Investigation of antibacterial activity against some *Vibrio strains* of red Areca nut (*Cyrtostachys renda*) ethanol extract

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Abstract
The Arecaceae family is known for its medicinal properties containing many natural substances with antibacterial activity. In this study, the seeds of red Areca (Cyrtostachys renda) belonging to the Arecaceae family were extracted from ethanol to preliminarily survey the chemical composition and to evaluate the antibacterial ability of the species Vibrio parahaemolyticus, Vibrio cholerae and Vibrio vulnificus. All these species cause acute hepatopancreatic necrosis disease (AHPNS) and white feces disease (WFD) in shrimp. The research results showed that the red Areca nut extract contains active ingredients such as phenol, tannin and flavonoid. The minimum inhibitory concentration (MIC) of the red Areca nut extract against V. cholerae, V. parahaemolyticus and V. vulnificus was 12.50 mg/ml, 3.13 mg/ml and 6.25 mg/ml respectively and the minimum bactericidal concentration (MBC) was 200.00 mg/ml, 16.67 mg/ml and 10.42 mg/ml respectively.

The results of the survey on the increase of V. cholerae, V. parahaemolyticus and V. vulnificus in TSA medium supplemented with red Areca nut ethanol extract over time (Time-kill curve assay) showed that red Areca nut extract had the best resistance to V. parahaemolyticus strain and completely killed V. parahaemolyticus at MIC concentration (3.13 mg/ml) after 2 hours of culture in living medium. Next, at 0.25MIC concentration (1.56 mg/ml), red Areca nut extract could completely kill V. vulnificus after 4 hours of culture in liquid medium. Finally, at MIC concentration (12.50 mg/ml), red Areca nut extract has the ability to completely destroy V.cholerae after 2 hours of culture in liquid medium.

Keywords: Red Aareca nut, *Cyrtostachys renda*, antibacterial, time-kill curve assay, *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus.*

Introduction

The seafood production industry in Vietnam, especially shrimp farming, has been growing strongly in recent years, playing an important role in increasing the country's export turnover. Along with the development of shrimp farming, the detection of epidemic diseases is a worrying issue because it causes serious economic damage and greatly affects the yield and quality of shrimp. The most common diseases that cause the greatest damage to shrimp today are acute hepatopancreatic necrosis syndrome (AHPNS) and white feces disease (WFD). This disease causes the destruction of the liver and intestine of shrimp. The disease will spread very quickly and lead to the death of a series of farmed shrimp in a short time, causing discomfort and danger to the economy and the farming ecosystem.

One of the causes of white feces disease in shrimp is the appearance of bacteria *Vibrio* at high concentrations and different proportions such as: *Vibrio parahaemolyticus* (40.48%), *Vibrio vulnificus* (6.25%), *Vibrioimius* (5.97%), *Vibrio damsel* (2.44%), *Vibrio anguillarum* (2.98%), *Vibrio cholerae* (18.75%), *Vibrio harveyi* (2.71%), *Vibrio splendens* (7.88%), *Vibrio fluvialis* (3.26%) and *Vibrio alginolyticus* (9.23%)[21] . Acute hepatopancreatic necrosis syndrome has been studied to be caused by *Vibrio parahaemolyticus*, which resides in the shrimp stomach and releases toxins that cause massive shedding of epithelial cells leading to shrimp death[5].

The antibiotics abusing in the prevention and treatment of shrimp diseases can cause antibiotic resistance and antibiotic residues in the product when the shrimp is processed. This not only causes difficulties in disease treatment but also affects the export of farmed shrimp to the world market. Therefore, the aquaculture industry is tending to use biological products derived from natural herbs to replace the use of antibiotics. Arecaceae is one of the large families containing many species with natural compounds with antibacterial activity; red Areca (*Cyrtostachys renda*) is also a species of the Areca family but has not been exploited and studied much. In this study, the nuts of red Areca were selected to examine the ability to resist some pathogenic *Vibrio* bacteria on shrimp attack, aiming to apply red Areca nuts as a source of herbal medicine in shrimp farming to limit the use of drugs and antibiotics.

