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Research Article

Extracts of *Moringa oleifera* and *Croton californicus* against infections of *Vibrio parahaemolyticus* (IPNGS16) in juvenile Pacific white shrimp (*Penaeus vannamei*)

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ABSTRACT. We evaluated the inhibition activity of extracts from two common plants, moringa (*Moringa oleifera*) and croton (*Croton californicus*), against *Vibrio parahaemolyticus* (IPNGS16), which causes acute hepatopancreatic necrosis disease (AHPND). The experiment was developed in three phases. First, extracts were prepared, and phytochemical screening of plants was performed. Second, microbiological tests were applied to calculate the minimum inhibitory concentration (MIC). Finally, two bioassays were performed on juvenile shrimp by administering the extracts (first) in shrimp feed at 24 and 72 h, pre-infection, and the second in feed and directly to the culture water. Based on preliminary results of antimicrobial activity, an extract concentration of 60 mg mL^{-1} inhibited *V. parahaemolyticus* (IPNGS16) at a final concentration of approximately 1×10^6 CFU mL^{-1}. Bioassays were carried out in order to determine the *V. parahaemolyticus* (IPNGS16) median lethal dose (LC_{50}) for juvenile *Penaeus vannamei* (0.20 ± 0.05 g); the LC_{50} was 85×10^3 CFU mL^{-1}. As a first test, the extracts were added to commercial feed at doses of 20, 40, and 60 mg mL^{-1}. After feeding, the shrimp were infected with *V. parahaemolyticus* (IPNGS16), and mortality was recorded. For the first infection time (24 h), survival was 46 and 33% with croton and moringa, respectively; for the second infection time (72 h), survival was 16 and 25% with croton and moringa. Application of antibacterial extracts directly to culture water were effective against *V. parahaemolyticus* (IPNGS16). The best result (94% survival) was obtained with a high dose (3.6 mg mL^{-1} or 30%) of moringa extracts added directly to culture water. Moringa methanol extracts produce active compounds capable of inhibiting replication of *V. parahaemolyticus* (IPNGS16) in shrimp aquaculture and reducing shrimp mortality.

Keywords: *Croton californicus*; *Moringa oleifera*; plant extracts; inhibition; early mortality syndrome; AHPND

INTRODUCTION

Globally, Pacific white shrimp (*Penaeus vannamei*) aquaculture is vulnerable to a *Vibrio parahaemolyticus* strain with a plasmid that is responsible for its entero-toxicity (Tinwongger et al. 2016), which causes acute hepatopancreatic necrosis disease (AHPND). The primary triggers are high-density shrimp culture, water polluted by nearby farms, and poor aquaculture practices that can create negative environmental consequences for water and wild communities in adjacent ecosystems. Infectious bacterial diseases may be prevented using shrimp culture plant products (Munaeni 2020) to avoid bacterial antibiotic resistance.

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The main symptom of AHPND is high mortality observed during the first 20-30 days of cultivation (Muthukrishnan et al. 2019), resulting in 40 to 100% mortality 12-24 h after symptomatic infection (Soto-Rodriguez et al. 2015, González-Meza et al. 2022). The *V. parahaemolyticus* strain harbors the plasmid that expresses the PirA and PirB proteins during the growing phase and is responsible for AHPND (Tinwongger et al. 2016). Recent studies (Muthukrishnan et al. 2019) have reported that the associated species *V. owensii* (Liu et al. 2015), *V. harveyi* (Kondo et al. 2015), *V. campbellii* (Dong et al. 2017), and *V. punensis* (Restrepo et al. 2018) all carry the PirA and B toxin plasmid (Muthukrishnan et al. 2019) and cause AHPND.

The process begins with bacteria growing in water debris and at the pond's bottom. They are transmitted orally, and infection starts in the digestive tract. In the acute phase, the bacteria produce toxins and hepato-pancreatic (HP) dysfunction, detachment of tubular epithelial cells, haemocytic inflammation, and HP necrosis (Muthukrishnan et al. 2019), followed by secondary bacterial infections in the terminal phase (Tran et al. 2013, Soto-Rodriguez et al. 2015). The usual response is the use of antimicrobial drugs, which in aquaculture have been associated with developing drug-resistant bacterial populations (DRBP) (Costa et al. 2017) and contamination of ecosystems adjacent to culture ponds.

Plant extracts have been presented as options to control and reduce bacterial populations and improve shrimp health in phytochemical and pharmacological studies (Jabamalai et al. 2011, Akinyeye et al. 2014). *Croton californicus* is a cosmopolitan plant found in the desert environments of the southwestern USA and northwestern Mexico (Williams et al. 2001). The plant's antibacterial activity is associated primarily with alkaloid metabolites (Pardo et al. 2014); triterpenes and flavonoids are the main metabolites detected. Another plant, *Moringa oleifera*, has been researched since the 1980s for its antimicrobial effect, which appears to be related to the presence of glycosides, 4-(α-L-rhamnosyloxy)-benzyl isothiocyanate, and 4-(α-L-rhamnosyloxy)-phenyl-acetonitrile (Costa et al. 2017). Moringa is useful for removing turbidity of contaminated water by coagulation (Golestanbagh et al. 2011) and biosorption of heavy metals in effluents (Araújo et al. 2010). It is an anti-inflammatory (Pereira et al. 2011) and has antibacterial activity. The phytochemicals in moringa pod husks have the potential for use against microbial drug-resistant pathogens (Arora & Onsare 2014), including *Vibrio* spp., and are thus promising for developing broad-spectrum drugs.

