Latin American Journal of Aquatic Research, 51(4): 543-555, 2023
DOI: 10.3856/vol51-issue4-fulltext-3051

Research Article

Therapeutic, histopathological, and non-specific immune status effect of *Rhizophora mangle* and *Laguncularia racemose* hydroalcoholic extracts against *Vibrio harveyi* and *Vibrio campbellii* in white shrimp (*Penaeus vannamei*)

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ABSTRACT. The objective of the research was to evaluate *in vitro* and *in vivo* antibacterial efficacy of *Rhizophora mangle* and *Laguncularia racemose* when added to the feed of *Penaeus vannamei* in an experimental infection with *Vibrio harveyi* (CAIM 1792) and *V. campbellii* (CAIM 333). Results show that the minimum inhibitory concentration for both extracts was 1.65 g mL^{-1}, with an inhibition zone of 25 ± 1 mm (*V. campbellii*); 18 ± 2 mm (*V. harveyi*) for *L. racemose* and of 21 ± 2 mm (*V. campbellii*); 20 ± 2 mm (*V. harveyi*) for *R. mangle*. *R. mangle* gave a higher total content of phenolic compounds (4.50 ± 0.26 mg GAE mL^{-1}) and flavonoids (2.60 ± 0.15 mg QE mL^{-1}). The challenge with *V. harveyi* resulted in 70% survival for organisms fed *L. racemose* extract and 53% for *R. mangle* extract. The challenge with *V. campbellii* resulted in 80% survival for organisms fed *L. racemose* and 90% *R. mangle*. Histopathological alterations were observed in the hepatopancreas with hemocytic infiltration within the intertubular connective tissue. Also, tubules with severe cell detachment and tubular atrophy were detected in the positive control organisms, and organisms treated with *R. mangle* and *L. racemose* 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 hydroalcoholic extracts of *R. mangle* and *L. racemose* reduced mortality, clinical signs, and organs and tissue alterations and improved *P. vannamei* immunity. It is possible to use these hydroalcoholic therapeutic extracts as treatments in shrimp-farm to enhance bacterial disease tolerance and prevent mortality.

Keywords: mangrove; hemocytes; extracellular-traps; shrimp; antibacterial; vibrio

INTRODUCTION

Shrimp farming has been affected by outbreaks of various diseases that have become one of the main challenges to be solved (Harlina et al. 2015, CESASIN 2017). Bacterial diseases include those caused by strains of *Vibrio harveyi* and *V. campbellii* that affect the Pacific white shrimp (*Penaeus vannamei*), such as "bolitas" síndrome (Mensor et al. 2001), systemic vibriosis (Kousoulaki et al. 2015), luminescent vibriosis (Flegel 2019), acute hepatopancreatic necrosis (AHPND) (Szóllósi & Szôllôsi-Varga 2002, Flegel, 2019), and "live reds" syndrome (Soto-Rodríguez et al. 2012, Tran et al. 2013), among others. The deteriorated environment has resulted in compromised health conditions of the cultured shrimp, exacerbated by the proliferation of opportunistic pathogens and other disease-carrying organisms in ponds. Antibiotics have been resorted to by some shrimp farm operators to combat this declining production of its environmental

Corresponding editor: Eduardo Ballester

hazards (Aybar-Parian & Ari-Condori 2018, Arulkumar et al. 2020).

Research using plant extracts for aquatic animals is increasing with the demand for eco-friendly and sustainable aquaculture. Plan extracts decrease the selective pressure for developing antibiotic resistance (Arulkumar et al. 2020).

Mangrove plants have been used in folklore medicines, and extract from mangrove species have proven inhibitory activity against human, animal, and plant pathogens. Several mangrove species produce bioactive compounds such as steroids, triterpenes, saponins, flavonoids, alkaloids, and tannins (Bandaranayake 2002), having therapeutic significance. It has also been reported that these are an excellent source of antiviral compounds compared to seaweeds and seagrasses (Premnathan et al. 1992). Extracts from different mangrove plants are reported to possess diverse medicinal properties such as antibacterial and anthelmintic (Bandaranayake 2002).

Screening plant species for antimicrobial activity in discovering new sources of economically valuable materials and metabolites with new therapeutic agents is important (Abeysinghe et al. 2006). The effect of *Rhizophora mangle* and *Laguncularia racemose* hydroalcoholic extracts studies on medicinal plants as a source of growth-promoting agents, antibacterial, antiviral, immunomodulator, and antioxidants have received a growing interest dealing with the undesirable effect of costly synthetic antibiotics (Benzie & Ystrain 1996, Azaza et al. 2008, Bermudez-Almada et al. 2014, Morales-Covarrubias et al. 2016, Blanco-Olano & Millones-Gómez 2020, Gamboa-Barraza et al. 2021). *R. mangle* (red mangrove RM) and *L. racemose* (white mangrove WM) are abundant in Mazatlan, Sinaloa, Mexico, and are a potential source of novel phytochemical compounds that could be used as pharmaceutical, herbal medicines. Therefore, this study aimed to evaluate the antibacterial activity of medicinal mangrove plants using different plant materials and screening them *in vitro* and *in vivo* for antibacterial activity and non-specific immune response in *P. vannamei*.

MATERIALS AND METHODS

Extract preparation

Mangrove plants such as *R. mangle* belongs to the family Rhizophoraceae with a vernacular name called "red mangrove," and *L. racemose* to the family Combretaceae with a vernacular name called "white mangrove" were collected from different localities in South Mazatlán, Sinaloa. The RM was collected from Estero el Infiernillo (23°13’06.7”N, 106°24’32.0”W), and WM from Isla de la Piedra (23°11'18"N, 106°19'46"W). The mangroves were taxonomically identification keys (Agraz et al. 2006, Rodríguez-Zúñiga et al. 2013), and voucher specimens were stored.