Material and Methods

Materials: *Vibrio vulnificus* ATCC 27562 PK/5 and *Vibrio parahaemolyticus ATCC 17802 PK* /5 were provided by My An Co. Ltd., Vietnam. *Vibrio cholerae* bacteria were provided by the Laboratory of Plant Biotechnology, Faculty of Biology - Biotechnology, University of Science - Vietnam National University, Ho Chi Minh City. Red Areca nuts

(*Cyrtostachys renda)* were harvested in Tien Giang province, Vietnam.

Preparation of ethanol extract: Red Areca nuts were harvested, the shells were peeled off to get the nuts and the nuts were dried away from sunlight until the mass remains constant. Red dried Areca nuts were ground into a fine powder and soaked in ethanol at room temperature. After 3 days, the extraction was filtered and evaporated to get the extract. Continue the soaking process a few more times until the sample was completely extracted. Ethanol extract was stored in dark conditions in the refrigerator. The test sample was mixed in absolute ethanol at a concentration of 1 mg/ml[1].

Identification of phenol using $FeCl_3$: Add 1 ml of 5% $FeCl_3$ solution to 1 ml of the liquid to be tested. Blue-black color confirms phenols.

Identification of quinones and coumarins using Bortrager's reagent with KOH: Add 1 ml of 5% KOH solution in methanol to 1 ml of the solution to be tested. Quinones and coumarins will give red, purple-blue or green colors.

Tannin identification: Add 1 ml of the solution to be tested to the mixture of NaCl (5 g), gelatin (0.5 g) dissolved in 100 ml of water. Positive reaction with tannin gives light yellow sediment, turning brown after a while.

Alkaloid identification: put a mixture of 1 ml of test solution and 1 ml of 1% sulfuric acid into a test tube to confirm alkaloid identification using Wagner's reagent. Dissolve 1.27 g I_2 and 2 g KI in 20 ml of tap water; mix the two solutions, add tap water to make 100 ml; drop 0.2 ml of reagent into the acid solution. The sample containing alkaloid will appear in the brown cabinet.

Flavonoids identification: Add 0.5 ml of concentrated H_2SO_4 to a 1 ml test tube of the test solution; flavones and flavonols give a deep yellow to orange color and have fluorescence; chalcones and auron give a deep red to blue-red color; flavanones give an orange to red color.

Saponin identification: Prepare 2 tubes, tube 1 contains 5 ml of 0.1N HCl (pH=1), 0.3 ml of sample test solution. Tube 2 contains 5 ml of 0.1N NaOH (pH=13), 0.3 ml of sample test solution; shake the mouth of the tube vigorously for 1 minute and allow to stand. Observe the bubble column in both tubes: the bubble column in both tubes is of equal height and the bubble has the same stability, the sample has triterpenoid saponin. Tube at pH =13 has a higher bubble column than tube at pH=1, then the sample has steroid saponin.

Survey of biological activities of *V. cholerae, V. parahaemolyticus, V. vulnificus* of red Areca nut extract by the dilution method to determine MIC and MBC values: MIC (Minimum inhibitory concentration) of an antimicrobial agent is the lowest concentration needed to stop a microorganism from developing noticeably throughout the course of an overnight incubation period. MBC (Minimum bactericidal concentration) of an antibacterial agent is the lowest concentration at which the bacteria are completely destroyed[4]. *V. cholerae* was cultured in TSA medium (Tryptic Soya Agar, Hi Media) at pH 7.3 ± 0.2. *V. parahaemolyticus* and *V. vulnificus* were cultured in TSA medium supplemented with 2% NaCl at pH 7.3 ± 0.2.

Tetracycline antibiotic (positive control) was dissolved in sterile water and the red Areca nut ethanol extract was dissolved in 3% DMSO. The test was carried out on 96-well plates. Before adding 100 µl of each test material to the wells, they were all sterile-filtered. The bacterial solution was adjusted to OD_{625} nm ≈ 0.1, equivalent to a bacterial density of ~10^8 cfu/ml, then diluted to achieve a density of ~10^6 cfu/ml. Next, 100 µl of the 10^6 cfu/ml bacterial solution was added to the 96-well plate. After incubating the test substance in the 96-well plate under optimal conditions at room temperature for 24 hours, the MIC value was determined using resazurin reagent (0.015%).

