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# Evaluation of medicinal plants and colloidal silver efficiency against *Vibrio parahaemolyticus* infection in *Litopenaeus vannamei* cultured at low salinity

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ABSTRACT: In shrimp aquaculture, reduction in the use of synthetic antibiotics is a priority due to the high incidence of resistant bacteria (*Vibrio*) in the white shrimp *Litopenaeus vannamei*. An increasing number of studies show bactericidal activity of natural treatments in aquaculture. The effectiveness of neem (*Azadirachta indica*) and oregano (*Lippia berlandieri*) aqueous extracts and colloidal silver against *V. parahaemolyticus* were evaluated in low salinity shrimp culture. Results show that aqueous extracts of oregano and neem each present a minimum inhibitory concentration (MIC) of 62.50 mg $ml^{-1}$ and inhibitory halos of 12.0 to 19.0 mm. Colloidal silver gave a MIC of 2 mg $ml^{-1}$, and the inhibitory halos were found to be between 11.8 and 18.8 mm, depending on treatment concentrations. An *in vivo* challenge test was conducted on white shrimp postlarvae cultured at low salinity (5 practical salinity units, PSU), and a significant increase ($p < 0.05$) in survival was demonstrated in the presence of the aqueous extracts (oregano 64%, neem 76% and colloidal silver 90%), when compared to the control (0%) in the challenge test. However, no significant differences were observed between treatments, suggesting that they all act as alternative bactericidal source agents against *V. parahaemolyticus* infections for *L. vannamei* postlarvae when cultured at 5 PSU.

KEY WORDS: Neem · Oregano · Colloidal silver · *Vibrio parahaemolyticus* · White shrimp · EMS · Acute hepatopancreatic necrosis syndrome · AHPNS



## INTRODUCTION

Antibiotic resistance in bacterial disease agents and the fact that vibrios can survive in a wide variety of salinities are stumbling blocks affecting penaeid shrimp production. Further studies need to be done in this area in order to reduce shrimp mortalities and provide support for shrimp aquaculture (Morales-Covarrubias 2010, Morales-Covarrubias & Gómez-Gil 2014). In the case of aquatic organisms, the control treatments used for common bacterial diseases include antibiotics such as enrofloxacin, florfenicol and oxytetracycline (Xu et al. 2006, Santiago et al. 2009). Recent reports indicate that acute hepatopancreatic necrosis syndrome (AHPNS), originally named early mortality syndrome (EMS), is caused by a resistant strain of *Vibrio parahaemolyticus* that produces a toxin capable of destroying shrimp tissue, primarily in R (resorptive), B (blister), F (fibrillar), and E (embryonic) cells, resulting in hepatopancreas (HP) dysfunction and massive mortalities (60–80%) during the first 30 d after stocking (Tran et al. 2013).

The World Health Organization (WHO) recognizes the therapeutic potential of herbal medicines and other traditional sanitary treatments, particularly for use in aquaculture (Torres et al. 2007). Since the end of December 2003, the Environmental Protection Agency (EPA, USA) has highlighted the need for further research using natural alternatives to synthetic antibiotics and their application in the production of aquatic

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organisms for human consumption (Mioso et al. 2014).

For over 2000 yr, medicinal plants have been used to combat pathogenic agents, and within the last decades, studies have focused on natural alternatives that can be employed as bactericidal treatment in aquaculture (Torres et al. 2007, Ramírez & Marín-Castaño 2009, García-Aguilar 2013).

To transform a medicinal plant for conventional medical use, the most common procedures are infusion and decoction to extract the active substances from the vegetable tissues. When the water ebullition point (100°C) is reached and the water makes contact with fresh or dry plants, the vegetable cells explode and discharge active substances. The scientific term for this is 'aqueous extracts', and the principal advantage of using this preparation method is provision of easy and fast *in situ* preparations (Citarasu 2010).

One plant considered an alternative medicine in aquaculture is neem *Azadirachta indica*; its principal active metabolite is nortriterpenoide (also known as azadiractine) with the presence of limonoides, which are structurally related and are biologically active (Hammer et al. 1999, López-Pantoja et al. 2007, Banerjee et al. 2013, Dhama et al. 2013a,b, Peña-Navarro et al. 2013). Another plant for consideration is oregano *Lippia berlandieri* Schauer, which possesses thymol and carvacrol as its bioactive substances, which both show significant bactericidal activity (Celikel & Kavas 2008). Silver (Ag) was also evaluated due to its established role as an efficient antimicrobial agent when in the form of colloidal silver ions (Morrill et al. 2013). 'The colloidal silver is a liquid solution created using electrolysis to suspend pure metallic elemental silver' (www.herbwisdom.com/herb-colloidal-silver.html, accessed February 2016). Colloidal silver suffocates single-celled organisms (bacteria, fungus, virus and yeast), disabling their oxygen metabolism enzymes (Vaseeharan et al. 2010). The aim of this study was to evaluate the bactericidal efficiency of neem and oregano aqueous extracts and of colloidal silver against *V. parahaemolyticus* under control conditions and in challenges on *Litopenaeus vannamei* postlarvae at low salinity.

