AquaTechnica 4(2): 109-123 (2022)
ISSN 2737-6095
DOI https://doi.org/10.33936/at.v4i2.4776
https://doi.org/10.5281/zenodo.6975284

Immunomodulatory and anti-vibrio properties of plant extract to manage the health of the shrimp *Penaeus vannamei*

Propiedades inmunomoduladoras y antivibrio de extractos de plantas para manejar la salud del camarón *Penaeus vannamei*

Gabriela Agurto-Rodríguez[1], Cristóbal Domínguez-Borbor[1], Cecilia Tomalá-Beltrán[1], Rosa Malavé[1], Jenny Rodríguez[1,2]
[1]Escuela Superior Politécnica del Litoral (ESPOL), Centro Nacional de Acuicultura e Investigaciones Marinas (CENAIM), Campus Gustavo Galindo Km. 30.5 Vía Perimetral, P.O. Box 09-01-5863, Guayaquil, Ecuador
[2]Facultad de Ciencias de la Vida (FCV), Escuela Superior Politécnica del Litoral, ESPOL, Guayaquil 090708, Ecuador
Correspondencia: *Jenny Rodríguez*, ***E-mail:*** *jenrodri@espol.edu.ec*

Original article | Artículo original

Keywords
GRAS plants
Shrimp immune system
Quorum sensing
Shrimp aquaculture
Vibriosis

ABSTRACT | Vibriosis is a multifactorial disease that causes economic losses in the shrimp farming industry. As part of an ecological approach for its control, aqueous extracts from plants generally recognized as safe (GRAS): *Allium sativum* (290000 µg mL^{-1}), *Camellia sinensis* (40000 µg mL^{-1}), *Morinda citrifolia* (250000 µg mL^{-1}) and Origanum vulgare (50000 µg mL^{-1}) were evaluated for their antibacterial, antioxidant and immunostimulant properties. The study began by evaluating the microbicidal properties of the extracts against shrimp pathogenic *Vibrio* spp. as well as their antioxidant characteristics. Subsequently, the minimum inhibitory concentration and the minimum bactericidal concentration (MBC) were determined in order to find sublethal working concentrations for a comprehensive anti-virulence strategy. The extracts had the ability to disrupt bioluminescence and/or biofilm formation over a wide range of dilutions below the MBC (about 10 to 50 times); 1450 to 145 µg mL^{-1} for *A. sativum*, 2000 to 200 µg mL^{-1} for *C. sinensis*, 12500 to 1250 µg mL^{-1} for *M. citrifolia*, and 2500 to 250 µg mL^{-1} for *O. vulgare*. All plant extracts exhibited antioxidant characteristics, differentially modulating superoxide generation depending on the plant extract, without significantly affecting superoxide generation rates; in this case, at the lowest concentrations tested. The most consistent results were obtained with *A. sativum* extract, which exhibited powerful properties to remove superoxide from unstimulated hemocytes at concentrations ranging from 14500 to 1.45 µg mL^{-1}. In experimental earthen ponds (400 m^2, four replicates per treatment), shrimp treated with plant extracts added to food at immunomodulatory concentrations (*A. sativum*: 36 µg per g, *M. citrifolia*: 31 µg per g) exhibited reduced melanosis, moreover shrimp treated with *A. sativum* had the highest harvest weight. These results indicate that aqueous extracts of *A. sativum*, *C. sinensis*, *O. vulgare*, and *M. citrifolia* can improve shrimp health due to their antioxidant, immunomodulatory, and antimicrobial properties.

Palabras clave
Plantas GRAS
Sistema Inmune de camarón
Detección de *cuorum*
Acuicultura de camarón
Vibriosis

RESUMEN | Las vibriosis son enfermedades multifactoriales que causan pérdidas económicas en la industria camaronera. Como parte de un enfoque ecológico para su control, extractos acuosos de plantas generalmente reconocidas como seguras (GRAS): *Allium sativum* (290000 µg mL^{-1}), *Camellia sinensis* (40000 µg mL^{-1}), *Morinda citrifolia* (250000 µg mL^{-1}) y *Origanum vulgare* (50 000 µg mL^{-1}), fueron evaluados por sus propiedades antibacterianas, antioxidantes e inmunoestimulantes. Se inició el estudio evaluando las propiedades microbicidas de los extractos contra *Vibrio* spp. patógenos del camarón, así como sus características antioxidantes. Posteriormente, se encontró la concentración inhibitoria mínima y la concentración bactericida mínima (MBC) para determinar concentraciones subletales como parte de una estrategia antivirulencia. Los extractos mantuvieron la capacidad de interrumpir la bioluminiscencia y/o la formación de biopelículas en un amplio rango de dilución por debajo del MBC (alrededor de 10 a 50 veces el MBC); 1450 a 145 µg mL^{-1} para *A. sativum*, 2000 a 200 µg mL^{-1} para *C. sinensis*, 12500 a 1250 µg mL^{-1} para *M. citrifolia* y 2500 a 250 µg mL^{-1} para *O. vulgare*. Todos los extractos de plantas exhibieron características antioxidantes, modulando de manera diferencial la generación de superóxido según el extracto de la planta, sin afectar significativamente las tasas de generación de superóxido, a las concentraciones más bajas ensayadas. Los resultados más consistentes se obtuvieron del ensayo realizado con extracto de *A. sativum*, el cual exhibió poderosas propiedades para eliminar el superóxido de los hemocitos no estimulados en concentraciones que oscilaron entre 14500 y 1,45 µg mL^{-1}. En estanques de tierra experimentales (400 m^2, cuatro repeticiones por tratamiento), los camarones tratados con extractos de plantas añadidos

al alimento en concentraciones inmunomoduladoras (*A. sativum:* 36 µg por g, *M. citrifolia:* 36 µg por g*)* exhibieron una melanosis reducida. Además, los camarones tratados con *A. sativum* tuvieron el mayor tamaño a la cosecha. Estos resultados indican que los extractos acuosos de *A. sativum, C. sinensis*, *O. vulgare* y *M. citrifolia* pueden promover la salud de los camarones debido a sus propiedades antioxidantes, inmunomoduladoras y antimicrobianas.

INTRODUCTION

The sustainability of the shrimp industry depends on disease control. Vibriosis is one of the diseases with a major impact on the shrimp industry. *Vibrio* spp. are common in *Penaeus vannamei* shrimp culture systems and species belonging to the Harveyi clade are among the most abundant. Luminescent and non-luminescent *Vibrio* strains belonging to this clade have caused severe losses in penaeid larviculture and grow-out ponds (Vandenberghe et al., 1999). *Vibrio parahaemolyticus*, producing PirA/B toxins, is the cause of acute hepatopancreatic necrosis disease (AHPND) and affects shrimp farming, causing huge losses (Han *et al*., 2015). Besides the tissue destruction by bacterial toxins, it has also been reported that vibriosis leads to oxidative stress in shrimp (Castex *et al*., 2010; Duan *et al*., 2015; Velázquez-Lizárraga *et al*., 2019), provoking additional tissue damage (Castex *et al*., 2010), and deleterious effects on shrimp immune system (Zhao *et al*., 2017). Moreover, the capacity of vibrios to persist in biofilms is considered a serious threat in shrimp culture systems (Beshiru & Igbinosa, 2018). Biofilm production is regulated by Quorum sensing (QS) (Parsek & Greenberg, 2005; Passos da Silva *et al*., 2017) Biofilms constitute reservoirs of *Vibrio* from which dispersal is possible, facilitating outbreaks, and easing horizontal gene transfer between vibrios (Kiran *et al*., 2014). To deal with vibriosis, shrimp farmers use antibiotics to mitigate the losses (Done & Halden, 2015; Loo *et al*., 2020), but their continuous and indiscriminate use could lead to the development of virulent strains (Akinbowale *et al*., 2007; Meek *et al*., 2015). Also, the presence of antibiotic residues in seafood augments the risk of bacterial resistance to antibiotics in humans (Done & Halden, 2015). Consequently, antibiotic traces are targeted in the quality analysis performed by shrimp importing countries (Productor, s. f.). To mitigate vibriosis and associated health disorders in shrimp culture, the use of alternative substances to antibiotics is being explored. Alternatives include immunostimulants (Mohan *et al*., 2019a; Mohan *et al*., 2019b; Wan *et al*., 2019), probiotics (Gullian & Rodríguez, 2001), and antimicrobial natural products (Asimi & Sahu, 2013; Stefanakis *et al*., 2014; Srinivasan *et al*., 2017; Elumalai *et al*., 2021). Natural products from plants have several compounds, such as flavonoids, terpenes, and phenols known for their many bioactivity properties (antimicrobial, antioxidant and/or immunostimulant); (Mendonça-Filho, 2006; Ravipati *et al*., 2012).