The reagent will turn from its original blue color to a light purple color at the MIC concentration, while values below the MIC will be dark pink. To find the MBC value, the dark blue inoculations at concentrations greater than the MIC value will be passed through the agar. The plates will be incubated for 24 hours and the value at which no live colonies are found will be recorded[11].

Investigation of the growth of *V. vulnificus, V. cholerae* and *V. parahaemolyticus* in TSA medium supplemented with red Areca nut ethanol extract over time (Time-kill curve assay): The best technique for determining bactericidal or fungicidal action is the growth curve (Time-kill curve). It is a powerful tool to collect information about the interaction between an antibacterial agent and a microorganism. Growth curve shows that the antibacterial effect is time- or concentration-dependent[6].

The initial bacterial density was 10^5 cfu/ml and was cultivated in a total of 10 ml of wild medium including the test substance. The ethanol extract of red Areca nut was dissolved in 3% DMSO and tetracycline antibiotic (positive control) was dissolved in stored water. The negative control was 3% DMSO mixed with stored water. All test substances were filtered through a sterile filter. At the MIC concentration, the test samples were introduced to the culture flask[6].

The culture system was operated and optimized at room temperature. At different incubation times (0, 4, 6, 8, 10, 12 and 24 h), 100 µl of the culture solution was dusted and spread on TSA agar medium for cultivation. To calculate the number of viable cells (cfu/ml) at the time of survey, the number of single colonies was counted following a 24-hour incubation period.

Statistical analysis: Data obtained from the tests were statistically processed using SPSS 20.0 and Microsoft Office Excel 2010 software. Experiment results were displayed as mean ±SD values.

Results and Discussion

Qualitative determination of the presence of some functional groups in the ethanol extract of red Areca nut by chemical reaction characterization: The results of determining the presence of functional groups in the ethanol extract of red Areca nut are presented in table 1 and figure 1.

The results in table 1 show that the nuts of the red Areca contain phenols, tannins and flavonoids. Of the two different reagents for qualitative flavonoid determination, only the concentrated H_2SO_4 reagent gave a positive result with a deep red color, indicating that the red Areca contains flavanoids. The results also show that the red Areca does not contain quinones, coumarins, alkaloids and saponins. Another study showed that the ethanol extract of the leaves of the red Areca also contains flavonoids, tannins and steroids[7]. Therefore, it can be seen that both the nuts and leaves of the red Areca contain flavonoids and tannins.

Investigation of biological activities of *V. vulnificus, V. cholerae* and *V. parahaemolyticus* of red Areca nut extract by the dilution method to determine MIC and MBC values: The results of the investigation presented in table 2 showed that for positive control, tetracycline had the best inhibitory effect on the growth of *V. vulnificus* (MIC 0.0002 mg/ml) followed by *V. parahaemolyticus* (MIC 0.0004 mg/ml) and finally *V. cholerae* (MIC 0.0008 mg/ml). Meanwhile, the red Areca nut extract had the lowest MIC value (3.13 mg/ml) against *V. parahaemolyticus* strain, meaning it had the best ability to inhibit the growth of this host strain followed by *V. vulnificus* (6.25 mg/ml) and finally *V. cholerae bacteria* (12.5 mg/ml). When considering the MBC value for the classification level, red Areca nut completely killed both *V. parahaemolyticus* and *V. vulnificus* strains at similar concentrations and was better than *V. cholerae* strain.

According to Mogana et al[10], the MBC/MIC ratio can be used to assess the extract's antibacterial activity. The extract is thought to have a bactericidal effect if the MBC/MIC ratio is less than 4 and an inhibitory effect on bacteria if the ratio is greater than 4. Based on the results in table 2, the red Areca nut extract has a complete killing effect on *V. vulnificus* (MBC/MIC = 1.67), but against *V. cholerae* (MBC/MIC = 16) and *V. parahaemolyticus* (MBC/MIC = 5.33), it had inhibitory effect. This result shows that the antibacterial ability of red Areca nut extract against different *Vibrio* species is not the same.

