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Research Article

Evaluation of aqueous extracts of *Gracilaria vermiculophylla* and *Ulva flexuosa* as treatment in challenged *Penaeus vannamei* with *Vibrio parahaemolyticus*

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ABSTRACT. This work looks at the antibacterial efficacy of *Gracilaria vermiculophylla* and *Ulva flexuosa* aqueous extracts when added to the feed of *Penaeus vannamei* in an experimental infection with *Vibrio parahaemolyticus* (M0904AHPND+strain). Results show that the minimum inhibitory concentration (MIC) for both extracts was 50 mg mL^{-1}, with inhibition zones of 18.00 ± 0.60 mm for *G. vermiculophylla* and 14.00 ± 0.29 mm for *U. flexuosa*. *G. vermiculophylla* gave a higher total content of phenolic compounds (10.58 ± 2.31 mg GAE g^{-1}) (gallic acid equivalent) and lower flavonoid content (10.32 ± 0.73 mg QE g^{-1}) in comparison to *U. flexuosa*, while using ABTS (2,2-diphenyl-1-picrylhydrazyl) and ferric reducing antioxidant power (FRAP) to measure antioxidant capacity showed that *G. vermiculophylla* gave a better result than *U. flexuosa*. The challenge with *V. parahaemolyticus* resulted in 67% survival for organisms fed *G. vermiculophylla* and 60% for those fed *U. flexuosa*, resulting in twice the amount of survival as opposed to 30% in the positive control at 24 h post-infection. Histopathological alterations in the hepatopancreas with hemocytic infiltration within the intertubular connective tissue were observed. Also, tubules with severe cell detachment and tubular atrophy were detected in the positive control organisms, and organisms treated with macroalgae only had vermiform structures in the tubular lumen, cell detachment, and infiltration hemolymph in intertubular connective tissue. According to the analysis of the studied variables, it can be concluded that the aqueous extracts of these macroalgae are a promising alternative for *V. parahaemolyticus* (M0904AHPND+strain) control in shrimp culture.

Keywords: macroalgae; *Vibrio parahaemolyticus*; *Penaeus vannamei*; phytogenics; AHPND; flavonoids

INTRODUCTION

Diseases affecting shrimp culture are mainly related to viruses and bacteria (Flegel 2012); since 2011, pathologies of bacterial origin have become more evident worldwide due to high mortalities affecting shrimp production units, leading to economic losses. One of the most serious diseases in shrimp farms is acute hepatopancreatic necrosis disease (AHPND), formerly known as early mortality syndrome (EMS), which is a bacterial shrimp disease due to the action of Pir A and B toxin secreted by *Vibrio parahaemolyticus* (Santos et al. 2020). *V. parahaemolyticus*, *V. campbellii*, *V. owensii*, and *V. punensis* have been proved to cause AHPND. However, the mechanisms underlying the burgeoning number of *Vibrio* species that cause AHPND is not known. All of AHPND-causing *Vibrio* bacteria (V_{AHPND}) harbor a highly homologous plasmid (designated as pVA1-type) carrying $pirAB^{vp}$ toxin genes (Dong et al. 2019)

Previous studies have reported shrimp pathogens belonging to the *Vibrio* genera showing resistance to antibiotics such as tetracycline and β-lactam antibiotics from aquaculture settings (Melo et al. 2011, Albuquerque-Costa et al. 2015). Karunasagar et al. (1994) reported mass mortalities of shrimp larvae due to antibiotic-resistant *Vibrio* infections, suggesting that antibiotic-resistant pathogens can be highly devastating.

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The source of antibiotic-resistant strains in shrimp farms could be the seawater collected for rearing. Draining antibiotics and other chemicals containing effluents into the sea may have contributed to the emergence of antibiotic-resistant *Vibrio* species in marine ecosystems (Srinivasan & Ramasamy 2009). Moreover, antibiotic residues in the tissues of aquatic animals can modify their intestinal flora and cause intoxication or allergies to the consumer (Alderman & Hastings 1998, Santiago et al. 2009, Morales-Covarrubias et al. 2011, 2012). As an alternative to this problem, the World Health Organization (WHO) recognizes the therapeutic potential of natural antibiotics extracted from plant sources for use in aquaculture (Abdallah 2011).

The Environmental Protection Agency (EPA, USA) has highlighted the need for research related to natural alternatives to synthetic antibiotics and their application in producing aquatic species that do not generate bacterial resistance. In the marine environment, natural products with pharmacological activities can be obtained, such as the bioactive compounds of sponges (Kotoku et al. 2017), soft corals (Lee et al. 2017), bryozoans (Ortega et al. 2017), mollusks (Chand & Karuso 2017), tunicates (Wang et al. 2017), echinoderms (Brasseur et al. 2017) and marine algae (Carvallo et al. 2013, Carroll et al. 2019) which can provide alternative treatments for diseases found in cultures of aquatic organisms.