## MATERIALS AND METHODS

### Preparation of plant extracts and colloids

The dry leaves of Mexican oregano *Lippia verlandieri* were collected from the municipality of Mapimí, Durango State, Mexico (25° 49′ 59″ N, 103° 50′ 52″ W) and were pulverized in a pestle and mortar to make infusion preparations (Meléndez-Rentería et al. 2009). Neem *Azadirachta indica* stems and leaves were collected in Mazatlan, Sinaloa State, Mexico (23° 4′ 25″ N, 105° 37′ 10″ W) and immediately prepared for infusions.

The oregano and neem infusions were prepared by placing 50 g of each pulverized plant in 200 ml of boiling water for 10 min, and leaving to cool. A concentrated infusion stock of 25% w/v was obtained. Colloidal silver was obtained from BIOINSEL S.A.de C.V. at a concentration of 15 ppm.

### *Vibrio parahaemolyticus* strain culture

The bacterium (01082013-LS) was isolated from a disease outbreak that produced high mortalities (90–100%) in a low salinity shrimp farm at Rosario, Sinaloa, Mexico (22° 57′ 43″ N, 106° 7′ 44″ W) (H. Ramirez pers. comm., 26 July 2013). Shrimp were disinfected with 70% ethanol and the cuticle removed, and the stomach and HP were dissected aseptically, weighed and homogenized in 1000 µl sterile 2.5% NaCl. A 100 µl aliquot of the homogenate was diluted in 900 µl with sterile 2.5% NaCl. Subsequently, 100 µl aliquots were taken from the homogenate as well as from the diluted aliquots and placed into the thiosulfate-citrate-bile-sucrose agar (TCBS) and marine agar (MA) Petri dishes and incubated at 30°C for 24 h to enumerate *Vibrio* spp. The green colony obtained from the TCBS Petri dish was purified in CHROMagar™ Vibrio (chromogenic medium for detection and isolation of *V. parahaemolyticus, V. vulnificus* and *V. cholerae*) and incubated at 30°C for 24 h. The pure isolates were used in a survival challenge test and cryopreserved at −80°C in tryptic soy broth (TSB; Bioxon) with 15% (v/v) glycerol.

For the survival challenge test, the bacteria were grown in TSB medium containing 2% NaCl and incubated on a rotary shaker for 18 h at 30°C. After incubation, bacterial concentration ($10^8$ cells $ml^{-1}$) was checked using a DS-11 spectrophotometer (De Novix) at an optical density (OD) of 600 nm. These suspensions were placed on TCBS agar after serial dilution to determine the colony forming unit (CFU) $ml^{-1}$ of the isolate.

### Confirmation of bacterial virulence (preliminary bioassay)

The virulence of bacteria was checked with a challenge test using 10 shrimp (*L. vannamei*), each

weighing 1.0 ± 0.3 g per aquarium. A positive control (bacteria only) and negative control (water only) with 3 replicates each were also included. Shrimp were kept in an experimental glass aquarium containing low salinity water (5 PSU) with aeration. Shrimp were infected by immersion in low salinity water containing $10^4$, $10^6$ and $10^{10}$ CFU ml$^{-1}$ of bacteria. Mortalities were monitored for 2 d. The moribund and surviving shrimp infected with the strain were collected for histopathological examination and polymerase chain reaction (PCR) of the stomach and HP. Pure isolates of the pathogenic strain (ID 01082013-LS) were molecularly identified by PCR in accordance with the methodology previously described by Flegel & Lo (2014), and the more sensitive AP4 method was also run (Dangtip et al. 2015) to detect AHPND. In addition, the typical histopathological lesions of the disease as described by Tran et al. (2013) were analyzed.