The rapid spread of aquaculture requires the development of new approaches aimed at measuring, controlling, and reducing the environmental impact. Despite the extensive scientific evidence on the bioactivity of moringa and other plant extracts as antibacterial agents (Moura et al. 2015, Onsare & Arora 2015), such studies have focused primarily on the *in vitro* use of extracts against multidrug-resistant *Vibrio* genus (Costa et al. 2017). Thus, the objective of the present study was to determine the inhibition capacity *in vivo* of extracts obtained from *M. oleifera* and *C. californicus* in *P. vannamei* shrimp infected with IPNGS16.

MATERIALS AND METHODS

Plant and bacterial material

For the experiments, the used *Moringa oleifera* (Verdcourt 1985) was obtained from the CIIDIR-Sinaloa experimental field (25°32'46.4"N, 108°28'52.7"W) and *Croton californicus* (Wiggins 1980) was collected from the Ahome Valley, Sinaloa, Mexico (25º99'01.92"N, 109º37'64.31"W). The collected species were validated (theplantlist.org) and separated by tissue of interest. Moringa seeds and pods were used, while croton petiole, leaves, and flowers were utilized without distinction. Surface sterilization was performed with 5% sodium hypochlorite. Each fraction was dried in the shade at room temperature (35ºC) and humidity (60%) until variation in weight was minimum. Later, each fraction was homogenized in an electric mill; the powder was sieved through a 10 μm mesh and preserved at -4ºC until use.

Vibrio parahaemolyticus (IPNGS16; isolated and characterized by López-León et al. 2016) was obtained from shrimp farms in Guasave, Sinaloa, Mexico. This *Vibrio* strain was used for bactericidal assays and infection challenges. Sub-cultures were preserved at -80ºC on trypticase soy agar (TSA) solid medium with 10% glycerol suspension. *V. parahaemolyticus* (IPNG S16) was activated in trypticase soy broth (TSB) medium with 2.5% NaCl, incubated at 33ºC for 24 h, and absorbance was read at 580 nm. In the exponential phase of kinetic growth, a stock was saved in Eppendorf tubes (approximately 1×10^6 CFU mL^{-1}) at the 0.5 McFarland standard.

Extract preparation

The solvents used to prepare the extracts were chloroform, ethanol (96%), methanol, and water added to increase polarity. For each extract, plant powder (5 g) was added to 25 mL (1:5, w/v) solvent; this was replicated three times. After extraction, the concen-

tration of extracts was 200 mg mL^{-1}. The mixture was held at 180 rpm for 1 h on an Orbital Shaker (S2030 Labnet International, Woodbridge, NJ, USA) and then allowed to stand for 24 h in the dark.

Following the resting period of each extract, the liquid fraction was separated from the sediment and centrifuged at 3900 rpm for 10 min, then filtered through Whatman N°1 filter paper. Only the chloroform extracts were filtered under an extraction hood (CFV-96, ECNO-Lab, Querétaro, Mexico). Extracts were concentrated in a rotary evaporator (Buchi R-3000, Buchi Labortechnik AG, Switzerland) to dryness at a temperature of 40°C (Amessis-Ouchemoukh et al. 2014, Sniegocki et al. 2014), and suspended in distilled water (15 mL) for ultraviolet light sterilization for 20 min. Finally, they were stored at -4ºC in tubes covered with aluminum foil, to be used within 12 weeks.

Antimicrobial activity and minimum inhibitory concentration (MIC)

The antimicrobial activity of the extracts against *V. parahaemolyticus* (García et al. 2006) was evaluated by TSA diffusion assay (Oliveira-Peixoto et al. 2011) to determine the diameter of the growth inhibition halo (Vieira et al. 2010) around the 6 mm test hole. To each hole, 100 µL (200 mg mL^{-1}) of the extract was added and distilled water as a control. The Petri dishes were incubated at 30°C for 24 h.

A combination extract-solvent was then used to calculate the MIC for *V. parahaemolyticus* (IPNGS16) growth inhibition. MIC was determined by the TSA dilution method (Arora & Onsare 2014, Kaur & Narang 2019). The initial extract solution (200 mg mL^{-1}) was distributed in dilutions of 20, 40, and 60 mg mL^{-1}. Tubes were inoculated with *Vibrio* (20 µL) at an estimated concentration of 1×10^6 CFU mL^{-1}. *V. parahaemolyticus* (IPNGS16) were incubated at 30°C for 24 h under the same conditions used for the subsequent shrimp infection test. Next, to calculate the CFU mL^{-1} of tubes with no apparent growth (Zhu et al. 2020), an aliquot was taken (100 µL), and the serial dilution method (Leyva-Madrigal et al. 2011, Zhu et al. 2020) was used to determine bacterial growth counts. The application of serial dilutions followed by viable counts in Petri dishes (Garre et al. 2019) is commonly used to approximate the microbial concentration. The MIC was determined as the minimum concentration of the extract that inhibited microbial growth completely (Chandra-Mohana et al. 2019).