During the last two decades, interest in using various extracts of marine algae as therapeutic or prophylactic agents in aquaculture has increased, mainly in diseases of bacterial origin because it has been shown that green, red and brown algae contain a wide variety of active metabolites with antibacterial, antiviral, antitumor and antioxidant properties (Hornsey & Hide 1974, Costa et al. 2010, Kelman et al. 2012, Farasat et al. 2014, Belattmania et al. 2016, Fatima et al. 2016, Osuna-Ruiz et al. 2016). Algal extracts can be administered to shrimp by injection, immersion, and feed supplement (Huang et al. 2006, Yeh et al. 2006). Hot water extract of the seaweed *Sargassum duplicatum* at a concentration below 10 μg g^{-1}, when injected into *Penaeus vannamei*, enhanced immune response against *V. parahemolyticus* infection (Yeh et al. 2006). Selvin et al. (2011) formulated a medicated diet using the secondary metabolites of marine algae, *Ulva fasciata*, which was highly effective in controlling shrimp bacterial infections at a dose of 1 g kg^{-1} of shrimp. Metabolites derived from marine algae are incorporated in shrimp feeds to improve the resistance of shrimp to infections. Dietary administration of the protein extract of red seaweed *Gracilaria fisheri* at 100 μg mL^{-1} in whiteleg shrimp exhibited better survival and had normal histological features of hepatopancreas when challenged with *V. parahaemolyticus* (Boonsri et al. 2017).

Marine algae are an excellent source of quorum quenching compounds that can reduce the biofilm formation of bacterial pathogens of shrimp. Ethanolic extract and furanone from *G. fisheri* inhibited biofilms of *V. parahaemolyticus* and *V. harveyi*, respectively, at sub minimum inhibitory concentrations (MIC) and reduced shrimp mortality that received these compounds through diet (Karnjana et al. 2019). A study conducted by Osuna-Amarillas et al. (2016) showed the antimicrobial property of the two extracts (acetone and methanol) of *Gracilaria vermiculophylla* on the growth of *V. parahaemolyticus*. The concentration the 50 μL with both extracts shows a higher inhibiting zone diameter (3.24 mm for the extract in methanol and 3.17 mm for the extract in acetone). In Mexico, *G. vermiculophylla* and *Ulva flexuosa* are widely distributed on the Pacific coasts (Rueness 2005, Abreu et al. 2011). This study's objective was to determine the effect of the aqueous extracts of *G. vermiculophylla* and *U. flexuosa* on the survival of *P. vannamei* infected with *V. parahaemolyticus* (M0904AHPND+strain), along with a wet-mount analysis and histopathological analysis.

MATERIALS AND METHODS

Marine algae collection and processing

During the hours of low tide, red algae (*G. vermiculophylla*) was collected from the Urías Estuary (23°10'47.81"N, 106°21'19.87"W) and green algae *U. flexuosa* from the intertidal zone of Playa Norte (23°12'29.04"N, 106°25'32.10"W), both sites located in Mazatlán, Sinaloa, Mexico. The samples were processed according to the methodology described by Thanigaivel et al. (2015). Each was rinsed in running water and dried in the shade at room temperature for five days, after which they were pulverized (Krups-Mod-GX410011MEX mill) and passed through an 850 μm sieve before being stored in paper bags at 7°C.

The technique reported by Morales-Covarrubias et al. (2016) was used to prepare the aqueous extracts: 5 g of each macroalga were added to 100 mL of distilled water at boiling point and boiled for 3 min, then filtered, and the concentrate was stored in an amber flask at 7°C.

Phytochemical composition and antioxidant capacity of aqueous extracts

The method proposed by Marigo (1973) was used to determine the total phenolic content (TPC) from the extracts of both macroalgae, whereas total flavonoids

content (TFC) were determined using the Luximon-Ramma et al. (2002) method, while sulfates content were determined by the method described by Dogson & Price (1962). Total uronic acids content was measured using the carbazole method (Bitter & Muir 1962), and the reduction of ABTS-free-radical scavenging activity 2.2´-azinobis (3-ethylbenzthiazoline)-6-sulphonic acid was determined by using the technique described by Przygodzka et al. (2014). The method described by Benzie & Strain (1996) as modified by Szôllôsi & Szôllôsi (2002) was used for ferric reducing antioxidant power (FRAP) assay.

