine the hepatosomatic index (HSI). Then, liver biopsy samples were frozen at −80 °C for analysis of metabolites.

Following injection with *Poly I:C* and HBSS, the fish were sampled for blood collection at 4 and 24 h after stimulation. Moreover, unstimulated individuals were also sampled on the day of injections and were regarded as time 0 h (control). For this challenge, parameters such as hematocrit, hemoglobin, as well as total red and white blood cell counts were taken in order to study the immune-modulatory effects of the experimental diets.

2.6. Growth Performance

Weight increase and feed consumption were used to calculate the following nutritional and biometrical indexes: weight gain (WG, g), weight gain (WG, %), condition factor (K, g cm^3), specific growth rate (SGR), and hepatosomatic index (HSI, %). These indexes were calculated according to the following Formulas (1)–(5)

$$\text{WG (g)} = \text{BMf (g)} - \text{BMi (g)} \tag{1}$$

$$\text{WG (\%)} = [(\text{BMf (g)} - \text{BMi (g)})/\text{BMi (g)}] \times 100 \tag{2}$$

$$\text{K (g cm}^{-3}) = [100 \times \text{BM (g)}/\text{BL}^3 \text{ (cm)}] \tag{3}$$

$$\text{SGR (\% day}^{-1}) = 100 \times [(\ln \text{BMf} - \ln \text{BMi})/\text{t (days)}] \tag{4}$$

$$\text{HSI (\%)} = (\text{BL}/\text{BMf}) \times 100 \tag{5}$$

where BL = fish body length; BM = fish body mass; BMf = final body mass; BMi = initial body mass.

2.7. Plasma and Hepatic Parameters

For the assessment of metabolite levels, previously frozen liver biopsies were finely minced on an ice-cold petri dish (~0.3 g) and subsequently homogenized by mechanical disruption (Ultra-Turrax, T25 basic, IKA®-Werke, Stanger, Espelkamp, Germany) with 7.5 volumes (*w*/*v*) of ice-cooled 0.6 N perchloric acid, and neutralized after the addition of the same volume of 1 M $KHCO_3$. Before centrifugation, an aliquot of each homogenate was separated for the measurement of triglycerides (TAG). The remaining homogenates were then centrifuged (30 min, 13,000× *g*, 4 °C), and the supernatants were recovered in different aliquots and stored at −80 °C until used in a glucose–glycogen metabolite assay.

All plasma and hepatic metabolites were analyzed using specific commercial kits from Spinreact (Barcelona, Spain) (Glucose-HK: Ref. 1001200; Lactate: Ref. 1001330; Triglycerides: Ref. 1001311) adapted to 96-well microplates. Liver glycogen levels were assessed using the method from Keppler and Decker [32], in which the glucose obtained after glycogen breakdown (after subtracting free glucose levels) was determined using the commercial kit described above for glucose. All assays were run on an Automated Microplate Reader (PowerWave 340, BioTek Instrument Inc., Winooski, VT, USA), controlled by KCjunior™ software version 1.4. All standards and samples were measured in quadruplicate and duplicate, respectively.

2.8. Haematological Analyses

The determination of circulating total red blood cells (RBC) and white blood cells (WBC) was performed by diluting previously collected blood with heparinized HBSS (30 units mL^{-1}) at 1/200 and 1/20, respectively. Cells were counted using a Neubauer hemocytometer under a light microscope after 2 min from pipetting to let cells settle [33]. Hematocrit values were immediately determined as the percentage of total blood volume (%) after sampling by placing fresh blood in capillary glass tubes and then centrifuging them for 10 min at 10,000× *g*. Hematocrit readings were performed with the aid of a microhematocrit reader. Hemoglobin determination was performed using a specific commercial kit from Spinreact (Barcelona, Spain) (Hemoglobin, Ref. 1001230) following the manufacturer's instructions and adapted to 96-well microplates.

Additionally, the mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were also calculated as follows (Equations (6)–(8)):

$$\text{MCV (mm}^3\text{)} = (\text{Ht}/\text{RBC}) \times 10 \tag{6}$$

$$\text{MCH (pg cell}^{-1}\text{)} = (\text{Hb}/\text{RBC}) \times 10 \tag{7}$$

$$\text{MCHC (g 100 mL}^{-1}\text{)} = (\text{Hb}/\text{Ht}) \times 100 \tag{8}$$

where Ht = hematocrit; RBC = red blood cells count; Hb = hemoglobin.

2.9. Statistical Analyses

Results are presented as means ± standard error of the mean (SEM). Statistical comparison for all given results was performed using a one-way ANOVA. Previously, both normality and equal variance were confirmed by Shapiro–Wilk and D'Agostino–Pearson tests, respectively. In addition, for all data sets, outliers were identified by the ROUT method at Q = 1%. All one-way ANOVA analyses were followed by a Tukey's post hoc test when significant differences were detected. Statistical analyses were performed using the GraphPad Prism® (v.6.0b) software (GraphPad Software, La Jolla, CA, USA), and significance for all tests was set at $p < 0.05$.

3. Results

3.1. Antimicrobial Activity of CEO

CEO inhibited the bacterial growth of the microorganisms, both in saline and ethanolic solution (Table 1). In the tests with saline, CEO inhibited *V. harveyi, P. damselae* subsp. *Piscicida,* and *V. fischeri* at a lower concentration than required to inhibit the growth of *A. hydrophila*. In the case of CEO ethanol extract, the MIC was higher in *V. harveyi* with respect to the other bacteria assessed.

Table 1. Inhibition of bacterial growth by CEO suspended in saline solution and dissolved in ethanol. The results were obtained in duplicate and expressed as the minimum inhibitory CEO concentration (MIC, mg mL^{-1}).

Extract	*V. harveyi*	*A. hydrophila*	*P. damselae* subsp. *piscicida*	*V. fischeri*
Saline solution	27.8	55.6	27.8	27.8
Ethanol	111.3	27.8	27.8	27.8

3.2. Acute Toxicity of CEO

Figure 1 shows the percentage of *V. fischeri*'s bioluminescence at different concentrations of CEO. The median effective concentration (EC_{50}) was 0.73 mg mL^{-1}, established by the interpolation of values fitted to the equation of a logarithmic regression line (y = −56.5 LN(x) + 39.022; R^2 = 0.94), and the maximal inhibition (EC_{100}) was obtained at 2.23 mg mL^{-1}.