Phytochemical screening

Qualitative tests were applied to extracts with halo inhibition and positive MIC to detect the following metabolites: alkaloids, anthraquinone glycoside, carbohydrates, cardiac glycosides, flavonoids, phenols, phlobatannins, saponins, steroids, tannins, and terpenoids.

Extracts and determinants were prepared according to standard methods (Iqbal et al. 2015).

Alkaloids: a few drops of Wagner's reagent were added to 2 mL of extract; presence was confirmed by the reddish-brown precipitate (Abdullahi 2013).

Anthraquinone glycoside: 0.5 mL of extract was boiled with Borntrager's reagent and 10 mL of sulfuric acid, filtered, and cooled, then placed in another test tube to which 5 mL of chloroform and 1 mL of NH_3 dilute were added (Joshi et al. 2013); the rose-pink confirmed presence to red color of the ammoniacal layer.

Carbohydrates: Molisch's reagent (20% alcohol and H_2SO_4) was added to 5 mL of extract; the violet confirmed the purple color between the two liquid layers.

Cardiac glycosides: following Kunatsa et al. (2020), a few drops of Kedde's reagent ($FeCl_3$ and concentrated H_2SO_4) were added to 0.5 mL of extract; the purple color confirmed presence.

Flavonoids: following Hossain et al. (2013), 2 mL of extract were subjected to the sodium hydroxide test (10% NaOH and HCl reagents); presence was confirmed by the appearance of an intense yellow color that became colorless with the addition of a few drops of diluted NaOH solution.

Phenols: following Kumar et al. (2019), 2 mL of extract were tested using potassium ferrocyanide reagent (1% ferric chloride and 1% potassium ferrocyanide solution, 1:1 ratio); the green color confirmed presence.

Phlobatannins: 2 mL of extract was subjected to the precipitate test following Sofowora (1996); the red precipitate determined its presence after adding several drops of dilute HCl.

Saponins: following Iqbal et al. (2015), 0.5 g of extract was added to 5 mL of distilled water in a test tube, and the solution was then shaken vigorously; the stable, persistent froth confirmed its presence.

Steroids: following Joshi et al. (2013), a Liebermann-Burchard reagent (three drops of chloroform, 2 mL of acetic anhydride, and 2 mL of sulfuric acid) was used to test 0.5 mL of extract; the green color confirmed presence.

Tannins: following Banso & Adeyemo (2010), Braymer reagent (0.1% ferric chloride in 100 mL of 95% alcohol) was used to test 0.5 mL of filtered extract; presence was confirmed by the brownish green or blueish black color.

Terpenoids: following Joshi et al. (2013), a Liebermann-Burchard reagent (three drops of chloroform, 2 mL of acetic anhydride, and 2 mL of sulfuric acid) was used to test 0.5 mL of extract; a reddish-brown precipitate confirmed presence.

Median lethal concentration (LC_{50})

The shrimp for all the experimental work were used following the protocols of the Official Mexican Standard (NOM-062-ZOO-1999).

A lethality test of *V. parahaemolyticus* (IPNGS16) against juvenile shrimp was performed using a negative control (without *Vibrio*) and densities of 10, 100, 250, and 500×10^3 CFU mL^{-1}. Ten healthy shrimp of similar weight (20 ± 3.6 mg) were used in each replicate, with three replicates per treatment. First, using chlorinated water (5%), the test conditions were 30 of water salinity under constant temperature (30ºC) and aeration. Following Joshi et al. (2014), under the same conditions (shrimp body weight and diluted seawater), the shrimp were then challenged by inoculating *V. parahaemolyticus* (IPNGS16) cells into the rearing water.

The experiment was maintained for 96 h and monitored twice daily; dead shrimp were recorded and removed. The final data were used to calculate the LC_{50} with the Probit analysis. The mean LC_{50}, standard deviation, and the confidence interval (95%) at 24 h were estimated using Minitab 17 (Minitab Inc., State College, PA, USA) when the cumulative mortality reached 50% of the experimental population (Finney 1971).

Shrimp and experimental conditions

White shrimp (*P. vannamei*) juveniles were obtained from Acuícola Montoya, EIRL (Sinaloa, Mexico). Polymerase chain reaction (PCR) analysis confirmed the absence of the white spot syndrome virus (WSSV), infectious hypodermal and hematopoietic necrosis virus (IHHNV), Taura syndrome virus (TSV), or infectious myonecrosis virus (IMNV) infections (Fierro-Coronado et al. 2019). The juvenile shrimp were reared to a size of 20 mg each at a density of 200 ind m^{-3} in the Laboratory of the Aquaculture Department at the National Polytechnic Institute of Mexico (IPN by its acronym in Spanish) to be used in the testing phase. Glass bottles with 3 L useful volume were used for rearing juvenile shrimp for the infection test. Before beginning the treatments, shrimp weighed 20 ± 3.6 mg each and were stocked at a density of 20 individuals per bottle.