Bacterial suspension preparation (inoculum)

A sample of *Vibrio parahaemolyticus* (M0904AH PND+strain) used in this experiment was isolated from shrimp farms affected by AHPND in northwestern Mexico and cryopreserved at -80°C (Soto-Rodriguez et al. 2015). The strain was recovered from cryovials, inoculated in 10 mL of tryptic soy broth (TSB) + 2.0% NaCl (TSB+ Bioxon), and incubated in a rotary shaker (nb-205L N-Biotek) at 30°C for 24 h. 100 µL was inoculated in 40 mL of TSB in triplicate; these samples were incubated in a rotary shaker at 30 ± 1°C for 24 h, bacterial growth was estimated by total viable count (TVC) on TCBS agar plates (BD Difco). Bacterial cells were washed by centrifugation (2330 *g* for 20 min at 20°C), and the optical density (OD600 nm) was adjusted to 1.0 (McFarland 1907).

MIC of aqueous extracts *G. vermiculophylla* and *U. flexuosa* against *V. parahaemolyticus* (M0904A HPND+strain)

Five concentrations (5, 30, 50, 70, and 90 mg mL^{-1}) of the aqueous extracts of the macroalgae were prepared in triplicate by adding 100 µL of the bacterial inoculum and then incubated at 30 ± 1°C for 24 h with constant stirring at 125 rpm. The samples were visually evaluated, and those that did not show turbidity were considered MIC (McDermott et al. 2005).

Minimum bactericidal concentration (MBC) of aqueous extracts *G. vermiculophylla* and *U. flexuosa* against *V. parahaemolyticus* (M0904AHPND+ strain)

Was determined in triplicate with the concentrations that did not produce turbidity (MIC), adding 100 µL of the bacterial inoculum in Petri dishes with tryptic soy broth (Bioxon© TSB, Mexico) and 2.0% NaCl, for 24 h, at 30 ± 1°C. The lowest concentration in which CFU did not occur was considered MBC (McDermott et al. 2005). Positive and negative controls were used to ensure adequate bacterial growth during the incubation period and sterility of the mediums (Lambert et al. 2001).

Sensitivity of aqueous extracts of *G. vermiculophylla* and *U. flexuosa* against *V. parahaemolyticus* (M09 04AHPND+strain)

The bactericidal capacity of the aqueous extracts of both macroalgae was determined by Bauer et al. (1966), with each experiment done in triplicate. A colony was selected and placed in a sterile saline solution until its turbidity matched a standard 0.5 McFarland (1907) solution. A 100 µL of bacterial suspension was added at a concentration of 1×10^8 CFU mL^{-1} and swabbed on the surface of Mueller-Hinton Agar (MHA) plates and supplemented with 2.5% NaCl and with pH adjusted to 8.4. Circles of sterile OXOID (OXOID antimicrobial susceptibility test discs) measuring 8 mm diameter were impregnated with three concentrations (10, 20, and 50 µL) in triplicate with the aqueous extracts of both macroalgae, including a negative control and incubated at 30 ± 1°C for 24 h. According to the test for bacterial sensitivity to antibiotics, effectiveness was classified according to the inhibition diameter proposed by Celikel & Kavas (2008), and it was achieved.

Food preparation and consumption assessment

Commercial feed pellets (Purina 35) were pulverized in a domestic mill, and a premix was made with 50 mL of aqueous extract (*G. vermiculophylla* and *U. flexuosa*) added. Distilled water was then added at 40°C. Pellets were reconstructed in the mill (3 mm in diameter), dried in the oven at 40°C for 12 h, and stored at 4°C. The same procedure was followed in the preparation of a control diet with no added aqueous extracts. The previously mentioned antibiogram procedure was applied to corroborate the antibacterial effectiveness, and the diameter of the inhibition halo was measured and recorded for each diet.

Food consumption and palatability were evaluated following the methodology described by Morales-Covarrubias et al. (2016). Individual shrimp were fed a fixed amount of food (3% wet body weight) with and without macroalgae extracts in a commercial diet (40% protein, 8% lipid). The food consumption was monitored for five consecutive days with six replicates for each treatment. After a 4 h period, solid waste (unconsumed food and feces) was siphoned out of each container, rinsed with distilled water to remove salts, and posteriorly dried using the same conditions for diet preparation. Finally, feces and unconsumed food were separated using a stereoscopic microscope (Olympus, USA) and weighed on an MT5 microbalance (Mettler Instrument Corp., Hightstown, NJ). The food and feces

were then weighed, and consumption was estimated using the following formula:

Weight of consumed food = weight of total food offered - weight of food recovered.