Hydroalcoholic extract from mangrove plants

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 hydroalcoholic extracts: 3 g of dried powder was extracted with 100 mL of ethanol (30%) for 24 h. It was filtered with eight layers of muslin cloth. The supernatant was collected, and the ethanol was evaporated to dryness. The concentrate was stored in an amber flask at 25°C until use (no longer than ten days). These preparations were examined for their toxicity, bactericidal activity, and protective effects from *V. harveyi* CAIM 1792 and *V. campbellii* CAIM 333 on injected administration in *P. vannamei*.

Bacterial inoculum

A sample of *V. harveyi* CAIM 1792 used in this experiment was isolated from shrimp farms affected by "bright red" in northwestern Mexico and cryopreserved at -80°C (Soto-Rodríguez et al. 2012), and the sample of *V. campbellii* CAIM 333 used in this experiment was isolated from the seawater of a shrimp broodstock tank from a hatchery in Sonora, México; its peak pathogenicity to crustaceans has been reported (Soto-Rodríguez et al. 2006). *V. harveyi* CAIM 1792 strains and *V. campbellii* CAIM 333 were plated in TSA + 2.0% NaCl and incubated for 24 h at 30ºC. Subsequently, the inoculum of each bacterium was prepared by resuspending a loop full of bacteria in sterile 2.5% NaCl and centrifuged at 4680 rpm for 10 min at 8ºC. Bacterial suspensions were adjusted to an optical density of 1.0 at 600 nm, equivalent to 0.5 MacFarland standard (McFarland 1907), and serially diluted to densities estimated at 10^8 CFU mL^{-1}, both suspensions were inoculated on agar TCBS plates to perform the experimental bioassay and determine the actual density of both *Vibrio* used to challenge the shrimp. For the negative control, the bacteria were autoclaved to inactivate them and were spread on an agar TCBS petri dish to confirm null growth.

Phytochemical composition and antioxidant capacity of hydroalcoholic extracts

The total tannin content in mangrove extracts was determined by the method reported by Makkar et al. (1993) with several modifications. This method is based on tannins binding with insoluble polyvinylpyrrolidone (PVP). The total flavonoid content was evaluated according to Luximon-Ramma et al. (2002). The free-radical scavenging activity of hydroalcoholic extracts was determined by the method described by Mensor et al. (2001). The scavenging activity of the 2,2'-azinobis [3-ethylbenzothiazoline]-6-sulfonic acid radical (ABTS) was determined according to the method described by Przygodzka et al. (2014) and the Ferric Reducing Antioxidant Power (FRAP) assay was performed according to the methods of Benzie & Strain (1996) as modified by Szôllôzi & Varga (2002).

Antimicrobial activity and minimum inhibitory concentration

The antibiograms were performed using the Kirby-Bauer technique described by Mayer-Bernal & Guzman (1984). Muller-Hinton (MH) medium with 2.5% NaCl was prepared according to the package instructions and distributed in sterile Petri dishes. It was seeded evenly over the surface of the medium with the applicator in three different directions; the surface was dried for 3-5 min keeping the box with the lid closed. The minimum inhibitory concentration (MIC) analysis described by Horna-Quintana et al. (2005) was performed. Serial dilutions were performed in tubes with 5 mL of MH broth with NaCl; three controls were used: positive control (MH broth to which 100 µL of bacterial suspension was added), negative control (MH broth without mangrove and bacterial suspension) and treatment (MH broth with bacterial suspension and mangrove extracts). After 24 h, turbidity was observed in the tubes and measured with a spectrophotometer (DS-11 spectrophotometer-De Novix) at 600 nm; the tubes with lower turbidity were seeded on TSA Agar plates with NaCl, as well as the positive and negative controls. This procedure was repeated for each bacterium to be analyzed.

Feed preparation

For each mangrove extract, the diets were prepared homogenizing paste with previously ground and sieved commercial feed Camaronina Purina® (35% protein), 1 g of grenetin per 500 g of ground feed, mangrove extract (50 mL) and distilled water (~450 mL). The control diet was processed similarly, substituting distilled water for the mangrove extract. Pellets were formed by passing the paste through a meat grinder (Moulinex™ domestic meat grinder ± 1.6 mm diameter), dried in a dehydrator (LBI, 30CH220V, Mexico) at 40°C for 12 h, and stored (Samsung, RT45VNSW5, Mexico) at 4°C. The antibiogram was performed again with the methodology described above and the following modification: the discs were replaced by a reconstituted pellet from each mangrove extract.

Experimental injection infection bioassay

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

A total of 200 juvenile *P. vannamei* were purchased from a local commercial hatchery with a certificate specifying that they were not detected with white spot syndrome virus (WSSV), infectious hypodermal and hematopoietic necrosis virus (IHHNV) and *V. parahaemolyticus*. The organisms were acclimated in Centro de Investigación en Alimentación y Desarrollo (CIAD) for one week in 1000 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 12:12h light: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, 30 shrimp (10% prevalence, Lightner 1996) were removed from the batch (200 juveniles) to determine their health status by bacteriological analysis, wet mount analysis (Lightner 1996, Morales-Covarrubias 2010), PCR commercial kits (IQ2000TM Kit: AHPND, WSSV, IHNV, necrotizing hepatobacterium (NHPB). GeneReach Biotechnology Corp., Taiwan) and histological analysis (Lightner 1996, Tran et al. 2013).

Bioassay (for effectiveness and survival record)

A bioassay was conducted 24 h in 10 L glass tanks with 10 shrimps (3-4 g, undetected pathogens, and intermolt stage) with three replicates per treatment and constant aeration. In total, four treatments were used: red and white mangrove hydroalcoholic extracts against *V. harveyi* CAIM 1792 and *V. campbellii* CAIM 333 in *P. vannamei* 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 salinity, pH 7.5-8.0, ammonium <0.1 mg L^{-1}, and oxygen 6-8 mg L^{-1}.