During the experiment, the water in each tank was maintained at a constant temperature (30ºC) and aeration (5 mg L^{-1}). The water quality parameters during the study ranged: pH 8.0-8.3, salinity at 30, nitrite (NO_2^-) 0.53-3.4 mg L^{-1}, and total ammonia nitrogen (TAN) 0.077-1.13 mg L^{-1}.

Treatments and experimental design

Only methanol extracts (<40 mg mL^{-1}) that met zero growth criteria of the MIC were used in the next two challenges.

The first bioassay consisted of two methanolic plants (moringa and croton) extracts added to the feed (20, 40, and 60 mg g^{-1}) at two feeding times (24 and 72 h pre-infection). Feed was administered daily to satiation four times at 07:00, 11:00, 15:00, and 19:00 h. The control diets contained 200 mg kg^{-1} enrofloxacin (ENR) (enro-blend® AQUA Laboratorio Avimex, S.A. de CV, Nuevo León, Mexico) without extract. There was also a positive infection (PI) which was given only commercial feed and *Vibrio*, and negative control (NC) was only given feed.

The second bioassay consisted of the following treatments: i) three concentrations (20, 40, and 60 mg mL^{-1}) of two methanolic plant extracts added to the feed (described in bioassay 1), ii) adding methanolic extracts (moringa and croton) at three concentrations to the culture water, and the controls with ENR, PI, and NC. Each was replicated three times. The experiment lasted eight days, monitoring the shrimps every 6 h to remove dead shrimp.

The feed was prepared using a reconstruction method. The water was inoculated directly with *V. parahaemolyticus* (IPNGS16) at the LC_{50} concentration. Commercial feed was pulverized to powder, mixed with the respective dose of crude extract plus water, and then dried in an oven for 8 h at 60°C.

Statistical analyses

The survival data were arccosine-transformed. A two-way ANOVA was used to evaluate the effect of application times and extracts; significant differences were identified using *post-hoc* Tukey tests. All analyses were performed using a 0.05 significance level.

RESULTS

Antimicrobial activity

In vitro growth of *Vibrio parahaemolyticus* (IPNGS16) challenged with croton methanol extracts presented mean inhibition halos of 10.7 ± 0.9 mm measured around the test hole, while no inhibition halo was present with ethanol extracts. Moringa methanol and

ethanol extracts showed mean inhibition halos of 13.8 ± 0.7 and 13.2 ± 0.7 mm, respectively. Other solvents and their respective controls exerted no inhibition. No significant difference in inhibition power was found between moringa pods and seeds. The pods were selected as they are the more abundant plant material available.

MIC of plant-solvent combinations on *Vibrio parahaemolyticus*

The MIC of the ethanol and methanol extracts and the corresponding controls (positive and negative) were determined. The number of colonies was countless (data not presented) in the dilutions of 20 and 40 mg mL^{-1}. The inhibition confirmation test was negative in all replicates for the 60 mg mL^{-1} concentration of moringa and croton methanol extracts.

Phytochemical screening

Qualitative analyses were conducted to detect extract metabolites' contents (Table 1). Alkaloids, saponins, and tannins were abundant in both solvents and plants. For croton, more metabolites were extracted with methanol solvents (Table 1).

Median lethal concentration (LC_{50})

Mean LC_{50} of *V. parahaemolyticus* (IPNGS16) in juvenile white shrimp was 85 ± 18×10^3 CFU mL^{-1} (mean and standard error) with a 95% confidence interval of 56-129×10^3 (Fig. 1). Mortality of Pacific white shrimp occurred between 12 and 48 h post-infection (HPI) and was zero for the negative control. Following the Probit analysis, goodness-of-fit tests (χ^2 = 0.682; P = 0.877) were performed on a log-normal distribution and the log-likelihood = -53.786.

Extract applications and shrimp survival

First bioassay

After 24 h of acclimation, shrimp were provided the corresponding feed with the extract added, and the water was inoculated with *V. parahaemolyticus* (IPNGS16) at the LC_{50} dose. The negative control group showed lowest mortality during the experiment (<10%), while the mortality of the PI was >75% with 24 h (Fig. 2a) and 72 h (Fig. 2c) pre-infection feeding. The shrimp-fed diets supplemented with ENR and extracts (moringa and croton) had better survival than the infected (PI) group (Figs. 2b,d). Survival was higher when the moringa and croton extract was fed 72 h prior to infection than in the ENR treatment (Fig. 2d).

Second bioassay

As in the first bioassay, after acclimatization, juveniles were subjected to the treatments (extract in feed, extract in culture water, ENR, PI, and NC) and finally inoculated with *V. parahaemolyticus* (IPNGS16). The most effective application of antibacterial methanolic extracts against infections of *V. parahaemolyticus* (IPNGS16) causing AHPND was directly in the culture water (Fig. 3). Furthermore, the results improved with a treatment dose higher (60 mg mL^{-1}) than the calculated MIC ($P < 0.05$) of moringa extracts added to culture water (Fig. 3). Survival was statistically similar to the NC ($P < 0.05$), and protection was better than the chemical control (ENR) for the last dose.