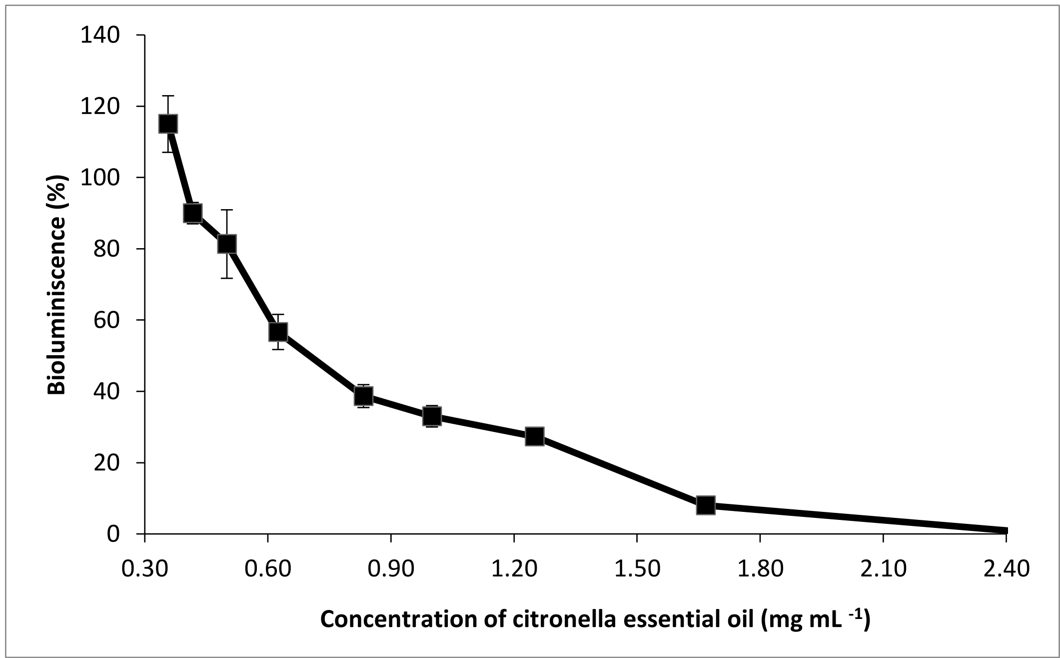

Figure 1. Percentage of *V. fischeri* bioluminescence with respect to the value of the controls, at increasing concentrations of citronella essential oil.

3.3. Growth Performance

No significant mortality was recorded during the experimental procedure, and survival was above 95% in all tanks. Different growth performance parameters are shown in Table 2. All dietary treatments exhibited a 2.4-fold increase in their initial body weights after 60 days of the feeding trial. According to growth rates and feeding conditions, inhibition of around 20% of the total potential weight gain (WG in %) was observed in the CEO1 group as compared to the CTRL and CEO2 groups. Although the specific growth

rate (SGR in % day^{-1}) of neither CEO1 nor CEO2 groups showed significant differences as compared to the CTRL group, the CEO2 group showed significantly higher SGR than the CEO1 group. No significant differences were detected, either in the condition factor (K) or the hepatosomatic index (HSI), while the experiment lasted.

Table 2. Biometrics, weight gain (WG, in g and %), specific growth rate (SGR), condition factor (K), and hepatosomatic index (HSI) in *S. aurata* individuals fed with different experimental diets (CTRL, CEO1, and CEO2) for 60 days. Data are presented as mean ± SEM (n = 9–54). Letters indicate significant differences among experimental diets (p < 0.05, one-way ANOVA followed by a Tukey's post hoc analysis).

	CTRL	CEO1	CEO2
Initial Weight (g)	17.9 ± 0.3	18.0 ± 0.3	17.9 ± 0.3
Final Weight (g)	44.8 ± 0.9	44.5 ± 1.0	44.6 ± 0.8
Initial Length (cm)	10.4 ± 0.1	10.4 ± 0.1	10.3 ± 0.1
Final Length (cm)	13.3 ± 0.1	13.2 ± 0.1	13.2 ± 0.1
WG (g)	27.6 ± 6.8	25.9 ± 7.4	27.3 ± 6.2
WG (%)	163.3 ± 6.7	142.7 ± 7.1	165.5 ± 6.5
SGR (% day^{-1})	1.6 ± 0.0 ^{ab}	1.4 ± 0.0 ^{a}	1.6 ± 0.0 ^{b}
K (g cm^{-3})	19.1 ± 0.2	19.2 ± 0.1	19.4 ± 0.2
HSI (%)	1.3 ± 0.1	1.3 ± 0.1	1.2 ± 0.1

3.4. Plasma and Liver Metabolites

Plasma glucose, triglycerides, and lactate values are shown in Table 3. The addition of CEO in experimental diets did not cause significant plasma glucose, triglycerides, or lactate changes. In the liver, the metabolic parameters assessed are shown in Table 3. Glycogen levels were significantly lower in the CEO2 group than in the CTRL group. No significant differences in hepatic free glucose nor triglycerides were detected among the experimental groups.

Table 3. Plasma (mM) and liver (mmol/g wet weight) parameters in *S. aurata* individuals fed with different experimental diets (CTRL, CEO1, and CEO2) for 60 days. Data are presented as mean ± SEM (n = 9). Letters indicate significant differences among experimental diets (p < 0.05, one-way ANOVA followed by a Tukey's post hoc analysis).