### Determination of minimum inhibitory concentration (MIC)

For oregano and neem, concentrations of 1.95, 3.90, 7.81, 15.62, 31.25, 62.50, and 125 mg ml$^{-1}$ were prepared from the infusion stock (25% w/v) and for colloidal silver of 0.125, 0.5, 1.0, 2.0, 3.0, 4.0 and 8.0 µg ml$^{-1}$ from the concentrated solution (15 ppm) were evaluated. To each of the aforementioned dilutions, 100 µl of the bacterial inoculum were added and incubated at 30°C for 24 h at a constant agitation speed of 125 rpm. The samples were then visually assessed for turbidity, and those which produced no turbidity were established as the MIC for the treatments; positive and negative controls were all conducted in triplicate.

### Determination of minimum bactericidal concentration (MBC)

Petri dishes containing TSB (DIFCO) supplemented with 2.5% NaCl were inoculated with the concentrations that did not present turbidity (MIC) and incubated at 30°C for 24 h. The lowest concentrations, where no CFU occurred, were considered as the MBC for each treatment. A positive and negative control were tested at the same time in order to demonstrate adequate microbial growth during the incubation period and media sterility, respectively (Lambert et al. 2001). All treatments were performed in triplicate.

### Inhibition rate of bacteria for oregano, neem and colloidal silver

The bactericidal capacity of the plant infusions and colloidal silver were analyzed. Petri dishes were inoculated with isolates of *V. parahaemolyticus*, using the Waune (2006) method to measure the inhibition rate of CFU. A bacteria inoculum (individual colony of the bacterial strain) was re-suspended in sterile 2.5% NaCl and turbidity was adjusted to the 0.5 McFarland (1907) standard. The entire agar surface of the Petri dish was spread with 100 µl of bacterial suspension ($1 \times 10^8$ CFU ml$^{-1}$). Circles of sterile Whatman™ paper No. 6 (Sigma Aldrich) measuring 8 mm in diameter were impregnated with the different test concentrations of neem and oregano extracts and colloidal silver solution and placed on the Petri dishes. Classification of bacterial sensitivity to antibiotics was related to the inhibition circle diameter (≤8 mm not sensitive, 9–14 mm sensitive, 15–19 mm highly sensitive, ≥20 mm extremely sensitive) (Morrill et al. 2013). A negative control was included using 2.5% NaCl. All assays were run in triplicate and incubated at 30°C for 24 h.

In a liter of distilled water, 38 g of agar powder (Mueller-Hinton) was mixed with 25 g of NaCl (2.5%) and placed in an autoclave at 120°C for 15 min. The agar was cooled to 40°C in a water bath before being poured into Petri dishes and then left at room temperature, ready for future use.

### Feed preparation

Commercial feed (Malta Texo™ Mexico; 40% protein and 8% lipids) was pulverized inside a domestic mill (Nixtamatic™). Plant extracts or colloidal silver (0.5 ml g$^{-1}$ feed) were added, along with warm water (40°C) when necessary and reconstituted in a Moulinex™ domestic meat grinder (±1.6 mm diameter). The resulting pellets were dried at an oven temperature of 38 ± 2°C for 12 h, then stored in plastic bags at 4°C. The same procedure, without the plant extract or colloidal silver, was followed for the control diet.

For effectiveness and doses corroboration, feed pellets treated with the plant extracts and colloidal silver were added and a bacterial inhibition rate assay was performed, again using 100 µl of bacterial suspension ($1 \times 10^8$ CFU ml$^{-1}$) inoculum spread over the entire surface of the agar in the Petri dish and incubated at 30°C for 24 h. The diameter of the inhibition halo for each treatment was measured.

Feed consumption and palatability was monitored for 5 consecutive days with 6 replicates for each treatment. Individual shrimp were fed a fixed amount of food (30% wet body weight) with and without plant infusion (neem and oregano) and colloidal silver in a commercial diet (40% protein, 8% lipid). After a 4 h period, solid waste (unconsumed food and faeces) were siphoned out of each container and rinsed with distilled water to remove salts and posteriorly dried using the same conditions as for diet preparation. Finally, faeces and unconsumed food were separated using a stereoscopic microscope (Olympus) and weighed on a MT5 microbalance (Mettler Instrument). The food and faeces were then weighed, and consumption was estimated using the following formula:

Weight of consumed food = Weight of total food offered − Weight of food recovered