DISCUSSION

Bacteria of the *Vibrio* genus occur naturally in the marine environment and are part of the marine invertebrate microbiota. Recently, more *Vibrio* species have been encoded by a plasmid (*V. owensii*, Liu et al. 2015; *V. harveyi*, Kondo et al. 2015; *V. campbellii*, Dong et al. 2017; and *V. punensis*, Restrepo et al. 2018) and have been recognized as shrimp pathogens capable of causing AHPND since they all contain the protein toxins (PirA and PirB toxin) of the plasmid (Muthukrishnan et al. 2019). *Vibrio* bacteria also are opportunistic pathogens of cultured aquatic organisms; thus, the use of antibiotics in shrimp cultivation is the first option to control infectious diseases (Munaeni et al. 2020). The negative aspects of using antimicrobials (e.g. antibiotics) in aquaculture include the need to use increasing amounts and the negative environmental impacts associated with bacterial populations resistant to chemical drugs.

In vitro Vibrio parahaemolyticus inhibition

Successful shrimp aquaculture requires adequate health status. Using plant extracts or powder for disease control in aquaculture is a viable alternative to antibiotics (Hossain et al. 2013, Fierro-Coronado et al. 2019, Howlader et al. 2020). The inhibition halos for moringa (13.8 ± 0.7 mm) and croton (10.7 ± 0.9 mm) was only effective with methanol extracts in the preliminary *in vitro* tests.

Other studies have examined *M. oleifera* extracts against *Vibrio parahaemolyticus* (Oliveira-Peixoto et al. 2011) and found mean inhibition halos of 21.9 mm for ethanol and 20.7 mm for aqueous extracts. For *V. cholerae* hexane and methanolic extracts, mean inhibition halo sizes were 22.2 and 13.8 mm, respectively (Walter et al. 2011). Croton ethanolic extracts

Table 1. Phytochemical screening of metabolites of *Moringa oleifera* and *Croton californicus* extracted with ethanol and methanol solvents. Metabolite presence (++), Metabolite absence (--).

Compounds	Moringa		Croton	
	Ethanol	Methanol	Ethanol	Methanol
Alkaloids	++	++	++	++
Anthraquinone glycoside	--	++	--	++
Carbohydrates	--	++	--	++
Cardiac glycoside	--	++	--	++
Flavonoids	--	++	--	--
Phenols	--	--	++	++
Phlobatannins	--	--	--	--
Saponins	++	++	++	++
Steroids	++	--	++	++
Tannins	--	++	++	++
Terpenoids	--	--	--	--

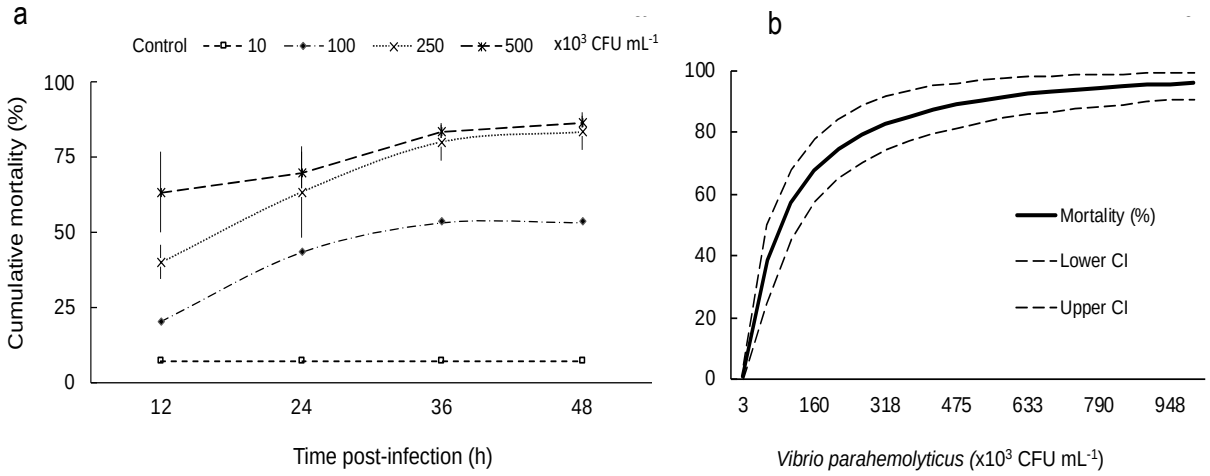

Figure 1. a) Cumulative mortality of *Penaeus vannamei* juvenile challenged with *Vibrio parahaemolyticus* (IPNGS16) causes AHPND. Control and treatments of 10, 100, 250, and 500×10^3 CFU mL^{-1}. Data represent the mean and standard deviation. b) Determination of the concentration of bacteria (LC_{50}) on a 24-h basis, using the data in a), and calculated as 50% mortality (continuous line) at the corresponding CFU mL^{-1}. The two other curves represent the 95% confidence interval.

showed significant sensitivity, and the mean inhibition halo was 17-21 mm in Gram-negative bacteria (*Escherichia coli* and *Stenotrophomonas maltophilia*); these bacteria are particularly interesting as they are now resistant to β-lactam antibiotics (Corrales-Ramírez et al. 2013). However, croton methanolic extracts were effective only against *V. parahaemolyticus* (IPNGS16) only *in vitro* tests. Methanolic extracts, in general, present greater antimicrobial activity (Mothana et al. 2008). These characteristics are due to the polarity of the compounds extracted by each of the solvents. The results of the preliminary tests (e.g. solvent selection) were necessary to estimate the MIC against *V. parahaemolyticus* (IPNGS16) for the complementary studies.