Experimental animals

A total of 200 juvenile *P. vannamei* were purchased from a local commercial hatchery with a certificate specifying that they were not detected of white spot syndrome virus (WSSV), infectious hemato-poietic necrosis virus (IHNV), and *V. paraha-menolyticus*. The organisms were acclimated in CIAD for one week in 600 L tanks with filtered (10 μm) seawater (33 of salinity) disinfected by UV radiation. Each tank had individual aeration, constant temperature (30 ± 1°C), and a photoperiod of 12 h light: 12 h dark. Shrimp were fed Camaronina™ daily, containing 35% protein and 9% lipids at 3% total biomass. Shrimp were fed three times a day.

Before the assay, 25 shrimp (10% prevalence, Lightner 1996) were removed from the batch (200 juvenile) to determine their health status by bacteriological analysis, wet mount analysis (Lightner 1996, Morales-Covarrubias 2010), PCR commercial kits (IQ2000^{TM} Kit: AHPND, WSSV, IHNV, necro-tising hepatobacterium (NHPB). GeneReach Biotech-nology Corp., Taiwan) and histological analysis (Lightner 1996, Tran et al. 2013).

Bioassay (for survival record)

A bioassay was conducted 24 h in 10 L glass tanks with 10 shrimps (3-4 g, not detected pathogens and intermolt stage) with three replicates per treatment and constant aeration. In total, two treatments were used: aqueous extracts of *G. vermiculophylla*, aqueous extracts *U. flexuosa* and two controls (positive and negative). Before the infection, an acclimatizing period of 24 h was allowed. The established control conditions during the test were: 30 ± 1ºC, 30 of salinity, pH 7.5-8.0, ammonium <0.1 mg L^{-1} and oxygen 6-8 mg L^{-1}.

Infection (bacterial inoculum)

Fifty milliliters of bacterial inoculum (concentration of 1×10^8 UFC mL^{-1}) was added directly into an experi-mental aquarium (concentration final in water 1×10^6 UFC mL^{-1}) containing 10 shrimps in the positive and negative control and treatments, and the same concentration of inactivated bacteria was added to the negative control at a temperature of 120°C for 15 min by autoclave. The first feeding was administered after 15 min of inoculation and then every 4 h until the end of the experiment (24 h).

Replicates from each treatment were used with 10 shrimps per replicate to evaluate survival rate, three for a total of 30 shrimps in each treatment and 120 shrimps overall. The survival rate was calculated as the survival probability at any particular time (S_t) (Goel et al. 2010). A total of 30 shrimp were used to evaluate the AHPND disease by wet mount and histological analysis. The surviving shrimp were also fixed in Davidson's solutions at the end of the experiment.

Wet mount analysis

Immediately after the survival challenge test, a wet mount analysis was made to assess if the surviving or moribund shrimps had an organ and tissue alterations. Their organs and tissue were removed, dissected, and squash mounted with sterile seawater then examined under the light microscope Olympus BX60 and photo-documented using an Olympus Infinity 2 camera (Lightner 1996, Morales-Covarrubias & Gomez-Gil 2014, Sriurairatana et al. 2014).

Histopathological analysis

Bioassay organisms displaying behaviors such as positioning in aquarium bottom, decubitus, and movement of the scaphognathite (moribund) were extracted and fixed with Davidson solution for conventional histological processes (Bell & Lightner 1988, Morales-Covarrubias 2010, Tran et al. 2013). Specimens were paraffin-embedded, cut into 4 μm sections, stained with hematoxylin and eosin, and reviewed under the light microscope to detect AHPND and modifications in the hepatopancreas (Bell & Lightner 1988, Tran et al. 2013, Soto-Rodriguez et al. 2015).

Lesion severity was graded according to the G-grading system (Lightner 1996), with G0 being negative and G4 as the highest severity of AHPND. Briefly, tissues graded as G0 are without lesions associated with AHPND; G1 is mild focal lesions; G2 and G3 are moderate, locally extensive to multifocal lesions; and G4 are severe, multifocal to diffuse lesions. Slides were observed under Olympus BX60 microscopy and photo-documented using an Olympus Infinity 2 camera.

Statistical analysis

Statistical analysis was conducted with R 3.3.1 software. Data were analysed using *post-hoc* ANOVA, *t*-student, Tukey and Kruskal-Wallis tests ($P < 0.05$), except the data for mortality and time of death, which were analyzed with a non-parametric estimator and the Kaplan-Meier (maximum likelihood) method to obtain survival curves (Goel et al. 2010).