Several plants, known as "Generally Recognized as Safe" (GRAS) for human food, are rich in bioactive molecules. Among these plants, *Allium sativum*, *Origanum vulgare*, *Camellia sinensis*, and *Morinda citrifolia* have documented activity against vibrios that cause diseases in aquaculture (Yano *et al*., 2006; Talpur & Ikhwanuddin, 2012; Morales-Covarrubias *et al*., 2016; Singh *et al*., 2016; Jahanjoo *et al*., 2018; Mukherjee & Bassler, 2019; Mohammadi *et al*., 2020). Additionally, these plants have antioxidant and immunostimulant properties (Ngo *et al*., 2007; de Queiroz *et al*., 2014; Hasanpour *et al*., 2017; Halim *et al*., 2017; Adineh *et al*., 2020; García Beltrán *et al*., 2020).

Aquaculture research on the use of bioactive extracts of plants has focused on specific characteristics or benefits of GRAS plants or their compounds, mostly in their microbicidal characteristics at lethal doses (Kakoolaki *et al*., 2016; Aftabuddin *et al*., 2017; Marisa Halim *et al*., 2017; Palanikumar *et al*., 2020; Reverter *et al*., 2021). These plants can be used as part of a holistic approach to treat vibriosis and the imbalances it causes on shrimp health (oxidative stress and immune suppression). In this study, we have determined that sublethal doses for pathogenic *Vibrio* spp. of shrimp (for a comprehensive anti-virulence strategy) of *A. sativum*, *O. vulgare*, *C. sinensis* and *M. citrifolia*, allow taking advantage of their anti-QS, antioxidant, and immunomodulatory characteristics, as part of a holistic approach for shrimp health management

MATERIALS AND METHODS

To assess the effectiveness of plant extracts, we started by evaluating their anti-vibrio properties (see below). Once confirmed that the aqueous extracts of plants had this bioactivity, we determined the Minimal Inhibitory Concentration (MIC), and Minimal Bactericidal Concentration (MBC) to find sublethal concentrations. Sublethal concentrations of plant extracts capable of inhibiting biofilm formation and bioluminescence of *Vibrio* spp. were taken as the basis for evaluating their immunomodulatory effect.

Preparation of plant extracts

Ten grams of *A. sativum* cloves were triturated with a mortar and macerated in 20 mL of distilled water and kept in constant motion in a shaker at 110 rpm (New Brunswick Scientific-Classic series, USA) for 12 h at room temperature (25 ºC). The extracts were then filtered with Whatman filter paper (0.7 µm, Whatman International Ltd., England). One gram of dry leaves of *C. sinensis* and *O. vulgare* were macerated in 10 mL of distilled water. The aqueous preparations were incubated with a lid (to minimize the evaporation) in a digital water bath (VWR Digital Dual Water Bath, Hampton) at 60 °C for 1h under shaking to 300 rpm. The plant extracts were filtered with Whatman filter paper. The juice from *M. citrifolia* was extracted by freezing and thawing the mature fruits before filtering with Whatman filter paper. The extracts were stored at 8 °C. To determine the concentrations of aqueous extracts, aliquots were lyophilized (Eyela, Japan), and the resulting dry powder was weighed. The concentrations of the aqueous extracts were 290000 µg mL^{-1} for *A. sativum*, 40000 µg mL^{-1} for *C. sinensis*, 250000 µg mL^{-1} for *M. citrifolia* and 50000 µg mL^{-1} for *O. vulgare*.

Estimation of bioactivities of aqueous extracts of plants

Antibiogram against Vibrio spp.

Three bacterial strains were used: *V. harveyi* Strain E22 isolated from diseased postlarvae with signs of "bolitas" syndrome in Ecuador (Vandenberghe *et al*., 1999); *V. parahaemolyticus* strain ATCC 27969, isolated from crab hemolymph (https://www.atcc.org/products/27969); and *V. vulnificus* strain S2, isolated from local shrimp larvae affected by Zoea Syndrome. Each bacterium strain was plated on Tryptic Soy Agar (TSA) enriched with sodium chloride (NaCl) 2%. Four colonies were inoculated in 9 mL of Trypticase Soy Broth (TSB) containing 2% NaCl and incubated at 28 °C until turbidity was visible (2-5 hours, depending on the type of bacterium). The turbidity was adjusted with a solution of 2% NaCl to reach a value of 0.5 on the McFarland scale (McFarland, 1907), equivalent to 1 x 10^8 Colony Forming Units (CFU) mL^{-1}. Before we validated the MacFarland method with the viable bacteria (CFU) count (Sutton, 2011). One mililiter of this bacterial suspension was spread on the whole agar surface, and after a few minutes, the excess liquid was removed, and the agar plates were left to dry for 5 min. In each agar plate containing the pathogenic vibrios, 5 discs of Whatman paper of 6 mm diameter were placed. Each Whatman paper disc before treated with 5, 10, 15, 20, and 25 µL of the plant extracts. The pH of each plant extract was neutralized with Hepes buffer to a final concentration of 10 mM, pH 7.0. Whatman disks were dried at room temperature (25 °C) for 5 min in a safety cabinet (type II) before being placed on the agar. Disks with oxytetracycline (3000 µg) were used as a positive control.

Determination of the Minimal Inhibitory Concentration (MIC) and the Minimal Bactericidal Concentration (MBC)

The determination of MIC was performed for the different bacteria, following the protocol described by Soković *et al*. (2010) with slight modifications. The bacterial inoculum at 1 x 10^8 CFU mL^{-1} was prepared as described before. A 1/100 dilution of the inoculum was carried out to get a concentration of approximately 1 x 10^6 colony CFU per mL. One hundred µL of this bacterial suspension was distributed into the wells in a 96-well microplate. The mother solutions of plant extracts were prepared by mixing 50 mL of each extract with 50 mL of TSB 2X. The attained concentrations of mother solutions were 1.16 x 10 mL^{-1} for *A. sativum*, 1.6 x 10^4 µg mL^{-1} for *C. sinensis*, 1.0 x 10^5 µg mL^{-1} for *M. citrifolia*, and 2.0 x 10^4 µg mL^{-1} for *O. vulgare*. To avoid the interference of low pH of plant extracts with the bacterial growth, the plant extracts were previously neutralized with Hepes buffer at pH 7 (50 mM final concentration). From these mother solutions,

several dilutions were prepared in TSB (Table 1). One hundred µL of each dilution was distributed by triplicate into the wells of the 96-well microplate, which was before inoculated with the bacterial suspension, thus obtaining a final volume of 200 µL in each well (Table 1). The microplates were incubated for 15 h at 28°C. The MIC corresponded to the wells with the lowest plant extract concentration where bacterial growth was not detected. One hundred microliter were extracted from the wells that exhibited no bacterial growth, were plated on Agar TSA with 2% NaCl and incubated for 15 h at 28°C. The lowest concentration of plant extract in culture wells, which upon sub-culturing on agar plates produced colonies less than 0.1% of the original inoculum, was considered the MBC.

Table 1. Final concentrations of each plant extract tested against bacteria to determine Minimal Inhibitory Concentration and Minimal Bactericidal Concentration.

N°	Volume per well (extract/bacterium)	*Allium sativum* (µg mL^{-1})	*Camellia sinensis* (µg mL^{-1})	*Morinda citrifolia* (µg mL^{-1})	*Origanum vulgare* (µg mL^{-1})
1	160/40	116000	16000	100000	20000
2	120/80	87000	12000	75000	15000
3	110/90	79750	11000	68750	13750
4	100/100	72500	10000	62500	12500
5	85/115	61625	11500	53125	10625
6	65/135	47125	6500	40625	8125
7	60/140	43500	6000	37500	7500
8	35/165	25375	3500	21875	4375
9	17,5/183	13050	1800	11250	2250
10	8,75/191	6525	900	5625	1125
11	1:10	14500	2000	12500	2500
12	1 : 100	7250	1000	6250	1250

Effects of plant extracts on Quorum Sensing indicators (biofilm formation and bioluminescence)

The ability of sublethal concentrations of plant extracts to interfere with two mechanisms, bioluminescence of *V. harveyi* and biofilm formation of all three vibrios, was estimated following the procedure described by Djordjevic *et al*. (2002). Each bacterial strain was plated on TSA with 2% NaCl and incubated for 18 h. After the incubation, the bacterial strains were inoculated in 9 mL of TSB and incubated using a water bath at 30 °C for 3 to 4 h (depending on the bacterium) until they reached turbidity of 0.5 on the McFarland scale (1 x 10 UFC mL^{-1}).