	CTRL	CEO1	CEO2
Plasma			
Glucose (mM)	3.1 ± 0.2	3.6 ± 0.2	3.3 ± 0.2
Triglycerides (mM)	2.4 ± 0.1	2.1 ± 0.1	2.0 ± 0.2
Lactate (mM)	2.2 ± 0.3	1.4 ± 0.1	1.1 ± 0.1
Liver			
Glucose (mmol g^{-1} w.w.)	36.3 ± 3.8	37.9 ± 4.5	41.2 ± 1.9
Glycogen (mmol g^{-1} w.w.)	285.4 ± 29.5 ^{a}	267.6 ± 20.5 ^{ab}	248.9 ± 20.6 ^{b}
Triglycerides (mmol g^{-1} w.w.)	7.2 ± 0.5	4.9 ± 1.0	5.1 ± 0.6

3.5. Hematological Parameters

The hematological profile is shown in Table 4. Total WBC numbers increased significantly in those individuals fed with the highest CEO-supplemented diets (CEO2), while the CEO1 group had intermediate levels, in between the CTRL and CEO2 groups. In contrast, RBC decreased significantly in individuals fed with CEO1 when compared to the CTRL dietary treatment. Remarkably, the CEO2 group presented intermediate levels, in between the CTRL and CEO1 groups. Moreover, a significant reduction in the mean corpuscular volume (MCV) was detected in the CEO2 group as compared to the CEO1 group. The addition of CEO in experimental diets did not cause significant changes in hemoglobin, hemat-

ocrit, mean corpuscular hemoglobin (MCH), nor in the mean corpuscular hemoglobin concentration (MCHC).

Table 4. Hematological parameters in *S. aurata* individuals fed with different experimental diets (CTRL, CEO1, and CEO2) for 60 days. Data are presented as mean ± SEM (n = 8–9). Letters indicate significant differences among experimental diets ($p < 0.05$, one-way ANOVA followed by a Tukey's post hoc analysis).

	CTRL	CEO1	CEO2
WBC ($\times 10^4$ cell μL^{-1})	2.3 ± 0.1 [a]	2.5 ± 0.1 [ab]	3.0 ± 0.2 [b]
RBC ($\times 10^6$ cell μL^{-1})	1.6 ± 0.2 [a]	1.1 ± 0.1 [b]	1.5 ± 0.1 [ab]
Hematocrit (%)	32.2 ± 2.0	29.1 ± 1.3	28.3 ± 1.3
Hemoglobin (g dL^{-1})	3.1 ± 0.3	3.1 ± 0.2	3.2 ± 0.1
MCV (mm^3)	209.9 ± 22.3 [ab]	256.6 ± 15.2 [a]	190.3 ± 10.0 [b]
MCH (pg $cell^{-1}$)	21.0 ± 2.8	27.2 ± 4.4	22.1 ± 1.8
MCHC (g 100 mL^{-1})	9.7 ± 0.7	9.8 ± 1.5	11.5 ± 0.8

3.6. Inflammatory Challenge after Feeding Trial

The in vivo experiment using the intraperitoneal administration of poly I:C in *S. aurata* juveniles previously fed with either CTRL or CEO2 was intended to study whether the CEO2 diet could modulate total WBC dynamics following an inflammatory stimulus. A significant progressive reduction in hematocrit was observed in the CTRL-fed individuals (0 h > 4 h > 24 h), injected either with sham or poly I:C (Figure 2A,B). No changes in this same parameter were observed for any of the CEO2-fed individuals. In contrast, the MCHC progressively increased just within CTRL-fed + poly I:C-injected individuals (Figure 2H). Note that this was not observed in any sham-injected group regardless of the diet (Figure 2G). Compared to CTRL-fed individuals, WBC were progressively reduced in CEO2-fed individuals (Figure 2D), both injected with poly I:C. In contrast, no similar responsive patterns were observed in WBC within sham-injected groups (Figure 2C). Additionally, MCV significantly increased in the CEO2-fed + poly I:C-injected individuals 24 h post-injection (Figure 2F), whereas no significant changes were observed in MCV within the sham-injected groups (Figure 2E).