### Survival *in vivo* challenge test

The bactericidal capacity of the plant infusions and colloidal silver were evaluated *in vivo* via survival assays using white shrimp postlarvae ($PL_{15}$) weighing between 1.0 and 2.5 g, cultured at low salinity (5 PSU). Before the assay, a representative number of shrimp (10%) were removed from the batch (total n = 500 $PL_{15}$) to determine their health status. Bacteriological analysis (Lightner 1996) to determine CFU, wet mount analysis (Morales-Covarrubias 2010), PCR commercial kits (IQ2000™ Kit: WSSV, IHHNV, NHPB; GeneReach Biotechnology) and histological analysis (Lightner 1996, Tran et al. 2013) were carried out to identify necrotizing hepatopancreatitis bacterium (NHPB), the white spot syndrome virus (WSSV), infectious hypodermal and hematopoietic necrosis virus (IHHNV) and protozoal diseases in the intermoult stage of the shrimp. After the shrimp were confirmed negative by batch analysis, 10 intermoult stage shrimp were stocked per aquarium containing 5 l of water (low salinity, 5 PSU) with 5 replicates for each treatment (neem, oregano, colloidal silver, positive control and negative control). Before infection, shrimp were allowed to acclimatize for a period of 24 h. The established control conditions during the test were 30 ± 1°C, 5 ± 1 PSU, pH 7.5–8.0, <0.1 mg $l^{-1}$ ammonium and 6–8 mg $l^{-1}$ oxygen.

The bacterial suspension (50 ml) containing approximately $1 \times 10^8$ CFU $ml^{-1}$ was added directly to each experimental aquarium containing 10 shrimp, apart from the negative controls. The first feed was given 25 min after the bacterial inoculation and every 4 h thereafter. Survival was recorded every 2 h until the end of the challenge (48 h).

### Histopathology analysis: sample preservation and processing

Histological sections were analyzed by light microscopy for AHPNS/EMS lesions and alterations in the HP and stomach. Tissues of moribund shrimp from all assays (lying on their side on the bottom of the tank with slight movement of the scaphognathite) were fixed in Davidson's solution. The specimens were embedded in paraffin; 4 µm sections were sliced and stained with hematoxylin eosin before being checked under the light microscope (Bell & Lightner 1988, Morales-Covarrubias 2010, Tran et al. 2013).

Immediately after the survival challenge test, diagnosis through wet mount analysis was done to assess if the surviving or moribund shrimp had organ and tissue alterations. Their organs and tissues were removed, dissected and squash-mounted with sterile seawater, then examined under the light microscope (Lightner 1996, Morales-Covarrubias & Gómez-Gil 2014, Sriurairatana et al. 2014).

### Statistical analyses

All comparisons tests were analyzed using Excel Solver2010™ software. In order to identify significant differences between survival models, we fitted both linear and exponential regression to the survival data, and the model selected as best was determined using the criterion of the least sum of squares. After that, an analysis of residual sum of squares was carried out in order to identify significant differences. This analysis compared the model of each treatment with a general model, and an *F* distribution or variance proportions were used to evaluate the statistical differences between the general model and each of the models (Haddon 2001). The equation used was:

$$F = \frac{\dfrac{\mathrm{RSS}_p - \sum \mathrm{RSS}_i}{\mathrm{df}_p - \sum \mathrm{df}_i}}{\dfrac{\sum \mathrm{RSS}_i}{\sum \mathrm{df}_i}} = \frac{\dfrac{\mathrm{RSS}_p - \sum \mathrm{RSS}_i}{3(K-1)}}{\dfrac{\sum \mathrm{RSS}_i}{N-3K}}$$

Fisher's distribution test, with $3(K–1)$ and $(N–3K)$ degrees of freedom (df). $K$ is the curve number compared and $N$ the number of samples; RSS is the residual sum of squares from a model ($i$) and the general model ($p$).

## RESULTS

### Determination of the MIC and MBC

The MIC of both oregano and neem was determined to be 62.5 mg $ml^{-1}$ and 2 µg $ml^{-1}$ for colloidal silver. The MBC values obtained for aqueous extract of oregano and neem were found to be 125 mg $ml^{-1}$ and for colloidal silver 2 µg $ml^{-1}$.

### Bacterial inhibition rate of oregano, neem and colloidal silver in Petri dishes and feed

The inhibition response for oregano and neem at 125 mg $ml^{-1}$ in Petri dishes was found to be 'highly sensitive' with inhibition halos of 18.8 and 19.0 mm diameter, respectively (Table 1). For the challenge test, shrimp were fed pellets including the 125 mg $ml^{-1}$ herbal extracts. For colloidal silver 6.0 and 8.0 µg $ml^{-1}$ in Petri dishes gave inhibition halos of 16.8 and 18.8 mm, respectively (Table 1).

The inhibition response obtained using feed pellets containing 8.0 µg $ml^{-1}$ of colloidal silver presented an inhibition halo of 11.3 mm, and pellets containing 125 mg $ml^{-1}$ of oregano and neem infusions produced inhibition halos of 9.2 and 9.5 mm diameter, respectively (Table 1).