RESULTS

Phytochemical composition of macroalgae

In this study, significant differences ($P < 0.05$) were obtained for TPC and TFC of macroalgae extracts. The aqueous extract of *G. vermiculophylla* produced 10.58 ± 2.31 mg GAE g^{-1} (TPC expressed as gallic acid equivalents mg^{-1} for each gram of dry extract) and for *U. flexuosa* 4.78 ± 0.67 mg GAE g^{-1}. However, the result for TFC expressed in equivalents mg^{-1} of quercetin extract (EQ) for each gram of dry extract showed that *G. vermiculophylla* extract contained less of this compound type when compared to *U. flexuosa* extract (10.32 ± 0.73 and 32.07 ± 0.99 mg EQ g^{-1}, respectively). Uronic acid was detected only in *U. flexuosa* extract (0.13%), while the amount of total sulfates found in extracts was 10.22% for *G. vermiculophylla* and 12.30% for *U. flexuosa* (Table 1).

Antioxidant capacity of aqueous extracts

The antioxidant activity of aqueous extracts of *G. vermiculophylla* and *U. flexuosa* were very similar for both species. The reduction of the radical ABTS mg^{-1} of dry extract for *G. vermiculophylla* was 21.73 ± 11.59 TEAC and 21.59 ± 15.22 TEAC for *U. flexuosa*, with no significant differences ($P < 0.05$) (Table 2).

Similarly, results obtained for FRAP indicate that *G. vermiculophylla* (46.78 ± 5.21 TEAC) has a similar reducing power as *U. flexuosa* (44.17 ± 2.42 TEAC) (Table 2).

MIC and sensitivity of aqueous extracts of *G. vermiculophylla* and *U. flexuosa* against *V. parahaemolyticus* (M0904AHPND+strain)

The experiment with the 5 mg mL^{-1} concentration produced bacterial growth, unlike in the case of 30, 50, 70, 90 mg mL^{-1} concentrations where no growth was observed (Table 3). The same number of concentrations were reseeded on agar Petri dishes (TSA with NaCl and TCBS) to substantiate these results, and the CFU of *V. parahaemolyticus* (M0904AHPND+strain) were counted, resulting in the MIC values for both *G. vermiculophylla* and *U. flexuosa* being 50 mg mL^{-1}, as no bacterial growth was detected on the agar Petri dishes.

The aqueous extract with the higher antibacterial activity was *G. vermiculophylla* with an inhibition halo of 18.00 ± 0.58 mm; however, this activity was lower for *U. flexuosa* (14.00 ± 0.29 mm), thereby inhibiting the growth of *V. parahaemolyticus* (M0904AHP ND+strain) (1×10^8 CFU mL^{-1}).

Evaluation of food consumption when treated with extracts and *in vivo* antibacterial capacity.

Favorable ingestion by the shrimp was observed as 30 min after feeding their intestine was full, hence why this is considered a positive acceptance of the food treated with the macroalgae extracts. Each shrimp consumed approximately 30 mg of food per day.

Wet mount analysis, clinical signs, and survival rate

All shrimp immersed in the positive control presented muscle opacity immediately after inoculation. After 30 min, the shrimp exhibited expansion of cuticular chromatophores (Fig. 1b), erratic swimming behavior, then settled to the bottom of the tank. After 3 h, the shrimp had an almost empty gut (whitish) and developed a pale HP (Fig. 1B) with moribund behavior, while mortalities of 20% (6) were recorded after 5 h and by 24 h only 20% shrimp survival, the negative control presented normal swimming behavior with no mortalities. In the *G. vermiculophylla* treatment, 12 h after inoculation, 10% (3) did not survive with 70% (21) survival after 24 h. The *U. flexuosa* treatment presented 6.6% (2) shrimp mortalities after 13 h of inoculation, with the survival of 60% (18) after 24 h. (Fig. 2).

Hepatopancreas wet mount analysis

Squash preparation of hepatopancreas (a soft consistency) showed vermiform bodies (Figs. 3a-b), hepatopancreas tubules cellular desquamation (Fig. 3c), and without feed residues, and the intestine showed whitish fluids.

Table 1. Phytochemical composition of the aqueous extracts of macroalgae. TPC: total content of phenolic compounds expressed as mg of gallic acid equivalent (GAE) per g of sample (dry weight). TFC: total flavonoid content expressed in mg of quercetin equivalents (QE) per gram of sample (dry weight). U: undetected. Different letters indicate significant differences ($P < 0.05$). Reported values correspond to the average ± standard deviation.

Macroalgae	TPC (mg GAE g^{-1})	TFC (mg QE g^{-1})	Uronic acids (%)	Sulfates (%)
G. vermiculophylla	10.58 ± 2.31^a	10.32 ± 0.73^b	U	10.22 ± 0.09^a
U. flexuosa	4.78 ± 0.67^b	32.07 ± 0.99^a	0.13 ± 0.06	12.30 ± 0.09^a

Table 2. Antioxidant capacity of aqueous extracts of macroalgae. Different superscript letters indicate significant differences ($P < 0.05$).