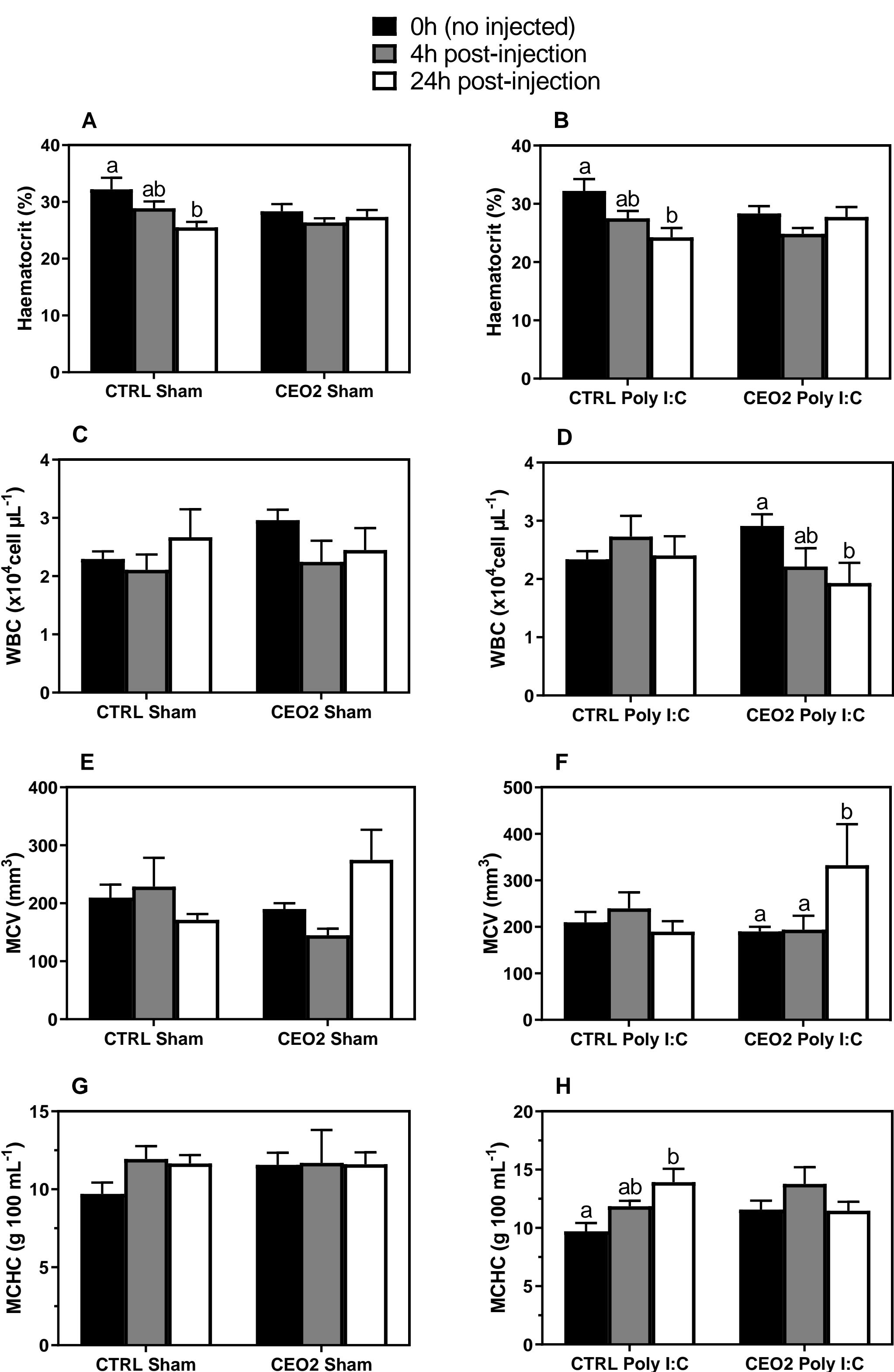

Figure 2. Hematological parameters in *S. aurata* either sham or poly I:C injected after 60 days of feeding with either CTRL or CEO2. Juveniles of gilthead seabream were sampled prior to injection (0 h), and then 4 and 24 h post-injection. Hematocrit response to sham (**A**) and poly I:C (**B**) injections; total white blood cells (WBC) response to sham (**C**) and poly I:C (**D**) injections; mean corpuscular volume (MCV) response to sham (**E**) and poly I:C (**F**) injections; and mean corpuscular hemoglobin concentration (MCHC) response to sham (**G**) and poly I:C (**H**) injections. Data are presented as mean ± SEM (n = 8–9). Letters indicate significant differences within injection and feed supplementation type among timings post-injection ($p < 0.05$, one-way ANOVA followed by a Sidak's post hoc analysis).

4. Discussion

4.1. Antimicrobial Activity of CEO

The cytotoxicity and antibacterial effects of several herbs have been previously examined [34,35], highlighting their potential use for controlling bacterial diseases in cultured fish. In our study, CEO inhibited bacterial growth, both in saline and ethanolic solutions. The antimicrobial activity of *C. nardus* essential oils was first reported by Hammer et al. [36]. The CEO inhibited the growth of *Acinetobacter baumanii*, *Candida albicans*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Serratia marcescens,* and *Staphylococcus aureus*. Recent studies have tested the antimicrobial activity of CEO against several fish pathogens [14,16]. The results achieved growth inhibition regardless of the bacteria being Gram-positive or Gram-negative. Specifically, MIC values ranged from 0.244 μg mL^{-1} up to 0.977 μg mL^{-1}. Since MIC results were obtained using an aqueous solution instead of ethanol, the following experiments were performed using the CEO diluted in an aqueous solution.

4.2. Acute Toxicity of CEO

The measurement of acute toxicity by the inhibition of *V. fischeri* bioluminescence using Microtox® is a well-established and standardized in vitro toxicity test, used in a variety of applications [37], including plant extract toxicity assessment [38–40]. Thus, the results obtained with this test can be extrapolated to the effect that the substances would have on animals. In our study, CEO showed an EC_{50} of 0.73 mg mL^{-1}. Remarkably, this result is lower than those obtained by Skotti et al. [33] with different plant extracts, in which the toxicity (EC_{50}) of *Melissa officinalis* L., *Origanum vulgare* L., *Origanum dictamnus* L., *Salvia officinalis* L., and *Hyssopus officinalis* L. lay between 8.61 and 224.87 mg mL^{-1}. This implies that CEO should be used in fish diets at concentrations lower than those described for these plant extracts, which could have adverse effects. For example, oregano EO has been used in sea bass feed at a 200 ppm of feed concentration for 60 days without generating mortality or adverse effects [41]. Similarly, Ghafarifarsani et al. [42] showed that a 5% inclusion of garlic and oregano extract in carp feed produced no adverse effects. Moreover, EC_{50} obtained for CEO in our study was higher (i.e., less toxic) than Greek herbs (e.g., *Artemisia dracunculus, Achillea millefolium, Matricaria chamomilla, Salvia pomifera, S. officinalis*, and *S. fruticosa*), which ranged from 0.005 up to 0.71 mg mL^{-1} [40]. Thus, the toxic activity of CEO implies that its effect must be taken into consideration when administered in fish feed, which justifies the in vivo studies conducted in this work.

4.3. In Vivo Effects of CEO

Medicinal plants in different formats (e.g., extracts, EO, aqueous, alcoholic, etc.) are used in aquaculture as chemotherapeutics and feed additives [43]. Several physiological effects have been linked to medicinal plants, including growth performance, immune stimulation, and disease resistance against fish pathogens [44–46].

The present and previous studies have already shown the potential toxicity of *Cymbopogon* spp. EO in both aqueous and ethanolic solutions against microorganisms.

After validating CEO in the initial in vitro test to recognize antimicrobial properties, it was decided to move forward to an in vivo test throughout its supplementation of a commercial diet.

Although the addition of any concentration of CEO to a diet apparently did not interfere with *S. aurata* growth (CTRL vs. CEO1/CEO2), remarkably, the SGR was found significantly reduced in the CEO1 group than in the CEO2 group. Moreover, HSI was also not influenced by diets containing *C. nardus* EO. Al-Sagheer et al. [23] showed that the use of lemongrass (*Cymbopogon citratus*) EO at 200 or 400 mg kg^{-1} of fish feed, respectively, improved growth performance, feed utilization, and the immunological parameters of *Oreochromis niloticus.* However, the employ of single or mixture EOs with no significant effects on the growth performance of *S. aurata* has also been previously reported [47–49].

Overall metabolism in *S. aurata* individuals fed with CEO dietary treatments remained unaffected, except for hepatic glycogen content, whose levels dropped in individuals fed with the highest dietary CEO level. Since those glycogen levels were in accordance with a normal range (200–300 mmol gw.w.$^{-1}$) observed in previous studies, and considering other harmful compounds that depleted the glycogen threshold content in the liver below 150 when supplied throughout the diet [50], it can be suggested that CEO at the levels tested in the present study appeared not to harm juvenile *S. aurata* energetic metabolism. Interestingly, a correlation between a drop in hepatic glycogen and the increase in WBC for the CEO2 group could be suggested since WBC have been shown to greatly augment the consumption of oxygen and sugar due to their intrinsic metabolism [51]. This evokes a prompt prophylactic reaction after 60 days of CEO2 feeding by an increase in leukocytes (since platelet counts were scarce) without significantly depleting the main metabolic tissue reservoirs.