### Survival challenge test

The average consumption of food with or without plant infusions or colloidal silver was not significantly different ($p < 0.05$) among treatments including the control. All shrimp immersed in the positive control presented muscle opacity immediately after inoculation. After 30 min the shrimp exhibited erratic swimming behavior, then settled to the bottom of the tank. After 2 h, the shrimp had an almost empty gut and developed a pale HP and showed moribund behavior, while mortalities of 44% (22) were recorded after 3 h, and by 18 h all shrimp had died. At the end of the testing period (48 h), the negative control presented normal swimming behavior with no mortalities. The oregano treatment presented 22% (11) shrimp mortalities after 10 h of inoculation, with cumulative mortalities of 36% (18) after 15 h. In the neem treatment, shrimp mortality was 16% (8) after 13 h with cumulative mortalities of 24% (12) after 28 h. In the colloidal silver treatment, 18 h after inoculation, 6% (3) did not survive with cumulative mortalities of 10% (5) after 28 h.

Table 1. Treatments and measurements of the inhibitory halos of oregano and neem prepared from infusion stock (25% w/v) and from colloidal silver (15 ppm solution). Solutions were impregnated onto circles of sterile Whatman™ paper No. 6 except where noted with a footnote

| Oregano | | Neem | | Colloidal silver | |
|---|---|---|---|---|---|
| Infusion (mg $ml^{-1}$) | Halo (mm) | Infusion (mg $ml^{-1}$) | Halo (mm) | Solution (µg $ml^{-1}$) | Halo (mm) |
| 63 | 12.0 | 63 | 12.4 | 3.0 | 12.0 |
| 75 | 14.0 | 75 | 14.0 | 4.5 | 13.8 |
| 100 | 17.0 | 100 | 17.0 | 6.0 | 16.8 |
| 125 | 18.8 | 125 | 19.0 | 8.0 | 18.8 |
| 125[a] | 9.2 | 125[a] | 9.5 | 8.0[a] | 11.3 |

[a]Included in the feed

### Fresh analysis

Diseased shrimp exhibited an atrophied and pale HP, empty stomach and midgut (Fig. 2B), and loss of tubule epithelial cells (Fig. 3A). Also vermiform bodies with and without feed residues (Fig. 3B) were observed. The intestines showed whitish fluids and several vermiform structures and sloughing of HP cells.

### Histology slides

Histopathologic examination revealed alterations such as hemolymph infiltration in gill and pyknotic nuclei in the diseased shrimp. The HP displayed severe cell sloughing from the proximal to the distal end region of the tubules into the opening of the tubular channel (Fig. 4). Cells with pyknotic nuclei and several vermiform structures were found in the tubule lumen (Fig. 5).

The lymphoid organ presented spheroids, vacuoles, hemolymph infiltration, and the antennal gland presented hemolymph infiltration. The medium and posterior intestine exhibited severe hemocytic enteritis, and the posterior blind distention and vermiform structures were detected within in the lumen intestine.

### Statistical analyses

Significant differences in survival were observed between shrimp from the bacteria-only (positive control) and the other treatments ($p < 0.05$), but no significant differences were observed between mortality rate and tissue and organ alterations between the

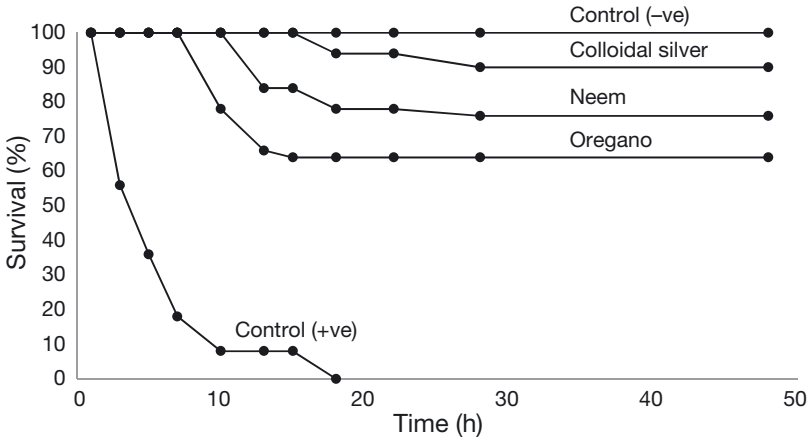


Fig. 1. Survival rate (%) against time (h) for postlarval *Litopenaeus vannamei* infected with *Vibrio parahaemolyticus* after inoculation with natural bactericides at low salinity (5 PSU)