One hundred microliters of neutralized plant extracts at different concentrations in a saline solution (2% NaCl) were distributed per well into a 96-well microplate. One hundred microliters of the bacterial suspension at a concentration of 1 x10^{6} CFU mL^{-1} (prepared as described before) were distributed into the wells of the microplate containing the extracts. The concentrations of plant extracts assessed were: 14500, 1450 and 145 µg mL^{-1} for *A. sativum*; 25000, 2500 and 250 µg mL^{-1} for *O. vulgare*; 20000, 2000, 200 µg mL^{-1} for *C. sinensis*; and 125000, 12500, 1 250 µg mL^{-1} for *M. citrifolia*.

Two controls (C) were performed: either 100 µL of 2% NaCl solution were used instead of plant extracts (C1), or the bacterial suspension was replaced by 100 µL of culture medium (C2). After overnight incubation, bacterial growth was estimated by recording the turbidity (after resuspending the cells) at 540 nm with a Synergy HT-BioTek (Winooski, Vermont, USA), and the luminescence registered (in a dark room).

After 20 h, the supernatants were withdrawn from the wells, and 200 µL of TSB (2% NaCl) per well were added. After 48 hours at 30°C, the supernatants were removed, and the wells were washed three times using 2% NaCl. The microplate was left to dry for 1 h at 30 °C. Next, 200 µL of Crystal Violet at 0.1% were added, and after 15 min, the microplate was washed again using tap water. The biofilm solubilization was carried

out using 200 µL of Tween 80 at 10%, and the reading was performed in the microplate reader Thermo Scientific Multiskan EX (Shangay, China) at 541 nm.

Antioxidant assay

The antioxidant capacity of each extract was determined using a colorimetric assay based on the method of total phenols (Walker & Everette, 2009). This method gauges the ability of the antioxidant to decolorize the 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical cation (ABTS•+), which is blue in solution (Walker & Everette, 2009). The stock solutions of plant extracts were tested, and ascorbic acid (10 mg mL^{-1}) was used as a positive control. The assay was performed in a 96-well microplate, and the optical density was read at 620 nm using the microplate reader Thermo Scientific Multiskan EX (Shanghai, China). A control treatment was performed using wells containing only plant extract without ABTS. No interference of the color of the extracts with the discoloration of ABTS was observed.

Anion (O_2^-) measurement

To analyze the effects of the sublethal concentrations of plant extracts on the shrimp immune system, the generation of superoxide anion (O_2^-) produced by shrimp hemocytes was assessed. Shrimp hemolymph was withdrawn from the ventral sinus located at the base of the first abdominal segment using 1 mL syringe containing 100 µL of sodium citrate solution (10%) as described by Muñoz *et al*. (2000). The O_2^- was measured by the reduction of Nitro blue tetrazolium (NBT), following the procedure optimized by Muñoz *et al*. (2000). The assay was performed in a 96-well microplate. Phorbol myristate acetate (PMA) (Sigma) prepared in dimethyl sulfoxide at a concentration of 30 µg mL^{-1} was used to elicit the respiratory burst. The plant extracts added to the primary culture of hemocytes were diluted in Hank's salts at different concentrations (starting from the concentrations inhibiting the formation of biofilms and bioluminescence). The O_2- was quantified as both basal activity (unstimulated) and stimulated activity with PMA. The results are expressed as a rate, dividing the optical density (620 nm) value of the sample stimulated with PMA against the value of the same sample without stimulation.

***In vivo* tests**

Pond trials

The experiment was carried out in 400 m^2 earthen ponds at the CENAIM experimental station, located 150 km from Guayaquil, for 117 days. Two plant extracts were chosen based on the results in vitro. Four replicates per treatment were employed, including the control. Each extract was applied to the commercial feed (28% of protein) in a single daily dose by soaking 110 mL^{-1} of seawater, containing plant extract (36 mg Kg^{-1} for *A. sativum* and 31 mg Kg^{-1} for *M. citrifolia*). Afterward, the feed was soaked with fish oil in order to avoid plant extracts lixiviation. In both cases, these concentrations were sublethal to vibrios but sufficient to create a protective layer on the feed, limiting bacterial adhesion. Probiotics P62-P64 (Gullian *et al*., 2004) were used in all ponds, including the controls. To limit the interference of plant extracts with the establishment of beneficial microflora, plant extracts were applied alternately with probiotics at weekly intervals starting the first day of culture and throughout the whole production cycle. Metabisulfite was not applied during the harvest, and the cephalothorax of the harvested animals was removed to reduce blackening. The following parameters were recorded: weight, survival rate, and yield. Finally, in order to evaluate the effect of plant extracts on the quality of the harvested shrimp, 30 animals were chosen randomly in order to perform a double-blind analysis of appearance (percentages of animals with telson dark pigment and body color) and palatability

Statistical analysis

A variance analysis (One-way ANOVA) was performed with the obtained data (Superoxide assays and pond trials). When significant differences were observed, Tukey's Honest Significant Difference (HSD) post hoc test was applied ($\alpha = 0.05$ significance level).

RESULTS

Antibiogram results

The four plant extracts produced a clearing zone by inhibiting bacterial growth in the agar plates for all three bacteria tested. Figure 1 and Table 2 show the results of the extracts of *Camellia sinensis* and *Origanum vulgare* against *Vibrio harveyi*.

Table 2. Inhibition zones produced by plant extracts against *Vibrio harveyi* using concentrations of 50000 µg mL^{-1} for *Origanum vulgare* and 40000 µg mL^{-1} for *Camellia sinensis*. Antibiograms are expressed as diameters (mm).

Aqueous Extracts	Volume (µg) by antibiogram				
	5 µL	10 µL	15 µL	20 µL	25 µL
Camellia sinensis	10 mm	13 mm	12 mm	15 mm	12 mm
Origanum vulgare	13 mm	15 mm	18 mm	20 mm	21 mm

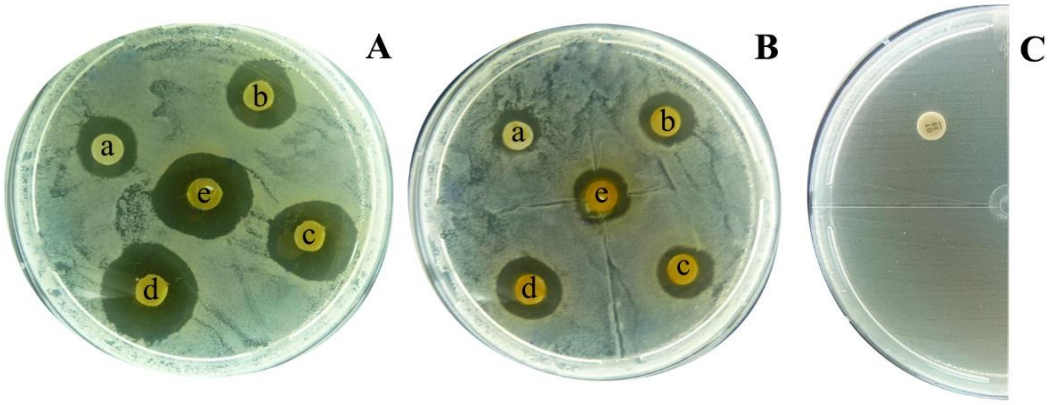

Figure 1. Antibacterial activity of aqueous extracts of *Origanum vulgare* and *Camellia sinensis* against *Vibrio harveyi*. **A.** *Origanum vulgare*, **B.** *Camelia sinensis*, and **C.** Positive control: Antibiotic oxytetracycline. Volume for disc: a) 5 µL; b) 10 µL; c) 15 µL; d) 20 µL and e) 25µL. Concentration 50000 µg mL^{-1} of *Origanum vulgare*, 40000 µg mL^{-1} of *Camelia sinensis* and 30000 µg of Oxytetracycline.

Minimal Inhibitory Concentration and Minimal bactericidal concentration against *V. vulnificus, V. parahaemolyticus* and *V. harveyi*

The MIC and the MBC of *A. sativum* were the same against the three *Vibrio* spp. tested (13050 µg mL^{-1}). For *O. vulgare*, no differences were observed between the MIC and MBC for *V. vulnificus* (7500 µg mL^{-1}) and *V. harveyi* (4375 µg mL^{-1}); but, against *V. parahaemolyticus*, the MIC was 10625 µg mL^{-1} and the MBC was 15000 µg mL^{-1}. For *C. sinensis*, the MIC and the MBC were the same against *V. vulnificus* (3500 µg mL^{-1}), *V. parahaemolyticus* (11500 µg mL^{-1}), and *V. harveyi* (6000 µg mL^{-1}). For *M. citrifolia*, the MIC and MBC were different for the three *Vibrio* spp. For *V. vulnificus*, the MIC and MBC were 62500 µg mL^{-1} and 100000 µg mL^{-1}, and for *V. harveyi*, the MIC was 53125 µg mL^{-1} and the MBC was 100000 µg mL^{-1}. An MBC was not found against *V. parahaemolyticus*, while the MIC was 62500 µg mL^{-1} (Table 3).