The effect of diet on the immune system has been widely recognized in farmed animals, and previous studies have already reported improved total WBC in fish fed a number of dietary additives such as amino acids and medicinal herbs [52–55]. In fact, increasing attention has been paid to the use of plant-derived products as prophylactic strategies to control diseases in farmed fish, as already reviewed by Vallejos-Vidal et al. [56]. In the present study, total WBC augmented in a dose-dependent manner in *S. aurata* fed with dietary CEO. These data suggest a potential effect of CEO in stimulating cellular-mediated immunity. According to previous studies and review articles, an increase in circulating WBC numbers can translate into improved disease resistance against pathogens [55,56]. Therefore, there is potential to further explore the effects of dietary CEO under more challenging scenarios as a way to verify its putative immunomodulatory potential.

It is also remarkable that the RBC appeared to drop in levels at the intermediate CEO dose, which could be circumstantial and not necessarily linked to CEO ingestion. We also observed that MCV was significantly higher in the CEO1 group, whereas it decreased significantly in the CEO2 group. Usually, when MCV is high, the RBC are abnormally large, whereas a low MCV indicates that RBC are abnormally small. Interestingly, hemoglobin levels remained unaffected, whereas the MCH (mean corpuscular hemoglobin) appears to have a non-significant increase in the CEO1 group. This observation might indicate that changes evoked by dietary CEO1 involve an RBC volume increase correlated with an increase in the amount of hemoglobin that these cell types contain (as suggested by MCH). Note that changes in RBC volume might imply vesicle formation in the cytoplasm, but not necessarily changes in hemoglobin content [57], as indicated by our results. Therefore, if these biophysical changes in volume (increment) somehow disrupt oxygen transport in the blood, it could putatively slow down juveniles' growth performance due to their more active daily metabolism, which could explain the significantly lower SGR observed within CEO1 as compared to the CEO2 group. However, we cannot rule out the possibility that some of the changes evoked by a potential immune response could be associated with changes in cell volume or plasma water content instead. Note that some works have addressed the relation between EO constituents (including the citronellal) and their effects on cardiovascular diseases [58].

In addition to studying the immunomodulatory effects of feeding *S. aurata* with CEO for 60 days, we also intended to verify the hematological response following an inflammatory insult by administering poly I:C. We observed that *S. aurata* fed with CEO2 supplementation seems to avoid some hematological responses only observed in CTRL-fed individuals, such as a progressive hematocrit reduction 24 h post-injection, as well as an increase in hemoglobin concentration specifically in poly I:C-injected specimens. In contrast, dietary CEO2 supplementation appears to evoke hematological and immune-related responses to the poly I:C injection, such as a progressive WBC reduction 24 h post-injection, and a significant increment in MCV, thus indicating an increase in RBC average volume. The decrease in peripheral WBC numbers is an important step of the innate immune response to different stimuli, and it has been observed as a result of WBC migration

to the inflammatory site. For instance, Machado et al. [59] also observed a significant reduction in circulating WBC levels after stimulation with a phlogistic agent. Moreover, Engelsma et al. [60] described a similar WBC response for common carp (*C. carpio* L.) after acute stress.

In summary, the present results on the inclusion of CEO in the diet reaffirm this compound as a potential candidate in the prevention of pathological outbreaks due to its antimicrobial properties in vitro; this is also true for the prevention of potential pathogen infections in vivo, based on differential responsive patterns observed during the inflammatory poly I:C injection challenge. Moreover, this compound has been successfully included in a commercial fish feed with non-apparent adverse effects and concomitantly appeared to increase blood leukocyte cell population at the highest concentrations (2 mL of CEO kg^{-1} fish feed) in just 60 days. At intermediate levels (CEO1), the specific growth rate of *S. aurata* juveniles seems to slow down. The lowest CEO concentrations might take longer to evoke the final physiological stage. Further research on supplying prophylactic fish feeds at different doses and for limited time windows should be considered in intensive aquaculture as an alternative to antibiotics or other more invasive treatments.

5. Conclusions

In conclusion, our results suggest that using citronella essential oil as a fish feed additive, at a rate between 1 and 2 mL per kg^{-1} of fish feed, might prevent bacterial outbreaks and improve potential in vivo disease resistance in cultured *S. aurata* without negatively affecting growth.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/fishes6040061/s1, Table S1: chromatographic analysis of essential oil.

Author Contributions: Conceptualization: B.C., S.A. and J.M.M.; methodology: B.C., S.A. and J.M.M.; validation: B.C., S.A. and J.M.M.; formal analysis: J.G.-M., A.B., Á.B.R. and B.C.; resources: B.C., S.A. and J.M.M.; data curation: J.G.-M., A.B. and B.C.; visualization: J.G.-M., A.B. and B.C.; supervision: B.C., S.A. and J.M.M.; project administration: J.M.M.; funding acquisition: S.A.; writing—original draft: J.G.-M.; writing—review and editing: all authors. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Ministry of Science of Spain (Plan Nacional de Investigación, AGL2010-17789 to S.A.), Ministerio de Ciencia e Innovación (PID2020-117557RB-C22 to J.M.M.), and the European Regional Development Fund.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics and Animal Welfare Committee of the Spanish Government (RD53/2013), and endorsed by the Regional Government.

Data Availability Statement: Data generated in this study are available on request from the corresponding author.

Acknowledgments: The authors wish to thank to Rosa Vázquez and staff for the use of the facilities of Servicios Centrales de Investigación en Cultivos Marinos SCI-CM, CASEM, University of Cádiz, Spain to carry out the experiments. We also thank the group of Francisco Javier Moyano of the University of Almería (Spain), who made the experimental aquafeeds. The authors are indebted to Professor Bernardo Baldiserotto (Universidade Federal de Santa Maria, Brazil) for the kind donation of the citronella essential oil.