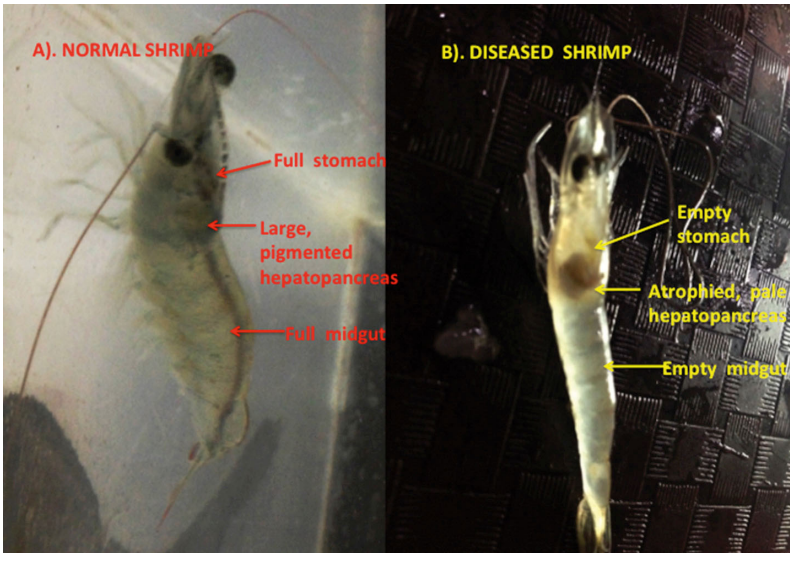


Fig. 2. *Litopenaeus vannamei* juvenile (A) normal shrimp and (B) shrimp affected by *Vibrio parahaemolyticus* (strain ID 01082013-LS)

medicated treatments. Linear and exponential regression were used to determine the survival rate of each treatment, and then adjusted for the RSS using the Newton algorithm included in the Excel Solver2010™ software. The exponential model gave the best fit in all treatments ($RSS_i < RSS_p$). No significant differences between curves ($p < 0.05$) were identified.

## DISCUSSION

MIC against *Vibrio parahaemolyticus* for oregano aqueous extracts was found to be lower (63 mg $ml^{-1}$) than cited by Paredes-Aguilar et al. (2007) for the *Vibrio* genus when using oregano essence oil. Hernández et al. (2005) reported a MIC of 500 mg $ml^{-1}$ and an MBC of 750 mg $ml^{-1}$ with *V. cholerae* using *Lantana achyranthifolia* oil, which belongs to the same family as oregano. Ávila-Sosa et al. (2010) reported a greater antimicrobial and bacteriological effect with the Mexican oregano. The inhibitory characteristics of oregano have been used to fight bacterial contamination in food for a long time (Rangel et al. 2008).

Banerjee et al. (2013) reported for neem extract an MIC of 31.3 mg $ml^{-1}$ for *V. parahaemolyticus* and 62.5 mg $ml^{-1}$ for *V. alginolyticus*, and an MBC