Each organism was injected with 100 µL in the third abdominal segment with a concentration of 1.25×10^6 CFU mL^{-1} for *V. harveyi* and 1.2×10^6 CFU mL^{-1} for *V.*

campbellii; for the negative control, shrimp was injected with heat-inactivated bacteria in solution. Feeding for shrimp in both challenges was 30 min post bacterial infection and then every four hours until the end of the challenge (24 h).

Three replicates from each treatment were used to evaluate the survival rate, with 10 shrimps replicates for 30 shrimps in each treatment. The survival rate was calculated as the survival probability at any particular time (St) (Goel et al. 2010). Thirty shrimps were used to evaluate the AHPND disease by wet mount and histological analysis. The surviving shrimps were also fixed in Davidson's solutions at the end of the experiment.

For a better description of the results, the following abbreviations were used: a: negative control, b: positive control of *V. harveyi*, c: treatment of *V. harveyi* with WM, d: treatment of *V. harveyi* with RM, e: positive control of *V. campbellii*, f: treatment of *V. campbellii* with WM, g: treatment of *V. campbellii* with RM.

Clinical signs and histological analysis

Immediately after the survival challenge test, a wet mount analysis was made to assess if the surviving or moribund shrimps had 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 2010).

Bioassay organisms displaying behaviors such as positioning in the 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). Specimens were paraffin-embedded, cut into 5 μm sections, stained with hematoxylin and eosin, and reviewed under the light microscope to detect alterations in organs and tissues and modifications in the hepatopancreas (Bell & Lightner 1988).

Hemolymph analysis

The hemolymph was directly from experimental and control animal groups using a pre-washed syringe with anticoagulant (Alsever solution (MAS)) in a ratio of 1:1.

Total hemocyte count (cells mL^{-1}) was performed using a Burker hemocytometer (Le Moullac et al. 1997). The hemocytes on the hemocytometer were observed at 100x magnification under a phase contrast microscope (Olympus BX60-USA) and counted manually in all 25 squares.

Differential hemocyte count (DHC) dense and semi-dense granulocytes, hyaline, were determined according to the method of Cheng et al. (2003).

The method proposed by Morales-Covarrubias et al. (2023) was used to determine the neutrophil extracellular traps (NET). The hemolymph was withdrawn from the ventral sinus of the organism (base of the first abdominal segment) and mixed with Alsever anticoagulant solution at a 1:1 ratio. The slides were observed for fluorescence microscopy (Olympus (BX60-USA) with excitation/emission at 358/461 nm.

Giemsa stain was prepared according to the package instructions (HYCEL CAT. 548) with modifications in the washed. The cells were washed one time with distilled water for 3 s. Cells were incubated at room temperature for 1 h. Slides were observed for microscopy Olympus (BX60-USA), adapted with a digital camera (Infinity 2).

Statistical analysis

A Kruskal-Wallis non-parametric analysis of variance ($P < 0.05$) was performed to evaluate the effect of mangrove extracts on organism mortality, and Tukey's multiple comparisons test ($P < 0.05$) all data were presented as means with standard deviation. The analyses were carried out with Sigma Stat version 3.0.

The data of cellular immunity were subjected to a completely randomized variance analysis design and then Duncan's multiple range test when significance was detected at $P < 0.05$ by employing Statistica 8 software.

RESULTS

Phytochemical composition and antioxidant capacity of hydroalcoholic extracts

In the hydroalcoholic extract of both mangrove species used in this study, statistically significant differences were found ($P < 0.05$) for the total content of polyphenols and flavonoids. The hydroalcoholic extract of RM presented a higher content of total polyphenols expressed as mg Gallic acid equivalents per milliliter of extract, concerning the hydroalcoholic extract of WM (4.50 ± 0.26 and 3.36 ± 1.29 mg GAE mL^{-1}, respectively). Likewise, the result for total flavonoid content expressed as mg quercetin equivalents per milliliter of the extract revealed that WM contained a lower amount of this type of compound concerning the extract of RM (1.96 ± 0.71 and 2.60 ± 0.15 mg QE mL^{-1}, respectively) (Table 1).

Antioxidant activity

The antioxidant capacity tests found data without and with significant differences ($P < 0.05$). Both extracts showed the same DPPH radical scavenging activity (0.12 ± 0.00 and 0.12 ± 0.01 TEAC mL^{-1}, respectively); in ABTS, the hydroalcoholic extract of RM was elevated compared to WM extract (0.74 ± 0.08 and 0.51 ± 0.07 TEAC mL^{-1}, respectively). On the other hand, the antioxidant activity measured by FRAP assay was higher for hydroalcoholic extract of WM compared to RM (6.56 ± 0.43 and 1.34 ± 0.62 TEAC mL^{-1}, respectively) (Table 1).

Antimicrobial activity and minimum inhibitory concentration (MIC)

The hydroalcoholic extract of both mangroves inhibited the bacterial growth of *V. harveyi*, with inhibition halos of 20 ± 2 mm with RM and 18 ± 2 mm with WM. For *V. campbellii*, the hydroalcoholic extract of RM presented inhibition halos of 21 ± 2 mm, and that of WM was 25 ± 2 mm. The MIC for both bacteria was 1.65 g mL^{-1} from the tubes with 5 mL of TS Broth and 100 µL of each bacterium at a 1×108 CFU mL^{-1} density. In the analyses with modified pellets of both extracts, for RM, 19 ± 1 mm inhibition halos were obtained in its challenge with the two bacteria. For WM, in *V. harveyi*, the inhibition halo was 17 ± 1 mm, while in *V. campbellii*, the halos were 23 ± 1 mm, respectively (Fig. 1).

Experimental injection infection bioassay and clinical symptoms

The average amount of food consumed with and without adding hydroalcoholic extract did not show significant differences ($P < 0.05$) among the treatments, including the control. During the experiment, shrimp on negative control showed large pigmented hepatopancreas, low cuticular chromatophores, full gut (Fig. 2a), and 100% shrimp survival (Fig. 3).