Effects of plant extracts on biofilm formation and bioluminescence at sublethal concentrations

The four plant extracts showed the ability to inhibit biofilm formation of *V. vulnificus*, *V. parahaemolyticus*, and *V. harveyi* at sublethal concentrations. The extracts' dilutions that disrupted biofilm formation without affecting bacterial growth (MIC and MBC) ranged between 9 and 90 times the MBC for *A. sativum*, 6 and 60 times for *O. vulgare*, 6 and 58 times for *C. sinensis*, and 8 and 80 times for *M. citrifolia*

(Table 3). Bioluminescence of *V. harveyi* was inhibited by *A. sativum* (at *O. vulgare* and *C. sinensis*) to the same effective concentrations against biofilms (Table 3).

Table 3. Comparison of MIC and MBC of plant extracts and concentrations that inhibit biofilm formation in *Vibrio vulnificus*, *Vibrio parahaemolyticus*, and *Vibrio harveyi*, and luminescence in *Vibrio harveyi*.
BF: biofilm. LM: luminescence
BG: Bacterial growth.

Extract	*Vibrio vulnificus*				*Vibrio parahaemolyticus*				*Vibrio harveyi*				
	BF		MIC (µg mL^{-1})	MBC (µg mL^{-1})	BF		MIC (µg mL^{-1})	MBC (µg mL^{-1})	BF		LM	MIC (µg mL^{-1})	MBC (µg mL^{-1})
	Concentration (µg mL^{-1})	Inhibition %			*Concentration* (µg mL^{-1})	Inhibition %			*Concentration* (µg mL^{-1})	Inhibition %			
Allium sativum	**1450**	57.2±17.24	13050	13050	**1450**	73.9±40.88	13050	13050	**1450**	50.7±14.13	Yes	13050	13050
	145	87.1±32.27			**145**	116±80.75			**145**	48.4±7.73	Yes		
Camellia sinensis	**2000**	100±6.00	3500	3500	**2000**	28±23.97	11500	11500	**2000**	36±6.51	No	6000	6000
	200	100±4.3			**200**	86.4±13.03			**200**	29.7±6.28	Yes		
Morinda citrifolia	**12500**	60±4.17	62500	100000	**12500**	75.8±3.46	62500	BG	**12500**	63.2±26.22	No	53125	100000
	1250	44.4±1.41			**1250**	92.7±13.82			**1250**	84.8±34.95	No		
Origanum vulgare	**2500**	22.5±2.62	7500	7500	**2500**	15.8±35.36	10625	10625	**2500**	27.8±6.17	No	4375	4375
	250	36.7±8.56			**250**	26.9±27.21			**250**	19.9±10.85	Yes		

Antioxidant activity

The antioxidant capacity, measured using the ABTS technique, indicated that all the tested aqueous solutions demonstrated significant antioxidant capacity ($p < 0.05$). The optical density values recorded were 1204±26 mOD for *A. sativum*, 89±2 mOD for *O. vulgare*, 57±10 mOD for *M. citrifolia*, 140±14 mOD for *C. sinensis*, 105±13 mOD for ascorbic acid (positive control), and 1915±67 mOD for the negative control (distilled water replacing plant extract).

Superoxide detection

Antioxidant properties of plant extracts affected the superoxide generation differently. The lowest concentrations of *A. sativum*, *C. sinensis*, and *O. vulgare* extracts reduce superoxide generation in unstimulated hemocytes without affecting their rates. *Camellia sinensis* and *O. vulgare* extracts also decreased superoxide generation in stimulated hemocytes (respiratory burst elicited by PMA) at the two lowest concentrations tested without affecting rates, while *M. citrifolia* extract significantly reduce superoxide elicited by PMA in hemocytes, negatively affecting superoxide generation rates. (Fig. 2). The highest concentration of *A. sativum* (144500 µg mL^{-1}), produced high rates of superoxide generation, while *C. sinensis* produced a strong response in superoxide generation in unstimulated and stimulated hemocytes at the highest concentration tested.

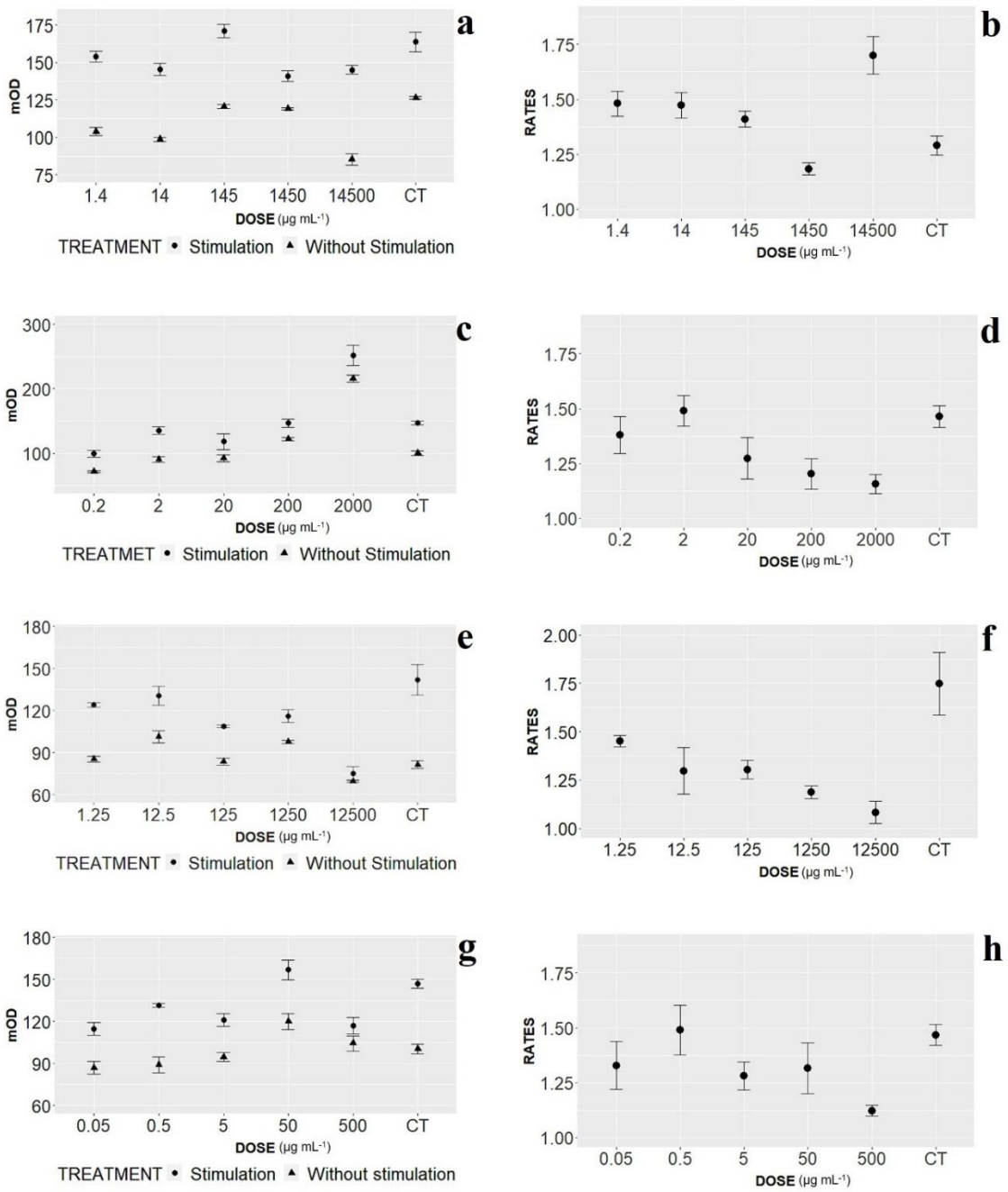

Figure 2. Superoxide anion generation by shrimp hemocytes in the presence of aqueous solutions of *Allium sativum*, *Camellia sinensis*, *Morinda citrifolia*, and *Origanum vulgare*, at several concentrations (µg mL^{-1}). In a, c, e, and g: Base activity and stimulated activity. In b, d, f, and h superoxide generation is expressed in rates. Rates have been calculated by dividing the optical density (620 nm) value of the sample stimulated with PMA against the value of the same sample without stimulation. Error bars represent the standard error of three replicates

Pond trial

Survival and yield were high in all ponds (treatments and control), and there were no significant differences among treatments. However, shrimps treated with *A. sativum* were larger than the shrimps from the control (P = 0.029) and those treated with *M. citrifolia* (P = 0.039). Considering the organoleptic characteristics, lower melanosis was observed in the animals treated with *A. sativum* and *M. citrifolia* (Fig. 3). A difference in taste was not detected among the treatments, and animals treated with *A. sativum* (Table 4), were visually more appealing, as they were more colorful and larger.