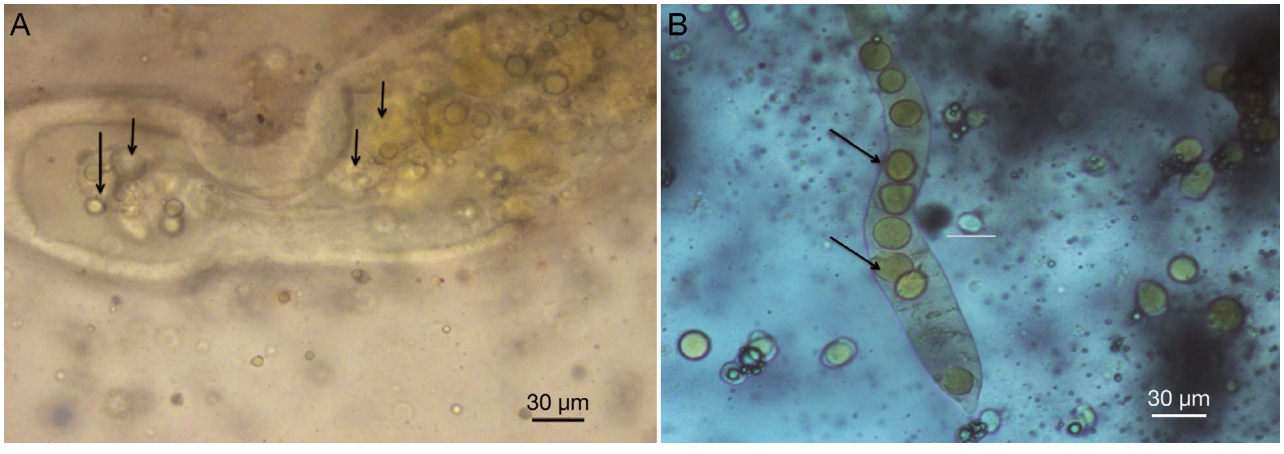


Fig. 3. Squash mount of (A) hepatopancreas tubules taken from juveniles of *Litopenaeus vannamei* with cellular desquamation (arrows), and (B) aggregated transformed microvilli containing cells (arrows)

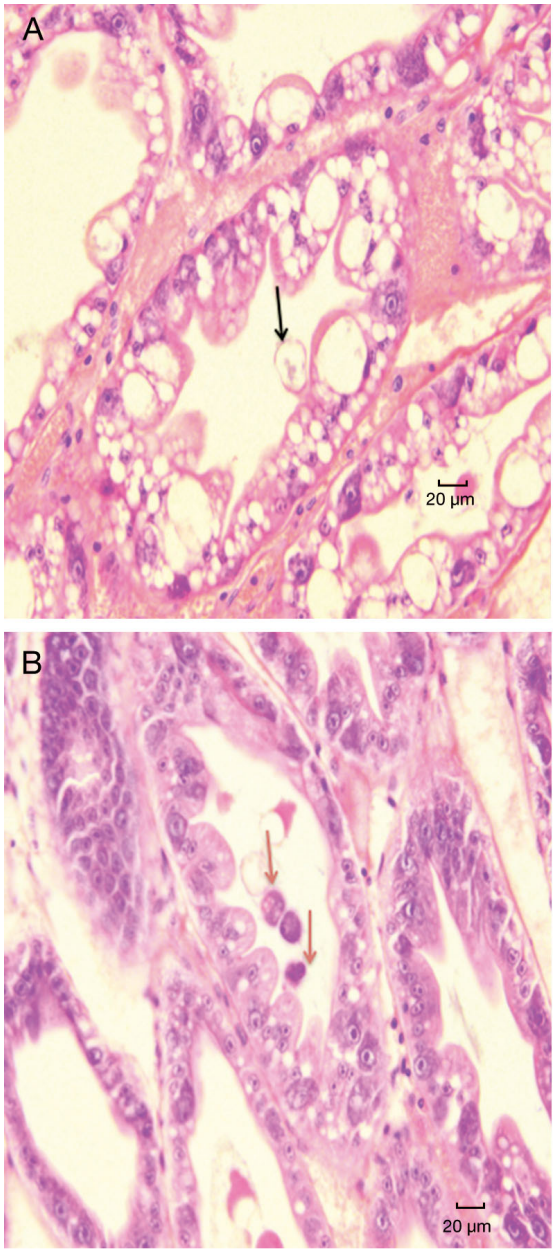


Fig. 4. Histological sections of hepatopancreas from *Litopenaeus vannamei* after immersion challenge. (A) Transversal view of tubules with elongation of epithelial cells (black arrow) toward the lumen. (B) Tubule epithelial cells are shown sloughing (red arrows) into the hepatopancreas tubule lumen. H&E stain. Scale bars = 20 µm

of 125 mg ml$^{-1}$ for *V. parahaemolyticus* and 250 mg l$^{-1}$ for *V. alginolyticus.* Similar results were also observed in our experiment when a MIC of 63 mg l$^{-1}$ was obtained against *V. parahaemolyticus*. According to Yamamoto (2004), an agent is considered bactericidal when the MBC does not exceed 4 times the MIC value.

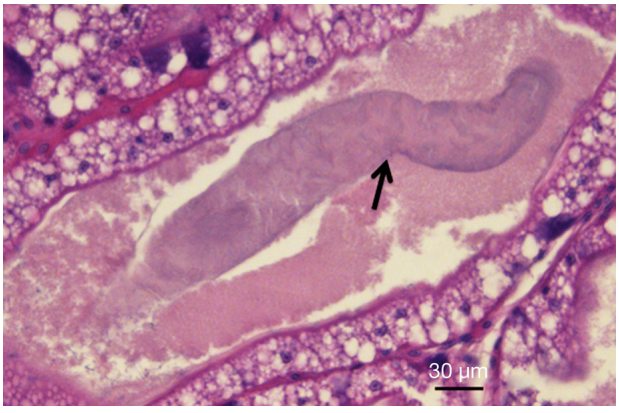


Fig. 5. Histological section of hepatopancreas from *Litopenaeus vannamei* after immersion challenge. Transversal view of tubules with highly condensed aggregated transformed microvilli in a tubule lumen (black arrow). H&E stain. Scale bar = 30 µm