All shrimp immersed in the positive control, 20% in treatment with WM and 10% in treatment with RM, presented muscle opacity (Fig. 2b) immediately after inoculation. After 30 min, the shrimp exhibited expansion of cuticular chromatophores (Fig. 2b), erratic swimming behavior, then settled to the bottom of the tank. After 6 h, the shrimp had an almost empty gut (whitish). They developed a pale hepatopancreas (Fig. 2b), transparent muscle (Fig. 2c), lateral decubitus swimming, and multifocal reddish coloration. At the same time, mortalities were recorded after 5 h, and by 24 h, only 20% of shrimp survived (Fig. 3) after 24 h. Significant differences in survival were observed between shrimp from both treatments only with *V.*

Table 1. Phytochemical composition and antioxidant capacity of red and white mangrove samples. TPC: total polyphenol content (mg of Gallic acid mL^{-1} extract), TFC: total flavonoid content (mg of quercetin mL^{-1} extract), TTC: total tannin content (mg of tannic acid mL^{-1} extract), Cx+c: carotenoids (µg of carotenoids mL^{-1} extract), TEAC mL^{-1}: mg of Trolox antioxidant activity mL^{-1} extract), DPPH, ABTS, FRAP: chromogenic compounds used to determine the ability of the phenolic compounds contained in the extracts to scavenge the free radicals generated. Different letters indicate significant differences ($P < 0.05$). Reported values correspond to the average ± standard deviation.

Phytochemical composition	Red mangrove	White mangrove	n
TPC (GAE mL^{-1})	4.50 ± 0.26^{a}	3.36 ± 1.29	6
TFC (QE mL^{-1})	2.60 ± 0.15^{a}	1.96 ± 0.71	6
TTC (TAE mL^{-1})	0.20 ± 0.01	0.32 ± 0.01^{a}	3
Cx+c (µg mL^{-1})	7.2 ± 1.8^{a}	1.0 ± 0.4	3
Antioxidant capacity (TEAC mL^{-1})			
DPPH	0.12 ± 0.00^{b}	0.12 ± 0.01^{b}	6
ABTS	0.74 ± 0.08^{a}	0.51 ± 0.07	6
FRAP	1.34 ± 0.62	6.56 ± 0.43^{a}	6

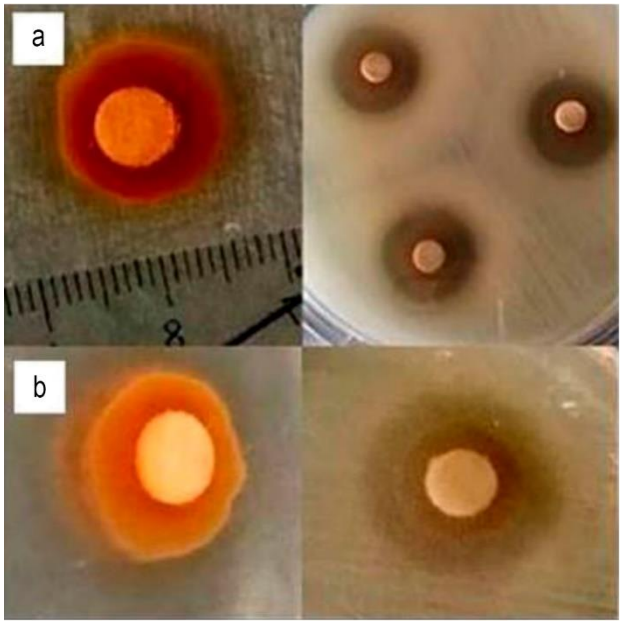

Figure 1. Inhibition zone diameter of red (left) and white (right) mangrove hydroalcoholic extracts against a) *Vibrio harveyi* and b) *V. campbellii*.

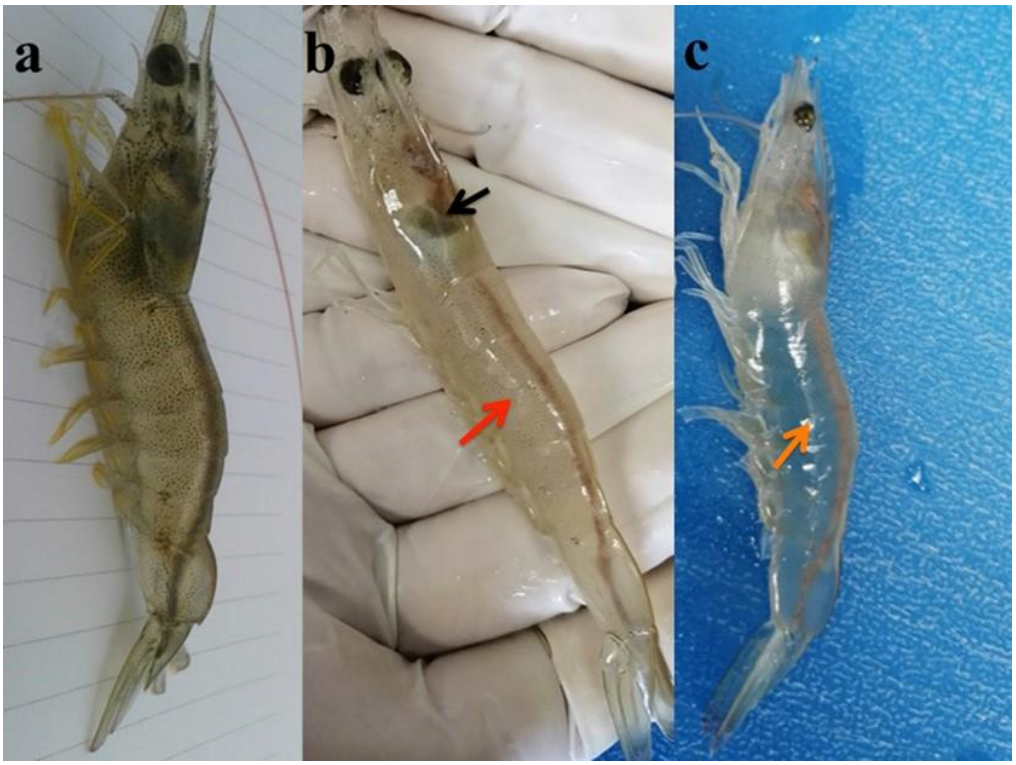

Figure 2. a) *P. vannamei* juveniles normal, large pigmented hepatopancreas, low cuticular chromatophores and full gut, and b-c) shrimps affected by *V. harveyi* and *V. campbellii* with pale hepatopancreas (black arrow-b), muscle opacity (red arrow-b) and transparent muscle (orange arrow-c).