Among the three *Vibrio strains* examined in this study, red Areca nut extract had the best inhibitory activity against *V. parahaemolyticus* (MIC 3.13 mg/ml, MBC 16.67 mg/ml). In addition, when comparing this result with the *V. parahaemolyticus* antibacterial activities of other published plant species, red Areca nut extract also has higher activity than leaf extract of others such as: *Azadirachta indica* and *Lippia berlandieri* (MIC 62.5 mg/ml, MBC 125 mg/ml)[12], *Phyllanthus amarus* (MIC 250 mg/ml, MBC 1000mg/ml)[14], *Azadirachta indica* (MIC 6.5 mg/ml, MBC 7 mg/ml)[9], *Foeniculum vulgare* (MIC 4 mg/ml, MBC 8 mg/ml)[3], *Myrtus communis* (MIC 200 mg/ml, MBC 200 mg/ml)[16], *Punica granatumv* (MIC 10 mg/ml)[17] and *Satureja bachtiarica Bunge* (MIC 12.5 mg/ml and MBC 25 mg/ml)[16].

Table 1
Qualitative results of the presence of some functional groups in the red Areca nut extract.

Functional group	Reagents	Red areca nut
Phenol	$FeCl_3$	+ (a dark blue)
Quinone, coumarin	Bortrager with KOH/methanol	-
Tannin	Gelatin + NaCl	+ (light yellow)
Alkaloid	Wagner	-
Flavonoids	Concentrated H_2SO_4	+ (dark red)
	NaOH 1%	-
Saponin	Foaming test with 0.1N HCl	-
	Foaming test with 0.1N NaOH	-

Note: (-): None. (+): Yes

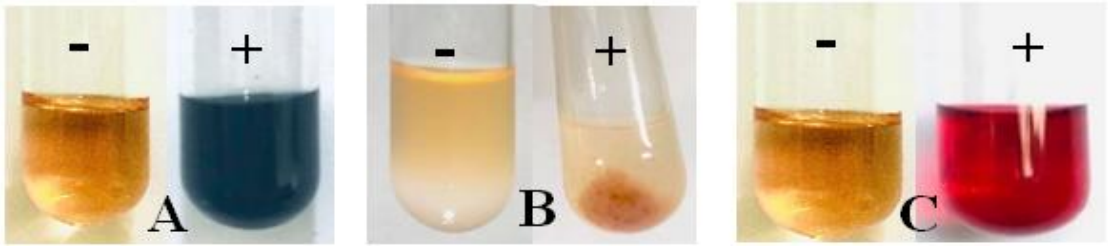

Figure 1: Ethanol extract of red Areca nut reacts positively with compounds such as (A) phenol, (B) tannin and (C) flavonoid. (-): negative control, (+): positive control.

Higher *V. parahaemolyticus* inhibitory activity than essential oil belongs to *Cuminum cyminum* nuts (MIC 12 mg/ml and MBC 25, mg/ml)[16] and *Allium sativum L.* bulb extract (MIC 25 mg/ml, MBC 25 mg/ml)[15]. Red Areca nut extract has lower inhibitory activity against *V. vulnificus* (MIC 6.25 mg/ml, MBC 10.42 mg/ml) than *V. parahaemolyticus* (MIC 3.13 mg/ml, MBC 16.67 mg/ml). On the other hand, when comparing red Areca nut with other plant species, the inhibitory activity against *V. vulnificus* of red Areca nut extract (MIC 6.25 mg/ml, MBC 10.42 mg/ml) was higher than that of *Allium sativum L.* bulb extract (MIC 8.33 mg/ml, MBC 12.5 mg/ml)[15], close to that of *Carica papaya* seed extract (MIC, 5.63 mg/ml) [13] and lower than that of *Alpinia galanga Linn.* extract (MIC 1.25 mg/ml, MBC 1.25 mg/ml)[22].

For *V. cholerae* strain (MIC 12.5 mg/ml, MBC 200 mg/ml), red Areca nut extract had a lower inhibitory activity than two strains *V. parahaemolyticus* and *V. vulnificus.* However, the inhibitory activity of red Areca nut extract against *V. cholerae* is still higher than the extracts of other plant species such as leaves of *Spondias mombin* (MIC 83.13 mg/ml)[18], leaves of *Albizia lebbeck* (MIC 24 mg/ml)[2] and *Allium cepa tubers* (MIC 19.20 mg/ml)[8]. In addition, the inhibitory activity of *V. cholerae* of red Areca nut extract also is the same with the extracts of *Zataria multiflora* leaves (MIC 12.5 mg/ml) [20] and *Allium sativum* L. tubers (MIC 12.5 mg/ml)[15].