MIC for inhibition of *V. parahaemolyticus*

Intensive aquaculture methods commonly used for the control of emergent and opportunistic pathogens in aquaculture include prophylactic chemotherapy or antibiotics (Munaeni et al. 2020). During the past few years, the advancement of responsible aquaculture practices has been enhanced by probiotics and medicinal plants to control pathogens.

Using ethanol extraction of moringa pods against species of the genus *Vibrio*, Nogueira-Brilhante et al. (2015) reported a MIC = 0.312-5 mg mL^{-1}. Antibacterial methanol extract showed a MIC ranging from 1.28 to 10.24 mg mL^{-1} on 73% of multiple-drug resis-

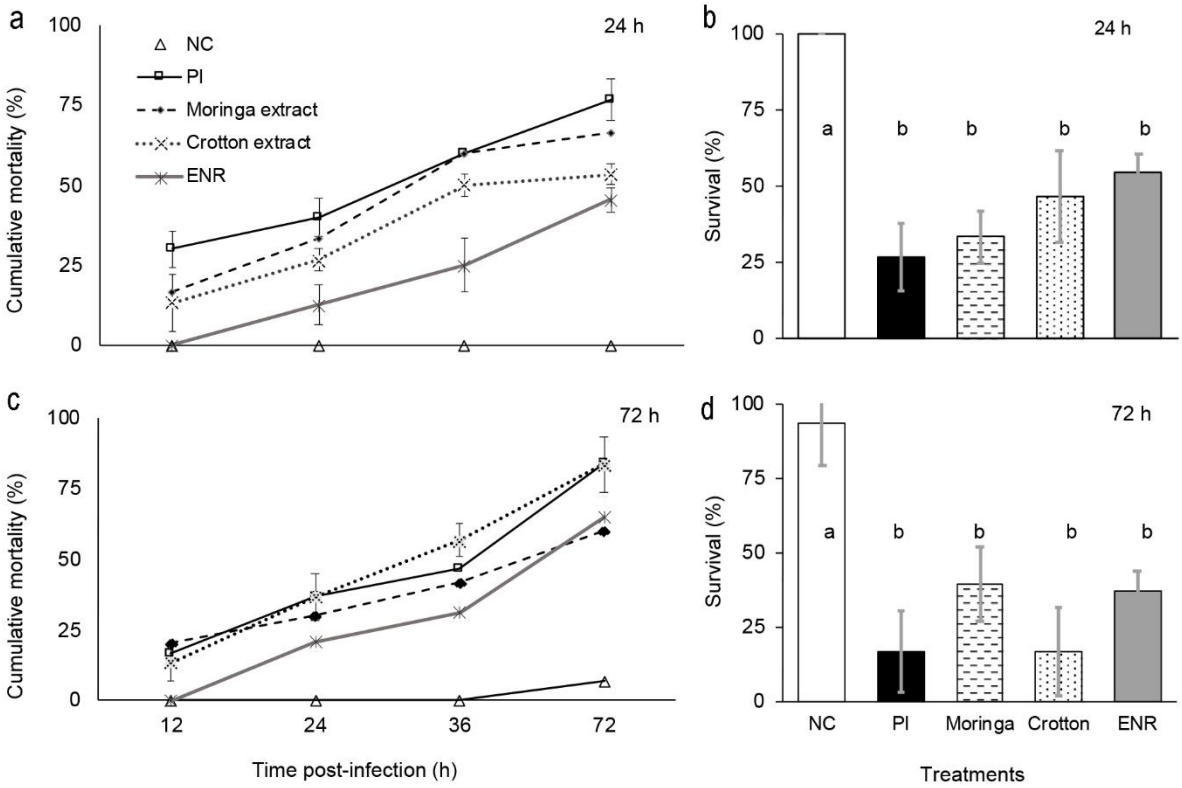

Figure 2. Cumulative mortality and final survival of *Penaeus vannamei* postlarvae after a-b) 24 and c-d) 72 h of feeding with extracts added to their food before exposure to *Vibrio parahaemolyticus* (IPNGS16), which causes AHPND (8.5×10^3 CFU mL^{-1}). Negative control (NC, triangle), positive infection (PI, solid line), methanolic moringa extracts (dashed line), croton methanolic extracts (dotted line), and enrofloxacin (ENR, solid gray line) antibiotic. Data shown are the average of three replicates. Error bars represent the 95% confidence interval of the data set. Different letters indicate significant differences among treatments ($P < 0.05$).

tant bacterial strains (Dzotam et al. 2016). For leaf extracts of antibacterial *Croton roxburghii* against *Staphylococcus aureus* and *Escherichia coli*, the MICs were 0.156 and 0.625 mg mL^{-1}, respectively (Panda et al. 2010). Quinteros-Espino (2017) determined that species of the genus *Croton* (*C. thurifer* and *C. collinus*) had no antimicrobial activity at concentrations ≤2 mg mL^{-1}. Based on nanoparticle (NP) technology the MIC value for ZnO-NP from *Croton bonplandianum* against *V. parahaemolyticus* was 0.625 mg mL^{-1} (Chandra-Mohana et al. 2019).