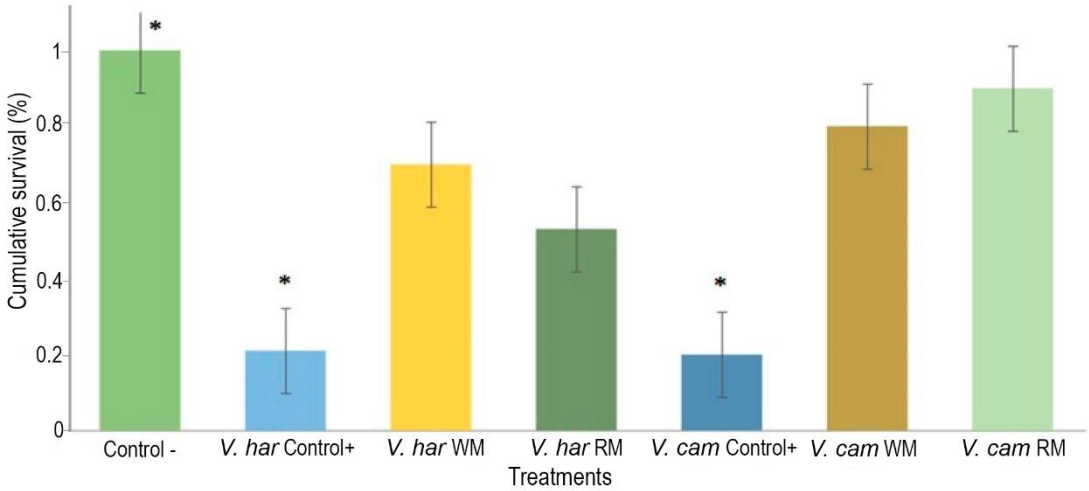

Figure 3. Cumulative survival in experimental bioassay infected with *V. harveyi* and *V. campbellii* and treated with mangrove hydroalcoholic extracts. A symbol (*) represents the groups with significant differences ($P < 0.05$). Reported values correspond to the average ± standard deviation. WM: white mangrove, RM: red mangrove, *V. har*: *Vibrio harveyi*, *V. cam*: *Vibrio campbellii*.

harveyi and *V. campbellii* (positive control) in Figure 3; $P < 0.05$, but no significant differences were observed between mortality rate and organ and tissue alterations between treatments with extracts of both mangrove species (Fig. 3).

Hemolymph analysis

For the organisms infected with *V. harveyi*, and treatments, the multiple comparison tests indicated the following: 1) total hemocytes count (THC) (Fig. 4a) in the negative control was significantly higher than the treatments (Fig. 4a; $P < 0.001$). The granular hemocytes, semi-granular hemocytes, phagocytosis, and traps extracellular in the negative control were significantly low than the treatments (Fig. 4b; $P < 0.001$). 2) The THC of the positive control was lower than the negative control and treatments analyzed (Fig. 4a; $P < 0.001$). The granular hemocytes, semi-granular hemocytes, phagocytosis, and extracellular traps (Fig. 5) were high on the treatments (Fig. 4b; $P < 0.001$), but between the two treatments (WM and RM), no significant differences were found (Fig. 4b; $P = 1.000$). No significant differences were found in the organisms infected with *V. campbellii* and treatments (WM and RM) ($P = 1.000$). The Tukey test indicated the following: a) the THC in the positive control was significantly low than the other treatments (Fig. 4a), and b) the THC did not significantly differ between the negative control and treatments (Fig. 4a; negative control *vs*. WM, $P = 0.874$ and negative control *vs*. RM, $P = 0.936$). The behavior of the granular hemocytes (Fig. 4b), semi-granular hemocytes, phagocytosis, and extracellular traps (Fig. 5) was the same as those infected with *V. harveyi*.

Histological analysis

The negative control showed the normal structure of gills with primary (Pri) and secondary (Sec) branched filaments with circulating hemocytes and hemocytic infiltration of apical branchial lamellae (Fig. 6a), the normal structure of tubules and epithelial cells in the hepatopancreas including a high level of lipid droplets (R-cell), secretory vacuoles (B-cell, Fig. 7b1). In contrast, the organisms infected with their respective bacteria (positive control) fixed in the 24 h displayed in gills hemolymph infiltration with hemocytes (Figs. 6b1-2, c1) and loss of structure in dendobranchia (Figs. 6b1-2, c2-3). The hepatopancreas (HP) showed atrophy tubules and sloughing of HP tubule epithelial cells in the medial region of the hepatopancreas (Figs. 7c1, 3). Very low B-cell and R-cell levels were observed (Fig. 7b2), and hemocytic infiltration surrounding tubules was common. At this point, bacterial colonization was observed with the presence of hemocyte nodule structure (Fig. 7b2).

The organisms treated with RM and WM showed low hemocytic infiltration in gills (Fig. 7c1-2) and the presence of normal cells in all organs and tissues; only in HP observed hemocytic infiltration (Fig. 7c2), surrounding atrophy tubules was common and low sloughing of HP tubule epithelial cells (Fig. 7c1) to the bacterial presence and host inflammatory response between and within HP tubules (Fig. 7c2).