In summary, the results showed that red Areca nut is a potential medicinal source in the treatment of diseases related to *Vibrio* strains that cause disease in shrimp. In particular, red Areca nut extract has the best inhibitory activity against *V. parahaemolyticus* comparing with the other surveyed *Vibrio* strains. This has demonstrated the potential value of red Areca nut in the treatment of shrimp diseases because *V. parahaemolyticus* is also the bacteria that causes acute hepatopancreatic necrosis disease (AHPNS)[5]. *V. parahaemolyticus* also causes the highest level of disease (40.48%) compared to other *Vibrio* that cause disease in shrimp[21]. In addition to this study, on the anti -*Vibrio* activity of red Areca nut, there is another study on the anti- *Staphylococcus aureus* ability of ethanol extract of red Areca leaves[7]. Therefore, further investigation of the antibacterial activity of red Areca nut on other bacterial species may yield very interesting results.

Investigation of the growth of *V. vulnificus*, *V. cholerae* and *V. parahaemolyticus* in TSA medium supplemented with red Areca nut ethanol extract over time (Time-kill curve assay):

Growth curve of Vibrio cholera as in table 3 and figure 2 displays the *V. cholerae* growth curve in TSA medium supplemented with 0.25MIC and MIC red Areca nut extract. In the negative control, the number of living cells decreased and a clear sign of the latent phase was observed when the bacteria were cultured in the nutrient medium for 0 to 2 hours.

Table 2
MIC and MBC results of 3 *Vibrio bacteria* for test substances

MIC and MBC	Bacterial strain	Tetracycline (mg/ml)	Red areca nut ethanol extract (mg/ml)
MIC	*V. cholerae*	0.0008[a]	12.50[a]
	V. parahaemolyticus	0.0004[b]	3:13[c]
	V. vulnificus	0.0002[c]	6.25[b]
MBC	*V. cholerae*	0.0063[a]	200.0[a]
	V. parahaemolyticus	0.0008[b]	16.67[b]
	V. vulnificus	0.0004[c]	10.42[b]
MBC/MIC	*V. cholerae*	7.88[a]	16.00[a]
	V. parahaemolyticus	2.00[b]	5.33[b]
	V. vulnificus	2.00[b]	1.67[c]

(Different samples represent significant differences (rows) at 95% confidence level)

Table 3
Log (cfu/ml) of viable cells of *V. cholerae* grown in TSA medium supplemented with red Areca nut at 0.25MIC and MIC concentrations.

Hour	Negative control	Tetracycline	MIC	0.25MIC
0	5.000 ± 0.000	5.000 ± 0.000	5.000 ± 0.000	5.000 ± 0.000
2	4.078 ± 0.162	4.408 ± 0.082	0.000 ± 0.000	3.087 ± 0.074
4	5.058 ± 0.526	4.208 ± 0.228	0.000 ± 0.000	2.997 ± 0.109
6	7.507 ± 0.638	3.951 ± 0.849	0.000 ± 0.000	4.815 ± 0.128
12	9.170 ± 0.502	4.420 ± 1.023	0.000 ± 0.000	6.017 ± 0.154
24	8.685 ± 0.596	6.735 ± 1.976	0.000 ± 0.000	9.601 ± 0.346