Table 4. Production variables at harvest of shrimp ponds treated with aqueous extracts of *Allium sativum* and *Morinda citrifolia* applied to commercial feed, plus a control treatment without aqueous extracts.

Characteristics	Treatment		
	Control	*Allium sativum*	*Morinda citrifolia*
Initial density (shrimp/m^2)	6	6	6
Final density (shrimp/m^2)	5±0.5	4.3±0.3	4.8±0.5
Weight (g)	10.2±0.8	12.7±1.4	10.4±1.0
Survival (%)	82±9	72±6	79±8
Yield (kg/ha)	503±33	548±43	497±87
Feed Conversion Ratio	1.6±0.1	1.5±0.1	1.6±0.3
Melanosis (percentage of telsons dark pigmented)	100%	30%	30%

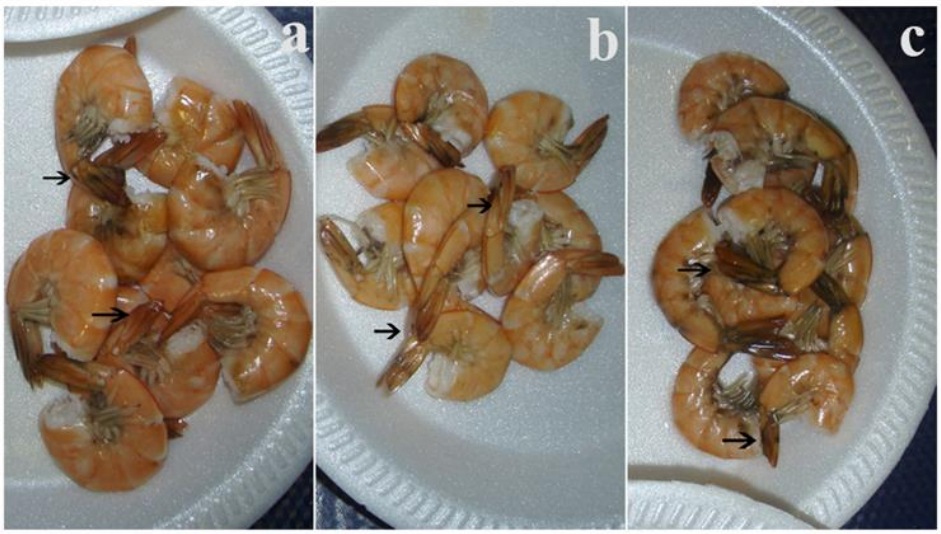

Figure 3. Appearance at the harvest of shrimp treated with plant extracts. a. *Allium sativum*; b. *Morinda citrifolia*; c. Control. Note the body color and level of melanization of the uropods (arrows).

DISCUSSION

In this study, we evaluated the effects of four GRAS plants *A. sativum*, *O. vulgare*, *C. sinensis*, and *M. citrifolia* aqueous extracts as tools to manage shrimp health. All four extracts exhibited antibacterial activity against pathogenic Vibrios of shrimp. At sublethal doses for pathogen *Vibrio* spp., the plant extracts exhibited the ability to disrupt the biofilm formation and luminescence, processes regulated by QS. At sublethal concentration, plant extracts also exhibit antioxidant properties, without affecting the ratios of superoxide generation in shrimp hemocytes. All plant extracts showed antibacterial activity against the three Vibrios. Curiously the inhibiting zone persisted, for several days, contrary to what is observed in the antibiograms performed with antibiotics, where the bacteria begin to invade the inhibition halos from 24 hours of exposure. The aquaculture literature has reported the bactericidal features of *A. sativum*, *O. vulgare*, *C. sinensis*, and *M. citrifolia* (Wei *et al*., 2008; Stefanakis *et al.*, 2014; Breyer *et al*., 2015; Kongchum *et al*., 2016; Chirawithayaboon *et al*., 2020; Fakharzadeh *et al*., 2020). In our study, we confirmed the anti-vibrio activity of aqueous extracts prepared with these plants. This was the preliminary step to studying its effect on QS indicators, such as bioluminescence, and biofilms, as well as antioxidant and immunomodulatory properties at sublethal concentrations.

Currently, QS disruption by using compounds at sublethal doses represents an alternative in microbiological control (Defoirdt *et al*., 2010; Lu *et al*., 2022), to avoid the selection pressure over the bacteria, minimizing the occurrence of bacterial resistance (Defoirdt *et al*., 2010). The ability of the tested extracts to inhibit the luminescence of *V. harveyi* and the biofilm formation of the three Vibrios. indicates that the four plant extracts can be applied in culture systems as bioproducts to manage bacterial diseases by limiting the risk of persistent vibriosis. Since infectious processes can induce oxidative stress, another aspect

addressed in this study was the antioxidant capacity of the plant extracts. (Duan *et al*., 2015; Arvaniti *et al*., 2019;). But oxygen radicals generated during the respiratory burst are potent antibacterial oxidizing agents, essential in host defense (Knight, 2000; Obeagu, 2018). Poor control of antioxidant mechanisms can increase the cost of the immune system, impair respiratory burst, and lead to tissue damage. Thus, in this study, to improve shrimp immune reactions, we tested concentrations of plant extracts that allow us to take advantage of their antioxidant properties without interfering with the oxidative reactions associated with the respiratory burst.

At sublethal doses, all the tested aqueous solutions exhibited significant antioxidant capacity. *Allium sativum* is a seleniferous plant (Rivlin, 2009), containing phenolic and organosulphur compounds (Phan *et al*., 2019), including the antioxidant diallyl sulfide (Phan *et al*., 2019; Herrera-Calderon *et al.*, 2021;). The antioxidant features of *M. citrifolia* (Ly et al., 2020; Pandy et al., 2020) are caused by the presence of lignans /neolignans (americanin, morindolin and others) (Nelson & Elevitch, 2006), whereas the antioxidant properties of *O. vulgare* and *C. sinensis* are explained by the presence of thymol, carvacrol and polyphenolic compounds (Ozdemir *et al*., 2018; Rani *et al.*, 2018). In addition, antioxidant compounds of crude plant extracts contain polysaccharides. These complex combinations of compounds have a deep immunomodulatory effect. Therefore, the effect of plant extracts on an effector of the immune response and the superoxide generation associated with respiratory burst were evaluated.

The generation of superoxide as basal activity decreased at low concentrations of *A. sativum*, *O. vulgare*, and *C. sinensis* extracts; however, this generation increased at higher concentrations of these extracts. The most consistent results were obtained from *A. sativum*, which showed powerful properties to scavenge superoxide from unstimulated hemocytes, while an increase in PMA-induced superoxide generation was observed at all concentrations tested (including the concentration inhibiting *Vibrio* spp. biofilm formation). Low basal superoxide generation in unstimulated hemocytes, accompanied by high responses under eliciting with PMA, results in enhanced rates of superoxide generation. These results could be explained by the antioxidant properties and immunostimulant polysaccharides present in *A. sativum* (Jiang *et al.*, 2022). Nya & Austin (2009) reported the stimulating effect of garlic on phagocytosis and the respiratory burst of trout. *Origanum vulgare* and *C. sinensis* extracts did not affect PMA-evoked superoxide generation rate in hemocytes, but *M. citrifolia* extract reduced rates mainly at high concentrations. These results can be explained by the potent antioxidant activity detected in this extract in the ABTS assay.