DISCUSSION

It is widely accepted that the application of herbal extracts provides multiple benefits for aquatic animals, including shrimps, such as improvement in the immune system and antioxidant status (Espinoza-Berrezueta 2017, Dotulong et al. 2018), antibacterial function particularly (Chen et al. 2003, Blanco-Olano et al. 2020), and survival rate and growth (Acevedo-Barrios et al. 2015, Espinoza-Berrezueta 2017). The present study proves that *L. racemose* and *R. mangle* extract contain various bioactive constituents, such as flavonoids, carotenoids, and tannins. These results confirmed previous phytochemical screening on a variety of mangrove species, such as *Avicennia officinalis* (Eswaraiah et al. 2020), *Suaeda maritime* (Falqueto et al. 2008), *Avicennia marina*, *A. germinans* and *L. racemose* (Flegel 2019), which primarily contained flavonoids, carotenoids, polyphenol, and tannins compounds. Among many species, there is great variability in the number of secondary metabolites due to ethnobotanical factors (Falqueto et al. 2008, Flegel 2019).

The polyphenol compounds are easily oxidized and converted from oxidase phenol into quinone oxidase that can kill pathogens and produce melanin (Eswaraiah et al. 2020). The results obtained in the phytochemical profiles of both mangrove extracts are similar to those reported by other authors (Makkar et al. 1993, Falqueto et al. 2008, Cruz et al. 2015, Da Silva 2019) in which flavonoids and tannins are always present and in high amounts (Glasenap et al. 2019). These compounds are linked to antioxidant and anti-inflammatory (Akter et al. 2010, Gawali & Jadhav 2011) properties, where they have a membrane stabilizing effect due to a charge binding between the plant extract components with the erythrocyte membrane, thus protecting the membrane from agents involved in its lysis (Islam et al. 2017).

The use of therapies with plant extracts in aquaculture has shown positive results and complements the possibility of using herbal medicine. Studies demonstrated immune system improvement by ingestion of feed added with plant extracts in Atlantic salmon *Salmo salar* (Palstra et al. 2018), red abalone *Haliotis rufescens*, rainbow trout *Oncorhynchus mykiss*, Nile tilapia *Oreochromis niloticus*, sea bream *Pagellus centrodontus* and sea bass *Dicentrarchus labrax* (Awad & Amani 2017), where all show a post-harvest benefit

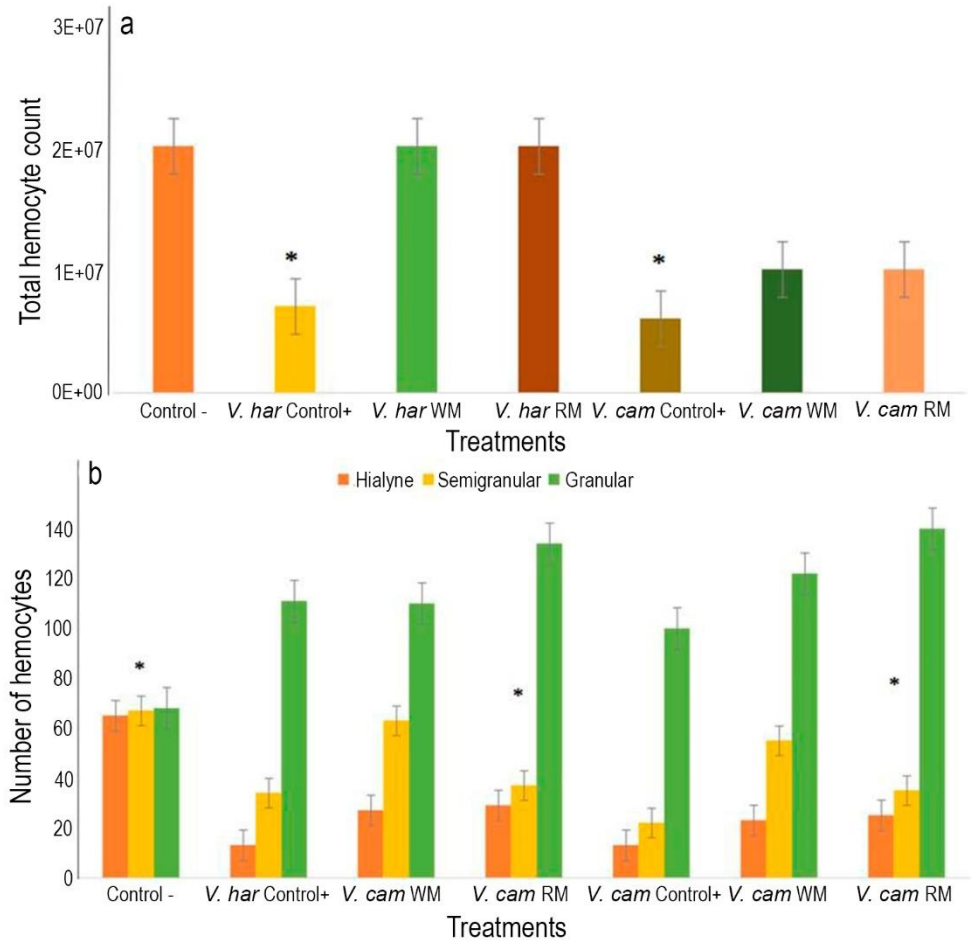

Figure 4. a. Total hemocyte count in experimental bioassay infected with *V. harveyi* and *V. campbellii* and treated with mangrove hydroalcoholic extracts, b., differential hemocyte count in experimental bioassay infected with *V. harveyi* and *V. campbellii* and treated with mangrove hydroalcoholic extracts. Symbol (*) represents the groups with significant differences ($P < 0.05$). Reported values correspond to the average ± standard deviation. WM: white mangrove, RM: red mangrove, *V. har*: *Vibrio harveyi*, *V. cam*: *Vibrio campbellii.*

for human consumption, as well as low or no impact on the environment.