The extracted phytoconstituents of the two plants may offer an antibiotic alternative to combat emerging antimicrobial resistance. The MICs for croton and moringa methanol extracts were completely effective at a concentration of 60 mg mL^{-1}.

Median lethal concentration (LC_{50})

Based on the Probit analysis, the lethal concentration (LC_{50}) of *V. parahaemolyticus* (IPNGS16) in juvenile Pacific white shrimp (20 ± 3.6 mg) was $85 \pm 18\times10^3$ CFU mL^{-1}. Determination of LC_{50} was significantly ($P < 0.05$) dependent upon shrimp age (weight). In a similar study on 452 ± 50 mg shrimp carried out using the same strain of *V. parahaemolyticus* (IPNGS16), Fierro-Coronado et al. (2019) reported an $LC_{50} = 6.5\times10^4$ CFU mL^{-1}. Velázquez-Lizárraga et al. (2019) challenged healthy *P. vannamei* weighing 12.26 ± 0.022 g with *V. parahaemolyticus* (IPNGS16); based on the Probit analysis, the LC_{50} = 660.95 CFU mL^{-1}, following exposure 24 HPI. In our work, LC_{50} was different for each experimental challenge with the same bacterial strain, likely due to differences in size and health status of shrimp.

Phytochemical screening

Using plants to obtain medicinal extracts for antibacterial purposes requires that the raw materials be readily available. The extracts should be biodegradable and not contaminate the environment when used in the prescribed manner (Munaeni et al. 2020).

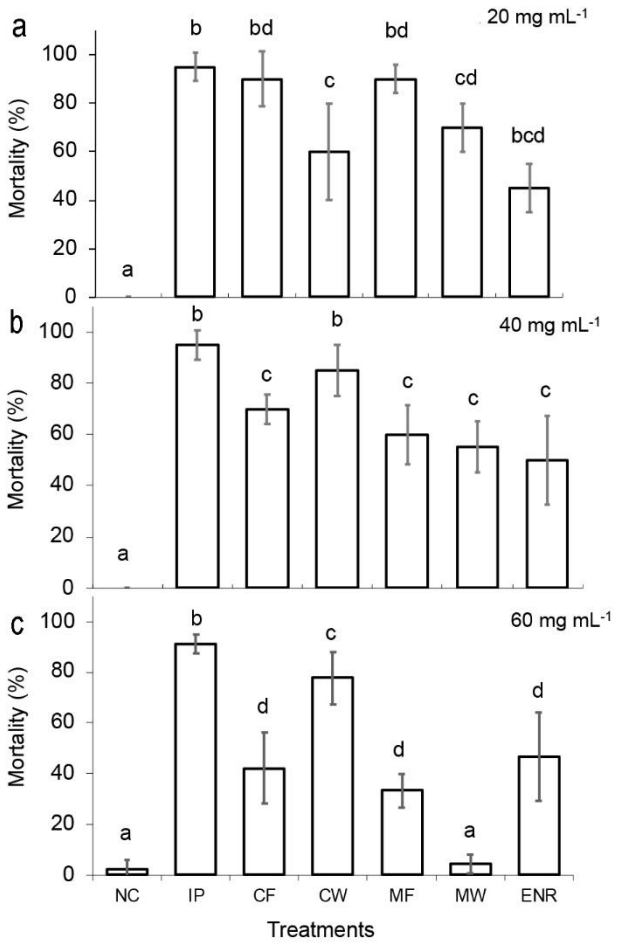

Figure 3. Cumulative mortality for *Penaeus vannamei* postlarvae exposed to *Vibrio parahaemolyticus* (IPNG S16) (8.5×10^3 CFU mL^{-1}) at extract concentrations in the feed of a) 20, b) 40, and c) 60 mg mL^{-1}. NC: negative control, PI: positive infection, CF: croton methanolic extract in feed, CW: croton methanolic extract in water, MF: moringa methanolic extract in feed, MW: moringa methanolic extract in water, and ENR: enrofloxacin. Data shown are the average of three replicates. Error bars represent the 95% confidence interval of the data set. Different letters indicate significant differences among treatments ($P < 0.05$).