Macroalgae	ABTS TEAC	FRAP TEAC
G. vermiculophylla	21.73 ± 11.59[a]	46.78 ± 5.21[a]
U. flexuosa	21.59 ± 15.22[a]	44.17 ± 2.42[a]

Table 3. Minimum inhibitory concentration results for aqueous extracts of *Gracilaria vermiculophylla* and *Ulva flexuosa*. +: bacterial growth, -: no bacterial growth.

Macroalgae	Bacteria (1×10^8 CFU)	Concentration (mg mL^{-1})				
		5	30	50	70	90
G. vermiculophylla	*Vibrio parahaemolyticus*	+	-	-	-	-
U. flexuosa	*Vibrio parahaemolyticus*	+	-	-	-	-

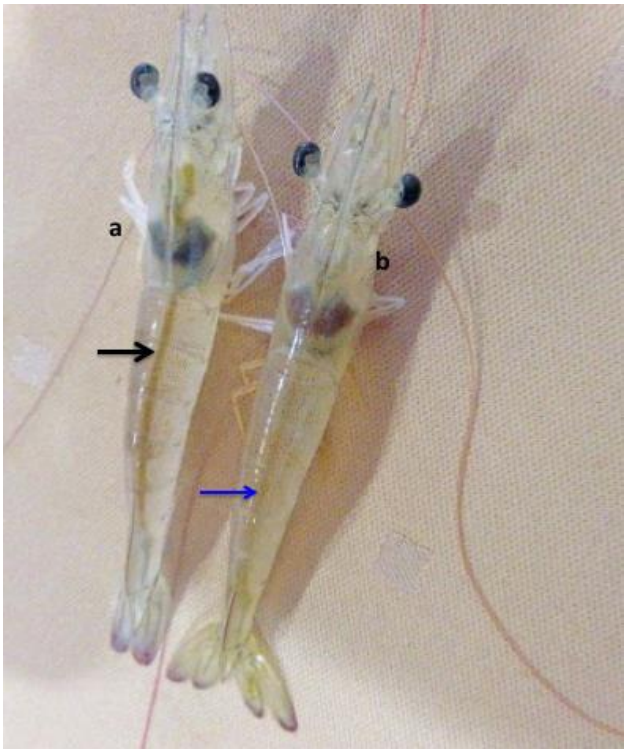

Figure 1. a) *P. vannamei* juveniles affected by *V. parahaemolyticus* (M0904AHPND+strain) with the expansion of cuticular chromatophores, empty gut (arrow blue), and pale hepatopancreas, compared to b) normal shrimp, low cuticular chromatophores, full gut, and large pigmented hepatopancreas.

Histological analysis

The hepatopancreas of the positive control organisms (24/30), *G. Vermiculophylla* (9/30), and *U. flexuosa* (12/30) showed G3-G4 lesions characteristic of the acute stage of AHPND. The tubular structure is lost due to severe necrosis and massive sloughing of the epithelial cells, causing necrotic cells to accumulate within the tubular lumen (Fig. 4). The organism's survival positive control (2/30), treated with *G. vermiculophylla* (21/30) and *U. flexuosa* (18/30), had the following alterations: vermiform structures (aggregated transformed micro-villi) in the lumen of the tubule, cell detachment (Fig. 4), and infiltration of hemolymph and hemocytes in intertubular connective tissue in hepatopancreas (G2-G3).

DISCUSSION

The phytochemical profiles and antioxidant capacity of both macroalgae were similar to those reported by Vijayavel & Martinez (2010), Thanigaivel et al. (2015) and Osuna-Ruiz et al. (2016), as the amount of flavonoids and tannins were always present. These compounds are linked to antioxidant (Rosales-Castro et al. 2009) and anti-inflammatory (Parvin et al. 2015) properties where they provide a stabilizing effect on the membrane, securing a load-bearing bond between the components of the plant extract with the hemocytes membrane (Oyedapo et al. 2010), this way protecting the membrane from agents/compounds involved in lysing (Rajauria et al. 2012).

Food administered with aqueous extracts of macroalgae reduced organ and tissue alterations and improved hemocyte response in organisms. Findings by Thanigaivel et al. (2014) indicate that shrimp treated with ethanolic extract of *Chaetomorpha antennina* infected with *V. parahaemolyticus* (with no toxigenic plasmid) did not damage or cause alterations to their hepatopancreas, gills, or muscle. *In vivo* trials by Thanigaivel et al. (2015) and Fatima et al. (2016) evaluated extracts of *Gracilaria folifera* and *Portieria hornemannii* against *Aeromona salmonicide* and *V. parahaemolyticus*, respectively, and both studies gave promising results.