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

References

1. Buján, N.; Toranzo, A.E.; Magariños, B. *Edwardsiella piscicida*: A significant bacterial pathogen of cultured fish. *Dis. Aquat. Org.* **2018**, *131*, 59–71. [CrossRef]
2. Abdel-Latif, H.M.R.; Khafaga, A.F. Natural co-infection of cultured Nile tilapia *Oreochromis niloticus* with *Aeromonas hydrophila* and *Gyrodactylus cichlidarum* experiencing high mortality during summer. *Aquac. Res.* **2020**, *51*, 1880–1892. [CrossRef]
3. Schreck, C.B.; Tort, L. The Concept of Stress in Fish. In *Biology of Stress in Fish: Fish Physiology*; Schreck, C.B., Tort, L., Farrell, A.P., Brauner, C.J., Eds.; Elsevier Inc.: London, UK, 2016; Volume 35, pp. 1–34. [CrossRef]

4. Bonga, S.E.W. The stress response in fish. *Physiol. Rev.* **1997**, *77*, 591–625. [CrossRef] [PubMed]
5. Andreoni, F.; Magnani, M. Photobacteriosis: Prevention and Diagnosis. *J. Immunol. Res.* **2014**, *2014*, 793817. [CrossRef]
6. Bulfon, C.; Volpatti, D.; Galeotti, M. Current research on the use of plant-derived products in farmed fish. *Aquac. Res.* **2015**, *46*, 513–551. [CrossRef]
7. Rahman, A.N.A.; ElHady, M.; Shalaby, S.I. Efficacy of the dehydrated lemon peels on the immunity, enzymatic antioxidant capacity and growth of Nile tilapia (*Oreochromis niloticus*) and African catfish (*Clarias gariepinus*). *Aquaculture* **2019**, *505*, 92–97. [CrossRef]
8. Immanuel, G.; Uma, R.P.; Iyapparaj, P.; Citarasu, T.; Peter, S.M.P.; Babu, M.M.; Palavesam, A. Dietary medicinal plant extracts improve growth, immune activity and survival of tilapia *Oreochromis mossambicus*. *J. Fish Biol.* **2009**, *74*, 1462–1475. [CrossRef] [PubMed]
9. Zeng, Z.; Zhang, S.; Wang, H.; Piao, X. Essential oil and aromatic plants as feed additives in non-ruminant nutrition: A review. *J. Anim. Sci. Biotechnol.* **2015**, *6*, 7. [CrossRef]
10. Citarasu, T. Herbal biomedicines: A new opportunity for aquaculture industry. *Aquac. Int.* **2009**, *18*, 403–414. [CrossRef]
11. Van Hai, N. The use of medicinal plants as immunostimulants in aquaculture: A review. *Aquaculture* **2015**, *446*, 88–96. [CrossRef]
12. Bandeira, G.; Pês, T.S.; Saccol, E.M.; Sutili, F.J.; Rossi, W.; Murari, A.L.; Heinzmann, B.M.; Pavanato, M.A.; de Vargas, A.C.; Silva, L.D.L.; et al. Potential uses of *Ocimum gratissimum* and *Hesperozygis ringens* essential oils in aquaculture. *Ind. Crop. Prod.* **2017**, *97*, 484–491. [CrossRef]
13. Abarike, E.D.; Jian, J.; Tang, J.; Cai, J.; Yu, H.; Lihua, C.; Jun, L. Influence of traditional Chinese medicine and Bacillus species (TCMBS) on growth, immune response and disease resistance in Nile tilapia, *Oreochromis niloticus*. *Aquac. Res.* **2018**, *49*, 2366–2375. [CrossRef]
14. Timung, R.; Barik, C.R.; Purohit, S.; Goud, V.V. Composition and anti-bacterial activity analysis of citronella oil obtained by hydrodistillation: Process optimization study. *Ind. Crop. Prod.* **2016**, *94*, 178–188. [CrossRef]
15. Nakahara, K.; Alzoreky, N.S.; Yoshihashi, T.; Nguyen, H.T.T.; Trakoontivakorn, G. Chemical composition and antifungal activity of essential oil from *Cymbopogon nardus* (Citronella grass). *Jpn. Agric. Res. Q.* **2013**, *37*, 249–252. [CrossRef]
16. Wei, L.S.; Wee, W. Chemical composition and antimicrobial activity of *Cymbopogon nardus* citronella essential oil against systemic bacteria of aquatic animals. *Iran. J. Microbiol.* **2013**, *5*, 147–152.
17. Pereira, F.D.O.; Mendes, J.M.; Lima, I.O.; Mota, K.S.D.L.; De Oliveira, W.A.; Lima, E.D.O. Antifungal activity of geraniol and citronellol, two monoterpenes alcohols, against *Trichophyton rubrum* involves inhibition of ergosterol biosynthesis. *Pharm. Biol.* **2014**, *53*, 228–234. [CrossRef]
18. Kaiser, K.L.E.; Ribo, J.M. Photobacterium phosphoreum toxicity bioassay. II. Toxicity data compilation. *Environ. Toxicol. Water Qual.* **1988**, *3*, 195–237. [CrossRef]
19. Backhaus, T.; Scholze, M.; Grimme, L. The single substance and mixture toxicity of quinolones to the bioluminescent bacterium Vibrio fischeri. *Aquat. Toxicol.* **2000**, *49*, 49–61. [CrossRef]
20. Parvez, S.; Venkataraman, C.; Mukherji, S. A review on advantages of implementing luminescence inhibition test (*Vibrio fischeri*) for acute toxicity prediction of chemicals. *Environ. Int.* **2006**, *32*, 265–268. [CrossRef]
21. Barbas, L.A.L.; Hamoy, M.; de Mello, V.J.; Barbosa, R.P.M.; de Lima, H.S.T.; Torres, M.F.; Nascimento, L.A.S.D.; da Silva, J.K.D.R.; Andrade, E.H.D.A.; Gomes, M.R.F. Essential oil of citronella modulates electrophysiological responses in tambaqui *Colossoma macropomum*: A new anaesthetic for use in fish. *Aquaculture* **2017**, *479*, 60–68. [CrossRef]
22. Aydın, B.; Barbas, L.A. Sedative and anesthetic properties of essential oils and their active compounds in fish: A review. *Aquaculture* **2020**, *520*, 734999. [CrossRef]
23. Al-Sagheer, A.A.; Mahmoud, H.K.; Reda, F.M.; Mahgoub, S.A.; Ayyat, M.S. Supplementation of diets for *Oreochromis niloticus* with essential oil extracts from lemongrass (*Cymbopogon citratus*) and geranium (*Pelargonium graveolens*) and effects on growth, intestinal microbiota, antioxidant and immune activities. *Aquac. Nutr.* **2018**, *24*, 1006–1014. [CrossRef]
24. Food and Agriculture Organization of the United Nations (FAO). *Global Aquaculture Production 1950–2016*; FAO: Rome, Italy, 2019.
25. Zorrilla, I.; Chabrillón, M.; Arijo, S.; Díaz-Rosales, P.; Martínez-Manzanares, E.; Balebona, M.; Moriñigo, M. Ángel Bacteria recovered from diseased cultured gilthead sea bream (*Sparus aurata* L.) in southwestern Spain. *Aquaculture* **2003**, *218*, 11–20. [CrossRef]
26. Arijo, S.; Chabrillón, M.; Díaz-Rosales, P.; Rico, R.; Martínez-Manzanares, E.; Balebona, M.C.; Toranzo, A.E.; Moriñigo, M.A. Bacteria isolated from outbreaks affecting cultured sole, *Solea senegalensis* (Kaup). *Bull. Eur. Assoc. Fish Pathol.* **2005**, *25*, 148–154.
27. Arijo, S.; Rico, R.; Chabrillón, M.; Díaz-Rosales, P.; Martinez-Manzanares, E.; Balebona, M.C.; Magariños, B.; Toranzo, A.E.; Moriñigo, M.A. Effectiveness of a divalent vaccine for sole, *Solea senegalensis* (Kaup), against *Vibrio harveyi* and *Photobacterium damselae* subsp. *piscicida*. *J. Fish Dis.* **2005**, *28*, 33–38. [CrossRef] [PubMed]
28. Kantachote, D.; Rattanachuay, P.; Tantirungkij, M.; Nitoda, T.; Kanzaki, H. Inhibition of shrimp pathogenic vibrios by extracellular compounds from a proteolytic bacterium *Pseudomonas* sp. W3. *Electron. J. Biotechnol.* **2010**, *13*, 8–9. [CrossRef]
29. Johnson, B.T. *Microtox® Acute Toxicity Test*; Springer: Berlin/Heidelberg, Germany, 2005; pp. 69–105.
30. Ajuzieogu, C.A.; Odokuma, L.O.; Chikere, C.B. Toxicity Assessment of Produced Water Using Microtox Rapid Bioassay. *South Asian J. Res. Microbiol.* **2018**, *1*, 1–9. [CrossRef]
31. Morgado, I.; Campinho, M.A.; Costa, R.; Jacinto, R.; Power, D. Disruption of the thyroid system by diethylstilbestrol and ioxynil in the sea bream (*Sparus aurata*). *Aquat. Toxicol.* **2009**, *92*, 271–280. [CrossRef]