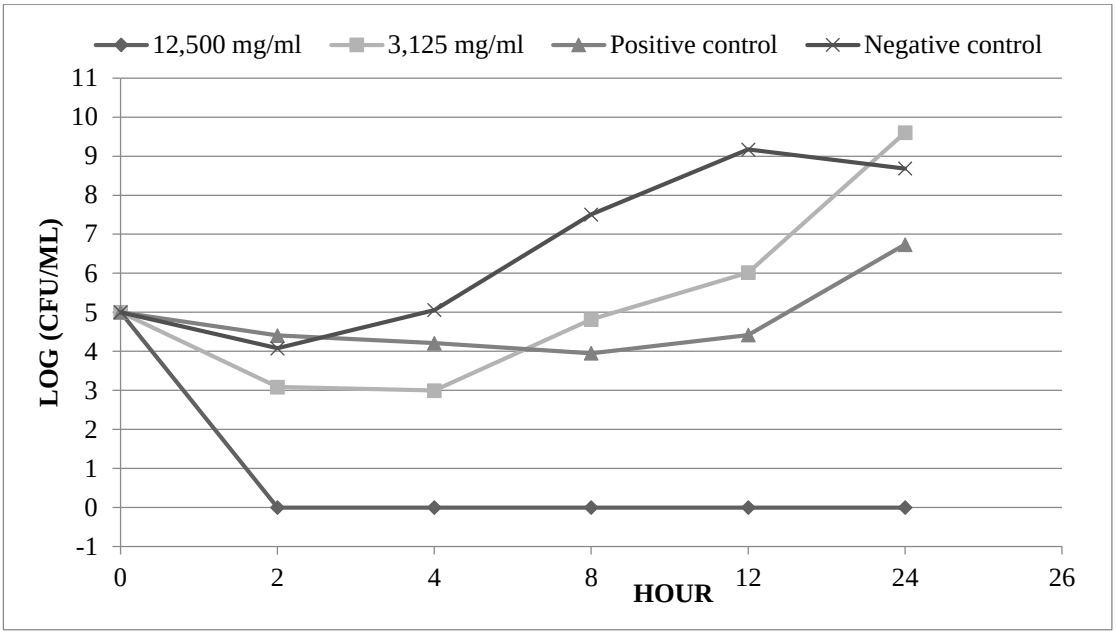

Figure 2: Growth curve of *Vibrio cholerae* in TSA medium supplemented with red Areca nut extract at concentrations of 0.25MIC and MIC.

Table 4
Log (cfu/ml) viable cells of *V. parahaemolyticus* were grown in TSA medium supplemented with red Areca nut extract at concentrations of 0.25MIC and MIC.

Hour	Negative control	Tetracycline	MIC	0.25MIC
0	5.000 ± 0.000	5.000 ± 0.000	5.000 ± 0.000	5.000 ± 0.000
2	3.688 ± 0.306	3.526 ± 0.093	0.000 ± 0.000	3.523 ± 0.023
4	4.474 ± 0.169	3.779 ± 0.421	0.000 ± 0.000	3.488 ± 0.007
6	6.129 ± 0.470	4.231 ± 0.664	0.000 ± 0.000	3.556 ± 0.004
12	7.790 ± 0.449	5.421 ± 1.816	0.000 ± 0.000	3.804 ± 0.113
24	8.357 ± 0.110	7.362 ± 0.238	0.000 ± 0.000	6.463 ± 0.055

The number of bacteria lost during this time was negligible and exhibited weak vitality, which is likely a result of their new surroundings. There was an increase in the number of bacteria between 2 and 12 hours which is indicative of the exponential phase. At 24 hours, the density of bacteria showed a decrease, indicating that *V. cholerae* began to enter the decline phase and *V. cholerae* had reached equilibrium in the time period after 12 hours before 24 hours.

For the positive control from 0 to 12 hours, bacterial growth showed inhibition of the tetracycline antibiotic. However, after 12 hours, the antibiotic, no longer had inhibitory activity against *V. cholerae*. Specifically, at 24 hours, the bacterial density in the antibiotic-containing medium was 6,735 log (cfu/ml), lower than the bacterial density in the nutrient medium without the inhibitor which was 8,685 log (cfu/ml). This result showed that at the MIC concentration, tetracycline had a low inhibitory effect on *V. cholerae*.

For red Areca nut extract, at MIC concentration, *V. cholerae* could not survive immediately after being cultured in the medium supplemented with red Areca nut extract. At 0.25 MIC concentration, the number of viable cells decreased in the medium from 0 to 4 hours, specifically at 4 hours log (cfu/ml) reached 2,997 compared to the growth curve of *V. cholerae* supplemented with antibiotics [4,208 log (cfu/ml)]. From 0 to 6 hours, the antibacterial ability of the extract was better than tetracycline antibiotics. After 6 hours, the bacterial density began to increase from 4,815 to 6,017 log (cfu/ml) at 12 hours, this value was still lower than the number of viable cells in TSA medium supplemented with excess inhibitors.

At 24 h, the regional density began to increase, indicating that the extract was no longer able to inhibit microflora growth after 24 h at 0.25MIC concentration. The results showed that red Areca nut extract at MIC concentration completely killed *V. cholerae* after 2 hours of culture.