In the present study, the histological structure in both treatments improved compared to the infected groups in the hepatopancreas, gills, and other organs and tissues, consistent with the survival percentage achieved after infection. Results confirm that using methanolic mangrove extracts for bacterial disease treatments is viable. Other authors have suggested that the addition of extracts of vegetable origin has a positive influence on *P. monodon* infected with *V. harveyi* (Aftab-Uddin et al. 2017); being probable that in addition to red and white mangrove, other mangrove species, such as *A. marina* are good candidates for treatment against *V. parahaemolyticus* infections in *P. vannamei* (Manilal et al. 2009).

Hydroalcoholic extracts and phytochemical antioxidants stimulated shrimps' cellular and humoral immune responses. The increase in phagocytosis was in line with the increase in total semi-granular hemocyte cells. Hemocyte is a component of immune cells that plays an important role in the process of cellular immunity of shrimps (Kumar et al. 2020). This stimulatory effect was confirmed in this experiment, whereas inducing shrimps with most of the hydroalcoholic extracts significantly increased the phagocytic percentage and extracellular traps 4 h after being challenged with *V. harveyi* and *V. campbellii* to the end of the experiment (24 h). Differential hemocytes were performed following Saptiani et al. (2020). Hyaline hemocytes and semi-granular hemocytes are involved in the phagocytosis process of

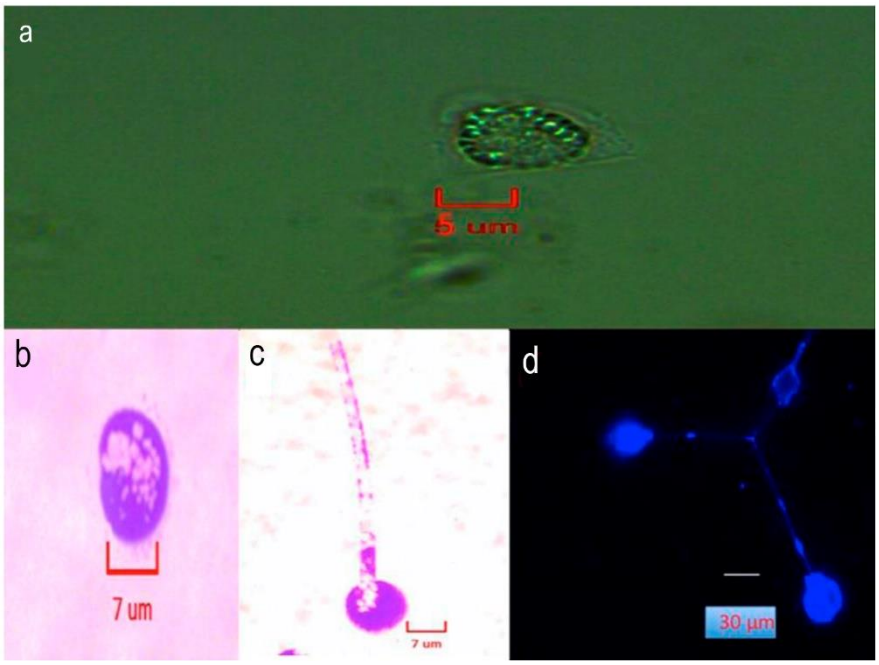

Figure 5. a). Wet mount of hemocytes composed granules in its cytoplasm of *P. vannamei*, b-c) Giemsa staining of hemocytes composed granules in its cytoplasm of *P. vannamei*, d) hemocytes formed extracellular traps (ETs) as revealed by DNA Giemsa staining, e) traps extracellular of white shrimp hemocytes entangle *V. harveyi* and *V. campbellii* cells. DAPI (blue) was used for DNA staining. Scale bar: a) 5 µm, b-c) 7 µm, and d) 30 µm.

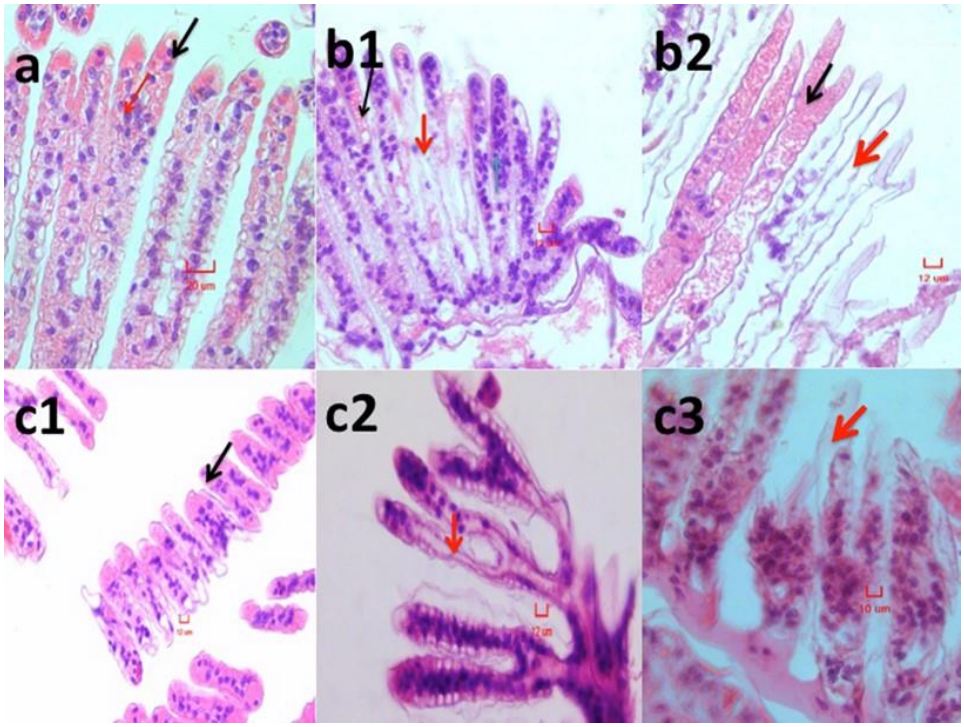

Figure 6. H&E stained photomicrographs collected from negative and positive control and hydroalcoholic extracts mangroves treatment inoculated with *V. harveyi* and *V. campbellii*: a) normal structure of gills with primary and secondary branched filaments with circulating hemocytes and hemocytic infiltration of apical branchial lamellae (black arrow) in negative control; b1-b2) gills hemolymph infiltration (black arrow) with hemocytes and loss of structure (red arrow) in positive control; c1) gills hemolymph infiltration (black arrow) with hemocytes and c2-c3) with low loss of structure (red arrow) in hydroalcoholic extracts mangroves treatments. Scale bar: a) 20 µm, b1-b2), c1-c2) 12 µm, and c3) 10 µm.