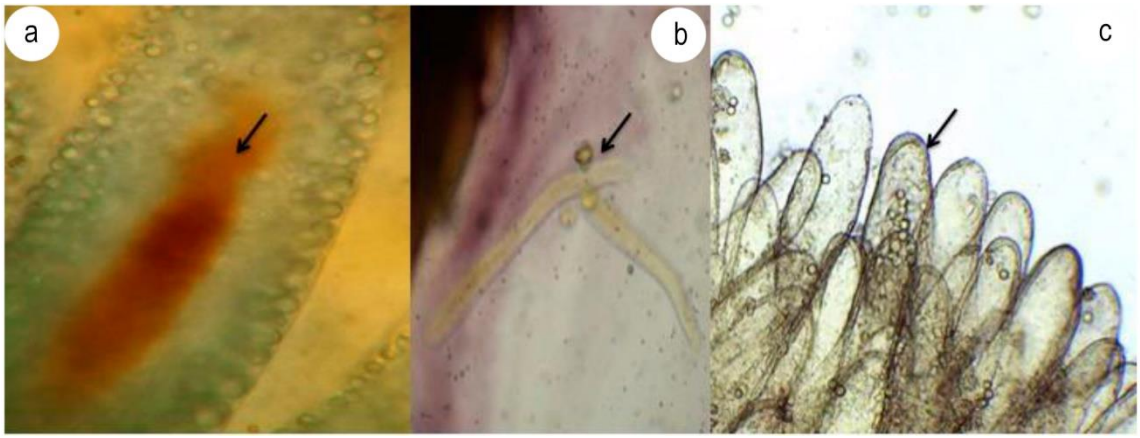

Figure 2. Microphotographs of *P. vannamei* challenged with *V. parahaemolyticus* (M0904AHPND+strain) at 24 h post-infection: a) wet mount preparation of hepatopancreas showed vermiform structures (aggregated transformed micro-villi) (arrow) in the lumen of the tubule, b) well-defined worm-like vermiform structure, and c) hepatopancreas tubules cellular desquamation (arrow). Scale bar = 30 μm.

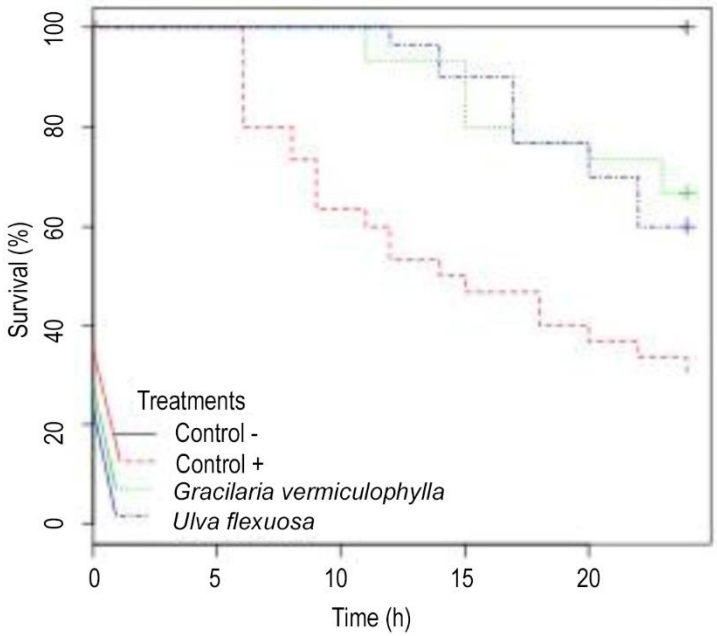

Figure 3. Survival curves (Kaplan-Meier) of *P. vannamei* juveniles infected with *V. parahaemolyticus* (M0904A HPND+strain).

As macroalgae act as immunostimulants, they have been associated with increased immune response in shrimp since stimulation enhances cellular and humoral defense parameters and decreases mortality when expo-posed to *Vibrio alginolyticus* (Huynh et al. 2011). Algal extracts have been tested to counteract shrimp bacterial diseases, but research has also been conducted to combat bacterial fish diseases, particularly tilapia (*Oreochromis mossambicus*). *In vivo* experiments by Thanigaivel et al. (2015) and Fatima et al. (2016) on extracts of *G. folifera* and *P. hornemannii* against *A. salmonicida* and *V. parahaemolyticus*, respectively, have provided favorable results. Some of the previously mentioned research suggests that food preparation containing bioactives obtained from macroalgae can be applied in the field on a large scale and that it is also a cost-effective and eco-friendly approach for disease management in aquaculture (Thanigaivel et al. 2015, Fatima et al. 2016).