Based on anti-vibrio activity, immunomodulatory performances, and antioxidant features at sublethal doses, the extracts of *A. sativum* and *M. citrifolia*, were selected to be applied in a preliminary assay performed in ponds. Both extracts were used at immunomodulating concentration. Considering in the calculation a food rate of 5% of the biomass, and losses by leaching and other causes (e.g., food consumption by fish present in ponds, such as *Poecilia reticulata*). No vibriosis outbreaks were observed in ponds, thus treatments were not challenged, but the antioxidant properties of both extracts had an unexpected positive effect on the quality of the final harvested product, observing a significant decrease in the melanosis in the harvested shrimp, which were not exposed to sodium metabisulfite. Also, shrimp treated with the extract of *A. sativum* were larger than those of the control, resulting in higher yields in the ponds. It cannot be ruled out that this effect on size was caused by enhanced growth from the added probiotics. Indeed, *A. sativum* has fructans, polysaccharides known as prebiotic (Jiang *et al.*, 2022). In the future, tests can be carried out combining probiotics and *A. sativum* extracts at sublethal concentrations, which would not exert a negative effect on probiotics. Despite the strong flavor of *A. sativum* and *M. citrifolia*, during a palatability test performed during the harvest, the volunteers did not find a difference in flavor between the treated and control shrimp; rather, they reported being more attracted by big red shrimp (ie, those treated with *A. sativum*). Color, taste, appearance, and organoleptic characteristics are critical in defining the quality of foodstuffs (Dixon, 1968) , and they significantly impact the marketing of the final product. These preliminary results illustrate the many applications that these natural products can have in shrimp aquaculture.

In conclusion, the extracts of GRAS plants can contribute to the control of vibriosis and other disorders, provoked by vibrio, also protecting shrimps from oxidative stress, and positively influencing their immune

system. Using GRAS plant extracts has the advantage of not presenting any risk to human health. In addition, sublethal doses applied to food do not affect the culture water quality. Another advantage is that they are effective at a wide range of sublethal concentrations, and therefore, their effectiveness can be guaranteed despite the possible variability in the chemical composition of the plants used. Finally, the plants tested are easily accessible at a low cost. As a result, aqueous plant extracts constitute promising tools that can be used to design health management strategies in shrimp cultures and thus avoid the use of antibiotics.

Acknowledgements

We thank Peter Bossier and Amanda Sliva for corrections and comments; they helped complete our manuscript. This study was supported by the project "Marine microbiology and immunomodulation with a focus on disease control of *Penaeus vannamei* shrimp"

Declarations of interest: none

REFERENCES

Adineh, H., Harsij, M., Jafaryan, H., & Asadi, M. (2020). The effects of microencapsulated garlic (*Allium sativum*) extract on growth performance, body composition, immune response and antioxidant status of rainbow trout (*Oncorhynchus mykiss*) juveniles. *Journal of Applied Animal Research*, 48(1):372-378. https://doi.org/10.1080/09712119.2020.1808473.

AftabUddin, S., Siddique, M. A. M., Romkey, S. S., & Shelton, W. L. (2017). Antibacterial function of herbal extracts on growth, survival and immunoprotection in the black tiger shrimp *Penaeus monodon*. *Fish & Shellfish Immunology*, 65:52-58. https://doi.org/10.1016/j.fsi.2017.03.050.

Akinbowale, O. l., Peng, H., & Barton, M. d. (2007). Diversity of tetracycline resistance genes in bacteria from aquaculture sources in Australia. *Journal of Applied Microbiology*, 103(5):2016-2025. https://doi.org/10.1111/j.1365-2672.2007.03445.x.

Arvaniti, O. S., Samaras, Y., Gatidou, G., Thomaidis, N. S., & Stasinakis, A. S. (2019). Review on fresh and dried figs: Chemical analysis and occurrence of phytochemical compounds, antioxidant capacity and health effects. *Food Research International*, 119,: 244-267. https://doi.org/10.1016/j.foodres.2019.01.055

Asimi, O., & Sahu, N. (2013). Herbs/spices as feed additive in aquaculture. *Scientific Journal of Pure and Applied Sciences*, 2:284-292. doi: 10.14196/sjpas.v2i8.868.

Beshiru, A., & Igbinosa, E. O. (2018). Characterization of extracellular virulence properties and biofilm-formation capacity of *Vibrio* species recovered from ready-to-eat (RTE) shrimps. *Microbial Pathogenesis*, :93-102. https://doi.org/10.1016/j.micpath.2018.04.015.

Breyer, K. E., Getchell, R. G., Cornwell, E. R., Wooster, G. A., Ketola, H. G., & Bowser, P. R. (2015). Efficacy of an Extract from Garlic, *Allium sativum*, Against Infection with the Furunculosis Bacterium, *Aeromonas salmonicida*, in Rainbow Trout, *Oncorhynchus mykiss*. *Journal of the World Aquaculture Society*, 46(3):273-282. https://doi.org/10.1111/jwas.12195.

Castex, M., Lemaire, P., Wabete, N., & Chim, L. (2010). Effect of probiotic *Pediococcus acidilactici* on antioxidant defences and oxidative stress of *Litopenaeus stylirostris* under *Vibrio nigripulchritudo* challenge. *Fish & Shellfish Immunology*, 28(4):622-631. https://doi.org/10.1016/j.fsi.2009.12.024

De Queiroz, Y. S., Antunes, P. B., Vicente, S. J. V., Sampaio, G. R., Shibao, J., Bastos, D. H. M., & Torres, E. A. F. da S. (2014). Bioactive compounds, *in vitro* antioxidant capacity and Maillard reaction products of raw, boiled, and fried garlic (*Allium sativum* L.). *International Journal of Food Science & Technology*, 49(5):1308-1314. https://doi.org/10.1111/ijfs.12428.

Defoirdt, T., Boon, N., & Bossier, P. (2010). Can Bacteria Evolve Resistance to Quorum Sensing Disruption? *PLOS Pathogens*, 6(7), e1000989. https://doi.org/10.1371/journal.ppat.1000989.

Dixon, M. P. (1968). Organoleptic qualities. *International Journal of Food Science & Technology*, 3(s1):423-429. https://doi.org/10.1111/j.1365-2621.1968.tb01484.x.

Djordjevic, D., Wiedmann, M., & McLandsborough, L. A. (2002). Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. *Applied and Environmental Microbiology*, 68(6):2950-2958. https://doi.org/10.1128/AEM.68.6.2950-2958.2002.

Done, H. Y., & Halden, R. U. (2015). Reconnaissance of 47 antibiotics and associated microbial risks in seafood sold in the United States. *Journal of Hazardous Materials*, 282:10-17. https://doi.org/10.1016/j.jhazmat.2014.08.075.

Duan, Y., Zhang, J., Dong, H., Wang, Y., Liu, Q., & Li, H. (2015). Oxidative stress response of the black tiger shrimp *Penaeus monodon* to *Vibrio parahaemolyticus* challenge. *Fish & Shellfish Immunology*, 46(2):354-365. https://doi.org/10.1016/j.fsi.2015.06.032.

Elumalai, P., Kurian, A., Lakshmi, S., Faggio, C., Esteban, M. A., & Ringø, E. (2021). Herbal Immunomodulators in Aquaculture. *Reviews in Fisheries Science & Aquaculture*, 29(1):33-57. https://doi.org/10.1080/23308249.2020.1779651.

Fakharzadeh, S. M. E., Haghighi, M., Rohani, S., Sharifpour, I., & Hamidi, M. (2020). An in vitro and in vivo study on antimicrobial activity of *Origanum vulgare* extract and its nano form against *Streptococcus iniae* in rainbow trout (*Oncorhynchus mykiss*). *Iranian Journal of Fisheries Sciences*, 19(5):2454-2463.

García Beltrán, J. M., Silvera, D. G., Ruiz, C. E., Campo, V., Chupani, L., Faggio, C., & Esteban, M. Á. (2020). Effects of dietary *Origanum vulgare* on gilthead seabream (*Sparus aurata* L.) immune and antioxidant status. *Fish & Shellfish Immunology*, 99:452-461. https://doi.org/10.1016/j.fsi.2020.02.040.

Gullian Klanian, M., & Rodríguez, J. (2001). *Estudio de las cualidades inmunoestimulantes de bacterias probióticas asociadas al cultivo de Litopenaeus vannamei*. Tesis de Pregrado. Escuela Superior Politécnica del Litoral, Guayaquil, Ecuador.

Han, J. E., Tang, K. F. J., Tran, L. H., & Lightner, D. V. (2015). Photorhabdus insect-related (Pir) toxin-like genes in a plasmid of *Vibrio parahaemolyticus*, the causative agent of acute hepatopancreatic necrosis disease (AHPND) of shrimp. *Diseases of Aquatic Organisms*, 113(1):33-40. https://doi.org/10.3354/dao02830.

Hasanpour, S., Salati, A. P., Falahatkar, B., & Azarm, H. M. (2017). Effects of dietary green tea (*Camellia sinensis* L.) supplementation on growth performance, lipid metabolism, and antioxidant status in a sturgeon hybrid of Sterlet (*Huso huso* ♂ × *Acipenser ruthenus* ♀) fed oxidized fish oil. *Fish Physiology and Biochemistry*, 43(5):1315-1323. https://doi.org/10.1007/s10695-017-0374-z.