A concentration of $10^8$ CFU ml$^{-1}$ *V. parahaemolyticus* was enough to produce 100% mortalities in *Litopenaeus vannamei*, and after 2 d of immersion, the same histological alterations were observed in the HP, gills, lymphoid organs and intestine as those reported by Tran et al. (2013) for AHPNS. Wang et al. (2008) also obtained 100% mortalities after 11 d post infection with *Lactococcus* spp. in *Macrobrachium rosembergii*, where histological damage was detected in the HP, again with results similar to those of our study.

Results demonstrate that 90% of the shrimp survive when colloidal silver is included in the *V. parahaemolyticus* challenge. Vaseeharanet al. (2010) reported 29% mortalities when the shrimp were exposed to *V. harveyi* and silver nanoparticles (AgNps) were administrated, in comparison to 91% mortalities in those that did not receive AgNps, thus suggesting that both silver forms (colloidal or AgNps) present bactericidal properties.

Shrimp inoculated by immersion displayed erratic swimming, empty intestines, and pale HP, signs which indicated *V. parahaemolyticus* virulence, with mortalities recorded 2 h post-inoculation and all shrimp dying after 10 h. Following an immersion challenge test, Tran et al. (2013) achieved a bacterial density of approximately 2 × $10^8$ cells ml$^{-1}$ with 100% mortalities from a pure strain of *V. parahaemolyticus*, with a typical response of pale atrophied HP, empty stomachs and midguts confirming the presence of AHPNS.

Sloughed E, R, F and B cells from the proximal to the distal region of the HP tubules was evident in our experiment. According to Tran et al. (2013), similar damage was observed when AHPNS from a pathogenic strain of *V. parahaemolyticus* affected shrimp.

Bacteria found in the detritus from the culture system orally colonized the digestive tract, produced toxins and caused HP cellular dysfunction, destroyed the E, R, F and B cells, sloughed the tubular epithelium cells from the proximal to the distal region and produced inflammation, hemocytic infiltration and necrosis. Sriurairatana et al. (2014) reported that when AHPNS appears, similar vermiform structures (aggregated transformed microvilli, ATM) are found in the HP and intestine, as observed in our study. Multiple functions are attributed to the HP: it serves as temporary food storage, produces and synthesizes protein, is involved with secretion of digestive enzymes and absorption of nutrients and is capable of metabolizing lipids and carbohydrate; therefore, any injures to this important organ could cause mortalities (Ceccaldi 1997, Lehnert & Johnson 2002, McGaw & Curtis 2013).

The 19 mm diameter inhibitory halo observed with 125 mg $ml^{-1}$ neem infusion in this study coincides with the findings of Banerjee et al. (2013), who reported a 19.6 mm halo for *V. parahaemolyticus* and 14.8 mm for *V. alginolyticus* with neem juice, which suggests that neem juice is a bactericidal agent that has inhibitory effects against *Vibrio* in shrimp.

Shrimp fed twice a day with 0.5 ml $g^{-1}$ feed of colloidal silver, oregano and neem extracts in their diet demonstrated an inhibition effect on the bacterial growth with mortalities of 10, 24 and 36%, respectively, after 2 d post-immersion. This may contribute to a reduction in the use of antibiotics with possible resistance by shrimp to enrofloxacin (Hung-Chiao et al. 2015). Vibrios can survive in a wide variety of salinities (Morales-Covarrubias & Gómez-Gil 2014): the actual diversification of shrimp cultures from hypersaline (40 PSU) to low salinity water (2 PSU) cultures and the overuse of antibiotics demonstrate the importance of using natural therapeutic methods to reduce mortalities and support shrimp aquaculture. Bacterial resistance to antibiotics has caused serious problems in many shrimp farms in Latin America (Morales-Covarrubias et al. 2011). The results obtained in this study demonstrated the feasibility of plant and colloidal silver use as alternative bactericidal sanitary treatments and provide a background for further studies.

## CONCLUSIONS

Colloidal silver and oregano and neem infusions have the capacity to inhibit growth of the pathogenic bacteria of shrimp. This study presents colloidal silver and plant infusion (oregano and neem) as alternative therapeutic treatments for the prevention of *Vibrio parahaemolyticus*, which causes AHPNS in shrimp culture.

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