The phytochemical composition of the genus *Croton* is diverse. The most abundant metabolites are terpenoids, flavonoids, and alkaloids (Salatino et al. 2007). Of these metabolites, only terpenoids are soluble in water. The metabolites in the methanolic extracts used in our study included carbohydrates, glycosides, phenols, saponins, and tannins. *Croton* spp. extracts have different immunomodulatory, anti-inflammatory, antiviral, antibacterial, and antioxidant properties (Corrales-Ramírez et al. 2013). Their antimicrobial activity is due to chloroquine acid, coberins A and B, 1,3,5-trimethoxybenzene, 2,4,6-trimethoxyphenol (Jones 2003), and metabolites of the following groups: alkaloids and terpenoids (Chandra-Mohana et al. 2019).

Moringa's most abundant secondary metabolites are glucosinolates, isothiocyanates, flavonoids, anthocyanins, proanthocyanidins, and cinnamates (Bennett & Chung 2001). In the phytochemical tests carried out in our study, glycosides, flavonoids, phenols, saponins, tannins, and terpenoids were present in ethanolic extracts, while methanol extracts contained alkaloids, saponins, and tannins. Previously, Nogueira-Brilhante et al. (2015) found that pod ethanol extracts were effective against *Vibrio* spp. and could inhibit *V. vulnificus*. The antibacterial properties of *M. oleifera* have been attributed to benzyl glucosinolates and their cognate isothiocyanate (Fahey 2005). The purified seeds of *M. oleifera* contain an uncommon glucosinolate with unique characteristics: (4-(α-L-rhamnosyloxy) benzyl) called glucomoringin (Galuppo et al. 2013). Based on *in vitro* experiments on other plants rich in purified glucosinolates (Cruciferae), Melrose (2019) reported that the bioactive form, allyl isothiocyanate [AITC] and sulphoraphanin [SFN], do not act in isolation. AITC and SFN are as potent as vancomycin in the treatment of antibiotic-resistant bacteria identified as "priority pathogens" by the World Health Organization (WHO) (Melrose 2019). Antimicrobial plant compounds are biodegradable and broadly effective. At present, it is important to develop strategies to minimize the impacts of pathogens while limiting the negative effects on the ecosystem, wild biota, and aquaculture production systems. The results showed that extracts applied directly to the culture water were the most effective method of employment to delay the exponential phase when protein toxins and the growth of the bacterium *V. parahaemolyticus* are generated in the culture medium.

Shrimp survival and effective applications

The use of plant extracts for disease prevention in aquaculture has been of interest for some time as an alternative to antibiotics, which can increase bacterial resistance in cultivated organisms (Costa et al. 2017). Moreover, antibiotic residues are toxic to humans and the aquatic environment (Paseka et al. 2020). Currently, the most effective application of extracts in water or feed is unknown; however, several hypotheses can be put forward to explain the inhibitory effect observed in application to water.

The shrimp-fed diets supplemented with ENR, moringa extracts, and croton extracts had better survival (36, 39, and 19%, respectively) than the PI

control group (16%). Compared with the ENR, survival was higher when the moringa methanolic extract protection time was longer (72 h) before infection. Based on the second challenge tests, the extracts in water were highly effective, with 95% survival. The results obtained in this study were validated with standard antibiotic enrofloxacin as an additional control. Similar results have been reported by Chandra-Mohana et al. (2019), wherein plant extracts effectively inhibited both Gram-positive and Gram-negative bacterial strains. For *in vitro* tests of moringa pods, Arora & Osare (2014) report MIC doses of 0.3-4 mg mL^{-1} against multi-drug resistant bacteria. The effective dose was >60 mg mL^{-1} when methanolic extracts were applied to culture water. Tinwongger et al. (2016) reported that the plasmid containing the toxic proteins PirA and PirB is expressed during the growing phase. One hypothesis is that moringa methanol extract may extend the lag phase of bacterial growth necessary to generate the toxins that cause damage to shrimp that feed on the pond bottom.

Free growth of *V. parahaemolyticus* (IPNGS16) in water caused AHPND virulence; thus, at the early stage of bacterial inhibition with active control is important. For example, moringa metabolites might lead to decreased transmission of AHPND among cultured organisms. In aquaculture, effective use of active plant metabolites in crude extracts requires a cost-effective extraction method avoiding the use of known toxic solvents, and development should be based on proven, more affordable, effective application methods.

CONCLUSION

Plant extracts are a viable alternative for treating pathogens such as *V. parahaemolyticus* (IPNGS16). Only the *M. oleifera* methanol extracts were effective for the survival of infected *P. vannamei* juveniles at a *V. parahaemolyticus* (IPNGS16) mean lethal concentration (LC_{50}) of 85×10^3 CFU mL^{-1}. The phytochemical components were alkaloids, glycosides, and saponins. For the *in vivo* challenge tests, inhibition of *V. parahaemolyticus* (IPNGS16) was only effective with the moringa methanolic extract dose at >60 mg mL^{-1} applied to the culture water. The results obtained in this study were validated with standard antibiotic enrofloxacin as an additional positive control. In the final challenge tests, the methanolic extracts applied to culture water were highly effective, with up to 95% survival, not significantly different from the control (NC). Currently, the extraction procedure is still expensive and therefore not feasible for use by shrimp farmers. More affordable extraction and application methods should be developed to mitigate this limitation of using natural extracts in shrimp aquaculture.

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