Herrera-Calderon, O., Chacaltana-Ramos, L. J., Huayanca-Gutiérrez, I. C., Algarni, M. A., Alqarni, M., & Batiha, G. E.-S. (2021). Chemical Constituents, *In Vitro* Antioxidant Activity and *In Silico* Study on NADPH Oxidase of *Allium sativum* L. (Garlic) Essential Oil. *Antioxidants*, 10(11):1844. https://doi.org/10.3390/antiox10111844.

Jahanjoo, V., Yahyavi, M., Akrami, R., & Bahri, A. H. (2018). Influence of adding garlic (*Allium sativum*), ginger (*Zingiber officinale*), thyme (*Thymus vulgaris*) and their combination on the growth performance, haemato-immunological parameters and disease resistance to *Photobacterium damselae* in Sobaity Sea Bream (*Sparidentex hasta*) Fry. *Turkish Journal of Fisheries and Aquatic Sciences*, 18(4):633-645. https://doi.org/10.4194/1303-2712-v18_4_15.

Jiang, X.-Y., Liang, J.-Y., Jiang, S.-Y., Zhao, P., Tao, F., Li, J., Li, X.-X., & Zhao, D.-S. (2022). Garlic polysaccharides: A review on their extraction, isolation, structural characteristics, and bioactivities. *Carbohydrate Research*, *518*, 108599. https://doi.org/10.1016/j.carres.2022.108599.

Kakoolaki, S., Akbary, P., Zorriehzahra, M. J., Salehi, H., Sepahdari, A., Afsharnasab, M., Mehrabi, M. R., & Jadgal, S. (2016). *Camellia sinensis* supplemented diet enhances the innate non-specific responses, haematological parameters, and growth performance in *Mugil cephalus* against *Photobacterium damselae*. *Fish & Shellfish Immunology*, 57: 379-385. https://doi.org/10.1016/j.fsi.2016.08.060.

Kiran, G. S., Lipton, A. N., Priyadharshini, S., Anitha, K., Suárez, L. E. C., Arasu, M. V., Choi, K. C., Selvin, J., & Al-Dhabi, N. A. (2014). Antiadhesive activity of poly-hydroxy butyrate biopolymer from a marine

Brevibacterium casei MSI04 against shrimp pathogenic vibrios. *Microbial Cell Factories*, 13(1):114. https://doi.org/10.1186/s12934-014-0114-3.

Knight, J. A. (2000). Review: Free radicals, antioxidants, and the immune system. *Annals of Clinical & Laboratory Science*, 30(2):145-158.

Kongchum, P., Chimtong, S., Chareansak, N., & Subprasert, P. (2016). Effect of Green Tea Extract on *Vibrio parahaemolyticus* Inhibition in Pacific White Shrimp (*Litopenaeus vannamei*) Postlarvae. *Agriculture and Agricultural Science Procedia*, 11:117-124. https://doi.org/10.1016/j.aaspro.2016.12.020.

Loo, K.-Y., Letchumanan, V., Law, J. W.-F., Pusparajah, P., Goh, B.-H., Ab Mutalib, N.-S., He, Y.-W., & Lee, L.-H. (2020). Incidence of antibiotic resistance in *Vibrio* spp. *Reviews in Aquaculture*, 12(4):2590-2608. https://doi.org/10.1111/raq.12460.

Lu, L., Li, M., Yi, G., Liao, L., Cheng, Q., Zhu, J., Zhang, B., Wang, Y., Chen, Y., & Zeng, M. (2022). Screening strategies for quorum sensing inhibitors in combating bacterial infections. *Journal of Pharmaceutical Analysis*, 12(1):1-14. https://doi.org/10.1016/j.jpha.2021.03.009.

Ly, H. T., Pham Nguyen, M. T., Nguyen, T. K. O., Bui, T. P. Q., Ke, X., & Le, V. M. (2020). Phytochemical Analysis and Wound-Healing Activity of Noni (*Morinda citrifolia*) Leaf Extract. *Journal of Herbs, Spices & Medicinal Plants*, 26(4):379-393. https://doi.org/10.1080/10496475.2020.1748159.

Marisa Halim, A., Lee, P.-P., Chang, Z.-W., & Chang, C.-C. (2017). The hot-water extract of leaves of noni, *Morinda citrifolia*, promotes the immunocompetence of giant freshwater prawn, *Macrobrachium rosenbergii*. *Fish & Shellfish Immunology*, 64:457-468. https://doi.org/10.1016/j.fsi.2017.03.045.

McFARLAND, J. (1907). The nephelometer: an instrument for estimating the number of bacteria in suspensions used for calculating the opsonic index and for vaccines. *Journal of the American Medical Association*, 49(14):1176-1178. https://doi.org/10.1001/jama.1907.25320140022001f.

Meek, R. W., Vyas, H., & Piddock, L. J. V. (2015). Nonmedical Uses of Antibiotics: Time to Restrict Their Use? *PLOS Biology*, 13(10):e1002266. https://doi.org/10.1371/journal.pbio.1002266.

Mendonça-Filho, R. R. (2006). Bioactive Phytocompounds: New Approaches in the Phytosciences. En *Modern Phytomedicine* (pp. 1-24). John Wiley & Sons, Ltd. https://doi.org/10.1002/9783527609987.ch1

Mohammadi, G., Rafiee, G., El Basuini, M. F., Van Doan, H., Ahmed, H. A., Dawood, M. A. O., & Abdel-Latif, H. M. R. (2020). Oregano (*Origanum vulgare*), St John's-wort (*Hypericum perforatum*), and lemon balm (*Melissa officinalis*) extracts improved the growth rate, antioxidative, and immunological responses in Nile tilapia (*Oreochromis niloticus*) infected with *Aeromonas hydrophila*. *Aquaculture Reports*, 18:100445. https://doi.org/10.1016/j.aqrep.2020.100445.

Mohan, K., Ravichandran, S., Muralisankar, T., Uthayakumar, V., Chandirasekar, R., Seedevi, P., Abirami, R. G., & Rajan, D. K. (2019a). Application of marine-derived polysaccharides as immunostimulants in aquaculture: A review of current knowledge and further perspectives. *Fish & Shellfish Immunology*, 86:1177-1193. https://doi.org/10.1016/j.fsi.2018.12.072.

Mohan, K., Ravichandran, S., Muralisankar, T., Uthayakumar, V., Chandirasekar, R., Seedevi, P., & Rajan, D. K. (2019b). Potential uses of fungal polysaccharides as immunostimulants in fish and shrimp aquaculture: A review. *Aquaculture*, 500:250-263. https://doi.org/10.1016/j.aquaculture.2018.10.023.

Morales-Covarrubias, M. S., García-Aguilar, N., Bolan-Mejía, M. D., & Puello-Cruz, A. C. (2016). Evaluation of medicinal plants and colloidal silver efficiency against *Vibrio parahaemolyticus* infection in *Litopenaeus vannamei* cultured at low salinity. *Diseases of Aquatic Organisms*, 122(1):57-65. https://doi.org/10.3354/dao03060.

Mukherjee, S., & Bassler, B. L. (2019). Bacterial quorum sensing in complex and dynamically changing environments. *Nature Reviews Microbiology*, 17(6):371-382. https://doi.org/10.1038/s41579-019-0186-5

Muñoz, M., Cedeño, R., Rodríguez, J., van der Knaap, W. P. W., Mialhe, E., & Bachère, E. (2000). Measurement of reactive oxygen intermediate production in haemocytes of the penaeid shrimp, *Penaeus vannamei*. *Aquaculture*, 191(1):89-107. https://doi.org/10.1016/S0044-8486(00)00420-8.

Nelson, S. C., & Elevitch, C. R. (2006). *Noni: The Complete Guide for Consumers and Growers*. PAR.

Ngo, S. N. T., Williams, D. B., Cobiac, L., & Head, R. J. (2007). Does garlic reduce risk of colorectal cancer? A systematic review. *The Journal of Nutrition*, 137(10):2264-2269. https://doi.org/10.1093/jn/137.10.2264.

Obeagu, E. (2018). *A Review on Free Radicals and Antioxidants*. 4:123-133. https://doi.org/10.22192/ijcrms.2018.04.02.019.