32. Keppler, D.; Decker, K. Glycogen: Determination with amyloglucosidase. In *Methods of Enzymatic Analysis*; Bergmeyer, H.U., Ed.; Verlag-Chemie: Deerfield Beach, FL, USA; Academic Press: Cambridge, MA, USA, 1974; pp. 1127–1131.
33. Stevens, M.L. *Fundamentals of Clinical Hematology*; W.B. Saunders: Philadelphia, PA, USA, 1997.
34. Cunha, J.; Heinzmann, B.M.; Baldisserotto, B.; Da Cunha, J.A. The effects of essential oils and their major compounds on fish bacterial pathogens—A review. *J. Appl. Microbiol.* **2018**, *125*, 328–344. [CrossRef]
35. Assane, I.M.; Valladão, G.M.R.; Pilarski, F. Chemical composition, cytotoxicity and antimicrobial activity of selected plant-derived essential oils against fish pathogens. *Aquac. Res.* **2021**, *52*, 793–809. [CrossRef]
36. Hammer, K.A.; Carson, C.F.; Riley, T.V. Antimicrobial activity of essential oils and other plant extracts. *J. Appl. Microbiol.* **1999**, *86*, 985–990. [CrossRef] [PubMed]
37. Abbas, M.; Adil, M.; Ehtisham-Ul-Haque, S.; Munir, B.; Yameen, M.; Ghaffar, A.; Shar, G.A.; Tahir, M.A.; Iqbal, M. Vibrio fischeri bioluminescence inhibition assay for ecotoxicity assessment: A review. *Sci. Total Environ.* **2018**, *626*, 1295–1309. [CrossRef] [PubMed]
38. Conforti, F.; Ioele, G.; Statti, G.A.; Marrelli, M.; Ragno, G.; Menichini, F. Antiproliferative activity against human tumor cell lines and toxicity test on Mediterranean dietary plants. *Food Chem. Toxicol.* **2008**, *46*, 3325–3332. [CrossRef]
39. Skotti, E.; Anastasaki, E.; Kanellou, G.; Polissiou, M.; Tarantilis, P.A. Total phenolic content, antioxidant activity and toxicity of aqueous extracts from selected Greek medicinal and aromatic plants. *Ind. Crop. Prod.* **2014**, *53*, 46–54. [CrossRef]
40. Sotiropoulou, N.-S.D.; Flampouri, E.; Skotti, E.; Pappas, C.; Kintzios, S.; Tarantilis, P.A. Bioactivity and toxicity evaluation of infusions from selected Greek herbs. *Food Biosci.* **2020**, *35*, 100598. [CrossRef]
41. Dinardo, F.R.; Deflorio, M.; Casalino, E.; Crescenzo, G.; Centoducati, G. Effect of feed supplementation with *Origanum vulgare* L. essential oil on sea bass (*Dicentrarchus labrax*): A preliminary framework on metabolic status and growth performances. *Aquac. Rep.* **2020**, *18*, 100511. [CrossRef]
42. Ghafarifarsani, H.; Hoseinifar, S.H.; Adorian, T.J.; Ferrigolo, F.R.G.; Raissy, M.; Van Doan, H. The effects of combined inclusion of *Malvae sylvestris*, *Origanum vulgare*, and *Allium hirtifolium boiss* for common carp (*Cyprinus carpio*) diet: Growth performance, antioxidant defense, and immunological parameters. *Fish Shellfish Immunol.* **2021**, *119*, 670–677. [CrossRef]
43. Tavares-Dias, M. Current knowledge on use of essential oils as alternative treatment against fish parasites. *Aquat. Living Resour.* **2018**, *31*, 13. [CrossRef]
44. Talpur, A.D.; Ikhwanuddin, M. *Azadirachta indica* (neem) leaf dietary effects on the immunity response and disease resistance of Asian seabass, *Lates calcarifer* challenged with *Vibrio harveyi*. *Fish Shellfish Immunol.* **2013**, *34*, 254–264. [CrossRef]
45. Awad, E.; Cerezuela, R.; Esteban, M. Ángeles Effects of fenugreek (*Trigonella foenum graecum*) on gilthead seabream (*Sparus aurata* L.) immune status and growth performance. *Fish Shellfish Immunol.* **2015**, *45*, 454–464. [CrossRef]
46. Gabriel, N.N.; Qiang, J.; He, J.; Ma, X.Y.; Kpundeh, M.D.; Xu, P. Dietary Aloe vera supplementation on growth performance, some haemato-biochemical parameters and disease resistance against *Streptococcus iniae* in tilapia (GIFT). *Fish Shellfish Immunol.* **2015**, *44*, 504–514. [CrossRef] [PubMed]
47. Saccol, E.; Parrado-Sanabria, Y.; Gagliardi, L.; Jerez-Cepa, I.; Mourão, R.; Heinzmann, B.; Baldisserotto, B.; Pavanato, M.; Mancera, J.; Martos-Sitcha, J. *Myrcia sylvatica* essential oil in the diet of gilthead sea bream (*Sparus aurata* L.) attenuates the stress response induced by high stocking density. *Aquac. Nutr.* **2018**, *24*, 1381–1392. [CrossRef]
48. Beltrán, J.M.G.; Silvera, D.G.; Ruiz, C.E.; Campo, V.; Chupani, L.; Faggio, C.; Esteban, M. Ángeles Effects of dietary *Origanum vulgare* on gilthead seabream (*Sparus aurata* L.) immune and antioxidant status. *Fish Shellfish Immunol.* **2020**, *99*, 452–461. [CrossRef] [PubMed]
49. Firmino, J.P.; Vallejos-Vidal, E.; Sarasquete, C.; Ortiz-Delgado, J.B.; Balasch, J.C.; Tort, L.; Estevez, A.; Reyes-López, F.E.; Gisbert, E. Unveiling the effect of dietary essential oils supplementation in *Sparus aurata* gills and its efficiency against the infestation by *Sparicotyle chrysophrii*. *Sci. Rep.* **2020**, *10*, 17764. [CrossRef]
50. Barany, A.; Guilloto, M.; Cosano, J.; de Boevre, M.; Oliva, M.; de Saeger, S.; Fuentes, J.; Martínez-Rodriguez, G.; Mancera, J. Dietary aflatoxin B1 (AFB1) reduces growth performance, impacting growth axis, metabolism, and tissue integrity in juvenile gilthead sea bream (*Sparus aurata*). *Aquaculture* **2021**, *533*, 736189. [CrossRef]
51. Glover, E.C.; Daland, G.A.; Schmitz, H.L. The metabolism of normal and leukemic leukocytes. *Arch. Intern. Med.* **1930**, *46*, 46–66. [CrossRef]
52. Harikrishnan, R.; Kim, M.-C.; Kim, J.-S.; Balasundaram, C.; Heo, M.-S. Protective effect of herbal and probiotics enriched diet on haematological and immunity status of *Oplegnathus fasciatus* (Temminck & Schlegel) against *Edwardsiella tarda*. *Fish Shellfish Immunol.* **2011**, *30*, 886–893. [CrossRef]
53. Kuang, S.-Y.; Xiao, W.-W.; Feng, L.; Liu, Y.; Jiang, J.; Jiang, W.-D.; Hu, K.; Li, S.-H.; Tang, L.; Zhou, X.-Q. Effects of graded levels of dietary methionine hydroxy analogue on immune response and antioxidant status of immune organs in juvenile Jian carp (*Cyprinus carpio* var. Jian). *Fish Shellfish Immunol.* **2012**, *32*, 629–636. [CrossRef]
54. Machado, M.; Azeredo, R.; Diaz-Rosales, P.; Afonso, A.; Peres, H.; Oliva-Teles, A.; Costas, B. Dietary tryptophan and methionine as modulators of European seabass (*Dicentrarchus labrax*) immune status and inflammatory response. *Fish Shellfish Immunol.* **2015**, *42*, 353–362. [CrossRef]
55. Machado, M.; Azeredo, R.; Fontinha, F.; Fernández-Boo, S.; Conceicao, L.; Dias, J.; Costas, B. Dietary Methionine Improves the European Seabass (*Dicentrarchus labrax*) Immune Status, Inflammatory Response, and Disease Resistance. *Front. Immunol.* **2018**, *9*, 2672. [CrossRef]