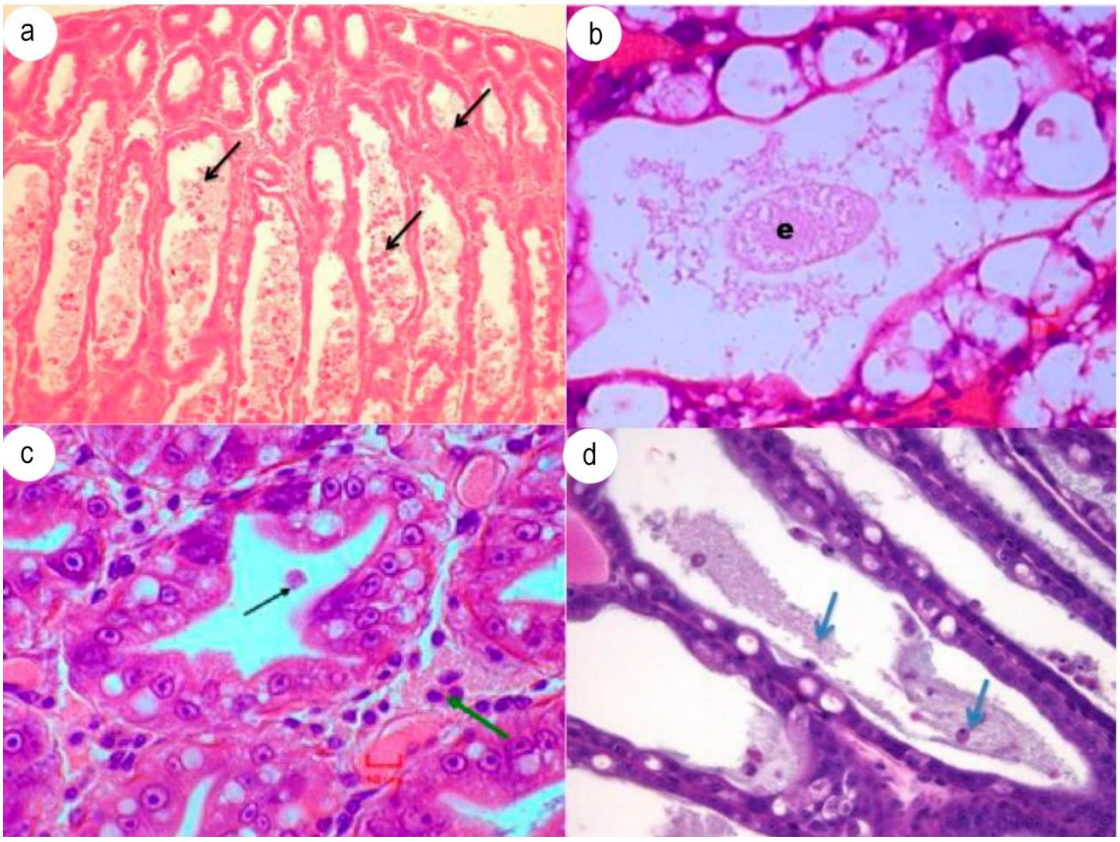

Figure 4. H&E stained photomicrographs of hepatopancreas collected from positive control, aqueous extract *Gracilaria vermiculophylla* treatment, and aqueous extract *Ulva flexuosa* treatment inoculated with *V. parahaemolyticus* (M0904AHPND+strain): a) hepatopancreatic tubules with necrotic and massive sloughed epithelial cells (black arrow) associated with the acute stage of acute hepatopancreas necrosis disease (AHPND) in positive control, b) hepatopancreas of treatments organisms showed vermiform structures (aggregated transformed micro-villi) in the lumen of the tubule (e), c) hepatopancreatic tubule with dead cells in the lumen of the tubule (Black arrow) with pyknotic nuclei (blue arrow) produced by epithelium desquamation, and hemocytic infiltration (green arrow), and d) tubules with dead cells in the lumen (blue arrow). Scale bar = 10 μm.

CONCLUSIONS

The results show that the aqueous extracts of the macroalgae *G. vermiculophylla* and *U. flexuosa* effectively against *V. parahaemolyticus in vitro* and *in vivo* experiments. The aqueous extract of *G. vermiculophylla* gave a higher amount of TPC than that obtained with *U. flexuosa* extract, while *G. vermiculophylla*, on the contrary, contained a lower amount of TFC when compared to *U. flexuosa*. Uronic acid was only detected in *U. flexuosa* extract, and the amount of total sulphates was fairly similar for both extracts. The antioxidant capacity measured by radical reduction ABTS and the ferric reducing antioxidant power revealed that extracts of both species have similar antioxidant activity. *G. vermiculophylla* extract gave an inhibition halo greater than *U. flexuosa*. The organisms treated with *G. vermiculophylla* and *U. flexuosa* showed fewer histological alterations in the hepatopancreas than those in the positive control organisms.

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