Ozdemir, N., Ozgen, Y., Kiralan, M., Bayrak, A., Arslan, N., & Ramadan, M. F. (2018). Effect of different drying methods on the essential oil yield, composition and antioxidant activity of *Origanum vulgare* L. and *Origanum onites* L. *Journal of Food Measurement and Characterization*, 12(2):820-825. https://doi.org/10.1007/s11694-017-9696-x

Palanikumar, P., Wahjuningrum, D., Abinaya, P., Babu, M. M., & Citarasu, T. (2020). Usage of plant natural products for prevention and control of white feces syndrome (WFS) in Pacific whiteleg shrimp *Litopenaeus vannamei* farming in India. *Aquaculture International*, 28(1):113-125. https://doi.org/10.1007/s10499-019-00448-5.

Pandy, V., Bonam, S. R., Raya, A. R., & Nadendla, R. R. (2020). Chapter 19—Morinda citrifolia Linn. (Noni) fruit, antioxidant effects, and neuroprotection. En C. R. Martin & V. R. Preedy (Eds.), *Oxidative Stress and Dietary Antioxidants in Neurological Diseases* (pp. 291-307). Academic Press. https://doi.org/10.1016/B978-0-12-817780-8.00019-0.

Parsek, M. R., & Greenberg, E. P. (2005). Sociomicrobiology: The connections between quorum sensing and biofilms. *Trends in Microbiology*, 13(1):27-33. https://doi.org/10.1016/j.tim.2004.11.007.

Passos da Silva, D., Schofield, M. C., Parsek, M. R., & Tseng, B. S. (2017). An Update on the Sociomicrobiology of Quorum Sensing in Gram-Negative Biofilm Development. *Pathogens*, 6(4):51. https://doi.org/10.3390/pathogens6040051.

Phan, A. D. T., Netzel, G., Chhim, P., Netzel, M. E., & Sultanbawa, Y. (2019). Phytochemical Characteristics and Antimicrobial Activity of Australian Grown Garlic (*Allium sativum* L.) Cultivars. *Foods*, 8(9):358. https://doi.org/10.3390/foods8090358.

Piyanut Chirawithayaboon, Nontawith Areechon, & Oraporn Meunpol. (s. f.). | Hepatopancreatic antioxidant enzyme activities and disease resistance of Pacific white shrimp (*Litopenaeus vannamei*) fed diet supplemented with garlic (*Allium sativum*) extract. *Agriculture and Natural Resources*, 54(4):377-386. https://li01.tci-thaijo.org/index.php/anres/article/view/247954.

El Productor, E. (06 enero 2020). *Prohíben ingreso de camarones de algunos exportadores de Malasia por presencia de antibióticos | Noticias Agropecuarias*. https://elproductor.com/2020/01/prohiben-ingreso-de-camarones-de-algunos-exportadores-de-malasia-por-presencia-de-antibioticos/

Rani, R., Arora, S., Kaur, J., & Manhas, R. K. (2018). Phenolic compounds as antioxidants and chemopreventive drugs from *Streptomyces cellulosae* strain TES17 isolated from rhizosphere of *Camellia sinensis*. *BMC Complementary and Alternative Medicine*, 18(1):82. https://doi.org/10.1186/s12906-018-2154-4.

Ravipati, A. S., Zhang, L., Koyyalamudi, S. R., Jeong, S. C., Reddy, N., Bartlett, J., Smith, P. T., Shanmugam, K., Münch, G., Wu, M. J., Satyanarayanan, M., & Vysetti, B. (2012). Antioxidant and anti-inflammatory activities of selected Chinese medicinal plants and their relation with antioxidant content. *BMC Complementary and Alternative Medicine*, 12:173. https://doi.org/10.1186/1472-6882-12-173.

Reverter, M., Tapissier- Bontemps, N., Sarter, S., Sasal, P., & Caruso, D. (2021). Moving towards more sustainable aquaculture practices: A meta- analysis on the potential of plant- enriched diets to improve fish growth, immunity and disease resistance. *Reviews in Aquaculture*, 13(1):537-555. https://doi.org/10.1111/raq.12485.

Rivlin, R. S. (2009). Can garlic reduce risk of cancer?123. *The American Journal of Clinical Nutrition*, 89(1):17-18. https://doi.org/10.3945/ajcn.2008.27181.

SINGH, M., Mallick, A. K., BANERJEE, M., & KUMAR, R. (2016). Loss of outer membrane integrity in Gram-negative bacteria by silver nanoparticles loaded with *Camellia sinensis* leaf phytochemicals: Plausible mechanism of bacterial cell disintegration. *Bulletin of Materials Science*, 39(7):1871-1878. https://doi.org/10.1007/s12034-016-1317-5.

Soković, M., Glamočlija, J., Marin, P. D., Brkić, D., & van Griensven, L. J. L. D. (2010). Antibacterial effects of the essential oils of commonly consumed medicinal herbs using an in vitro model. *Molecules (Basel, Switzerland)*, 15(11):7532-7546. https://doi.org/10.3390/molecules15117532.

Srinivasan, R., Santhakumari, S., & Ravi, A. V. (2017). *In vitro* antibiofilm efficacy of Piper betle against quorum sensing mediated biofilm formation of luminescent *Vibrio harveyi*. *Microbial Pathogenesis*, 110:232-239. https://doi.org/10.1016/j.micpath.2017.07.001.

Stefanakis, M. K., Anastasopoulos, E., Katerinopoulos, H. E., & Makridis, P. (2014). Use of essential oils extracted from three *Origanum* species for disinfection of cultured rotifers (*Brachionus plicatilis*). *Aquaculture Research*, 45(11):1861-1866. https://doi.org/10.1111/are.12137.

Sutton, S. (2011). Measurement of Microbial Cells by Optical Density. *Journal of Validation Technology*, 4;46-49.

Talpur, A. D., & Ikhwanuddin, M. (2012). Dietary effects of garlic (*Allium sativum*) on haemato-immunological parameters, survival, growth, and disease resistance against *Vibrio harveyi* infection in Asian sea bass, *Lates calcarifer* (Bloch). *Aquaculture*, 364-365:6-12. https://doi.org/10.1016/j.aquaculture.2012.07.035.

Vandenberghe, J., Verdonck, L., Robles-Arozarena, R., Rivera, G., Bolland, A., Balladares, M., Gomez-Gil, B., Calderon, J., Sorgeloos, P., & Swings, J. (1999). Vibrios associated with *Litopenaeus vannamei* larvae, postlarvae, broodstock, and hatchery probionts. *Applied and Environmental Microbiology*, 65(6):2592-2597. https://doi.org/10.1128/AEM.65.6.2592-2597.1999.

Velázquez-Lizárraga, A. E., Juárez-Morales, J. L., Racotta, I. S., Villarreal-Colmenares, H., Valdes-Lopez, O., Luna-González, A., Rodríguez-Jaramillo, C., Estrada, N., & Ascencio, F. (2019). Transcriptomic analysis of Pacific white shrimp (*Litopenaeus vannamei*, Boone 1931) in response to acute hepatopancreatic necrosis disease caused by *Vibrio parahaemolyticus*. *PLOS ONE*, 14(8):e0220993. https://doi.org/10.1371/journal.pone.0220993.

Walker, R. B., & Everette, J. D. (2009). Comparative reaction rates of various antioxidants with ABTS radical cation. *Journal of Agricultural and Food Chemistry*, 57(4):1156-1161. https://doi.org/10.1021/jf8026765

Wan, Q., Li, N., Du, L., Zhao, R., Yi, M., Xu, Q., & Zhou, Y. (2019). Allium vegetable consumption and health: An umbrella review of meta-analyses of multiple health outcomes. *Food Science & Nutrition*, 7(8):2451-2470. https://doi.org/10.1002/fsn3.1117.

Wei, L. S., Musa, N., Sengm, C. T., Wee, W., & Shazili, N. a. M. (2008). Antimicrobial properties of tropical plants against 12 pathogenic bacteria isolated from aquatic organisms. *African Journal of Biotechnology*, 7(13):2275-2278. https://doi.org/10.4314/ajb.v7i13.58974.

Yano, Y., Satomi, M., & Oikawa, H. (2006). Antimicrobial effect of spices and herbs on *Vibrio parahaemolyticus*. *International Journal of Food Microbiology*, 111(1):6-11. https://doi.org/10.1016/j.ijfoodmicro.2006.04.031.

Zhao, W., Wang, L., Liu, M., Jiang, K., Qi, C., Yang, G., & Wang, B. (2017). The effects of *Vibrio parahaemolyticus* on hepatopancreas antioxidant enzyme activity and gene expression of *Litopenaeus vannamei. Journal of Fishery Sciences of China 24(6):1261-1270 DOI:10.3724/SP.J.1118.2017.16221.*

Recibido: 23-06-2022
Aprobado: 31-07-2022
Versión final: 04-08-2022