56. Vallejos-Vidal, E.; Reyes-López, F.; Teles, M.; MacKenzie, S. The response of fish to immunostimulant diets. *Fish Shellfish Immunol.* **2016**, *56*, 34–69. [CrossRef] [PubMed]
57. Malka, R.; Delgado, F.F.; Manalis, S.R.; Higgins, J.M. In Vivo Volume and Hemoglobin Dynamics of Human Red Blood Cells. *PLoS Comput. Biol.* **2014**, *10*, e1003839. [CrossRef] [PubMed]
58. De Andrade, T.U.; Brasil, G.A.; Endringer, D.C.; Da Nóbrega, F.R.; de Sousa, D. Cardiovascular Activity of the Chemical Constituents of Essential Oils. *Molecules* **2017**, *22*, 1539. [CrossRef] [PubMed]
59. Machado, M.; Azeredo, R.; Domingues, A.; Boo, S.F.; Dias, J.; Conceicao, L.; Costas, B. Dietary tryptophan deficiency and its supplementation compromises inflammatory mechanisms and disease resistance in a teleost fish. *Sci. Rep.* **2019**, *9*, 7689. [CrossRef]
60. Engelsma, M. Multiple acute temperature stress affects leucocyte populations and antibody responses in common carp, *Cyprinus carpio* L. *Fish Shellfish Immunol.* **2003**, *15*, 397–410. [CrossRef]