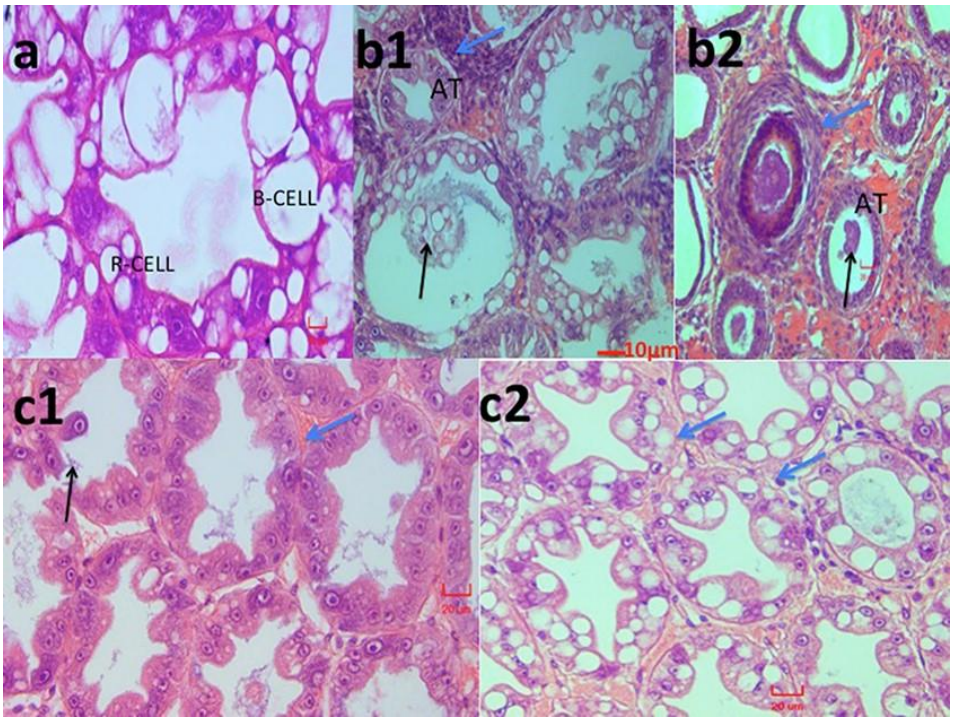

Figure 7. H&E stained photomicrographs collected from negative and positive control and hydroalcoholic extracts mangroves treatment inoculated with *V. harveyi* and *V. campbellii*: a) normal structure of tubules and epithelial cells in the hepatopancreas including a high level of lipid droplets (R-cell), secretory vacuoles (B-cell) with hemolymph infiltration in negative control; b1) hepatopancreatic tubules with hemolymph infiltration, nodule formation (blue arrow), necrotic massive sloughed epithelial cells (black arrow) and tubular atrophy (AT) in positive control; b2) hepatopancreas with hemolymph infiltration, nodules formation (blue arrow) and necrotic massive sloughed epithelial cells (black arrow); c1-c2) hepatopancreatic tubules with hemolymph and hemocytes infiltration (blue arrow) and low loss of structure (black arrow) in hydroalcoholic extracts mangroves treatments. Scale bar: a-b1):10 µm, b2-c1-c2): 20 µm.

shrimp antigen. An increase in hemocyte cells in the present study indicates that mangrove hydroalcoholic extract acts as an immunostimulator (Saptiani et al. 2020). The increase in phagocytosis was in line with the increase in total semi-granular hemocyte cells. Phagocytosis is part of hemolymph in the invertebrate defense system, and it acts as an early internal defense mechanism against invaders by circulating hemocytes (Liu et al. 2020). The extracellular traps increase aligned with the total granular and semi-granular hemocyte cell increase. The impact of extracellular traps derives from the combined antimicrobial activities of granular components, histones, and some cytoplasmic proteins. Eosinophils and mast cells are granulocytes closely related to neutrophils, granulocyte homologs in lower vertebrates, and even plants release extracellular traps (Borregaard 2010, Brinkmann & Arturo 2012). The main job of neutrophils is to eliminate microbes. Extracellular traps probably evolved to rein in infections by, as their name indicates, "trapping", preventing dissemination, inactivating virulence factors, and exterminating microbes. Trapping microbes prevents their dissemination from the initial infection site. Microbes most likely stick to NETs through charge interactions (Urban et al. 2009, Brinkmann & Arturo 2012).

CONCLUSIONS

The present study concluded that the hydroalcoholic extracts of RM and WM supplied via the feed effectively ameliorate the pathogenicity effect of *V. harveyi* and *V. campbellii*. The hydroalcoholic extracts reduced mortality, clinical symptom, alteration reduction in organs and tissues, and improved the cell immunity of *P. vannamei*. Thus, it is possible to use the hydroalcoholic extract of *R. mangle* and *L. racemose* as treatments on the shrimp farm to enhance bacterial disease tolerance and prevent mortality.

ACKNOWLEDGMENT

This work is part of the first author's graduate thesis project funded by self-generated funds at CIAD (CIAD-20657-MCMS).

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Received: March 29, 2023; Accepted: May 29, 2023

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