Growth curve of Vibrio parahaemolyticus: Figure 3 and table 4 display the *V. parahaemolyticus* growth curve in TSA medium with red Areca nut extract added at 0.25MIC and MIC concentrations. For the negative control, when the bacteria were cultured in the nutrient medium, from 0 to 4 hours, the number of living cells decreased from 5 to 4,474 log (cfu/ml), the bacteria were in the latent phase.

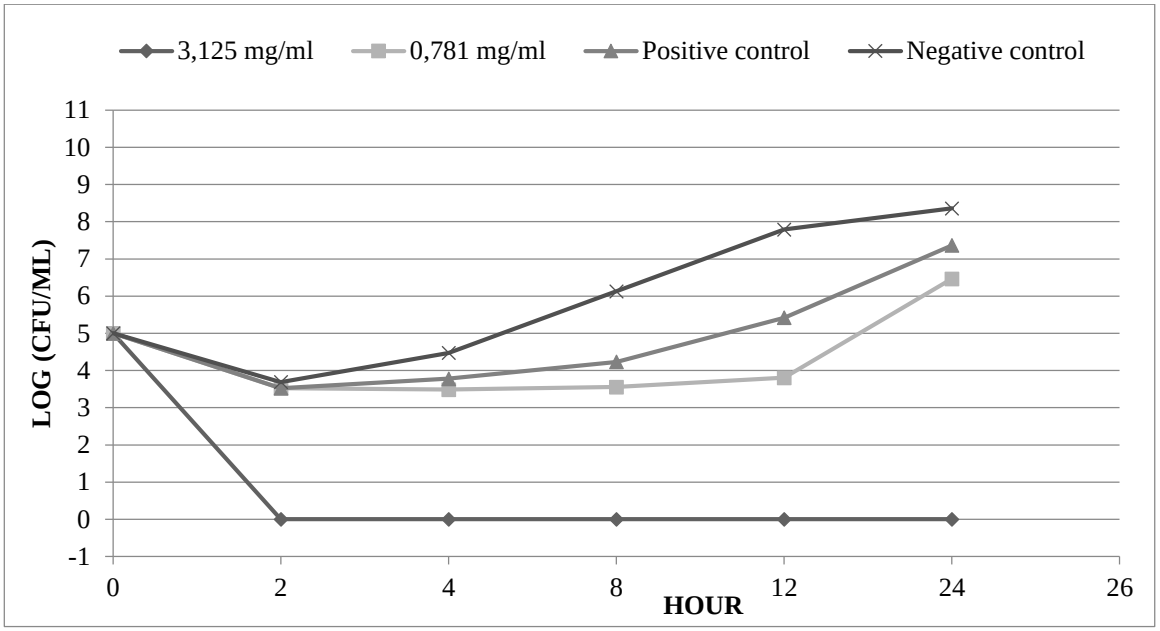

Figure 3: Growth curve of *Vibrio parahaemolyticus* in TSA medium supplemented with red Areca nut extract at concentrations of 0.25MIC and MIC.

From 4 to 12 hours, the density of *V. parahaemolyticus* began to increase, at 12 hours it was 7,790 log (cfu/ml), this is the stage when the bacteria were in the exponential phase. At 24 hours, the number of living cells reached 8,357 log (cfu/ml) with a slight drift to 12 hours, showing that *V. parahaemolyticus* was in the equilibrium phase.

For the positive sample, from 0 to 6 hours, the growth curve of *V. parahaemolyticus* showed inhibition by tetracycline antibiotics, with bacterial densities ranging from 5 to 4,231 log (cfu/ml). After 6 hours, the bacterial density began to increase but was not as high, indicating that the antibiotic was no longer able to inhibit bacteria as in the first hours and the bacteria had begun to adapt to the environment. Specifically, at 24 hours, the bacterial density was 7,362 log (cfu/ml), still lower than the bacterial density grown in alkaline TSA medium (8,357 log (cfu/ml)) without adding inhibitors. This result showed that the antibiotic still had the ability to inhibit the low growth of *V. parahaemolyticus* at MIC concentrations in the first 12 hours.

For red Areca nut extract, at MIC concentration, after 2 hours, the bacteria were completely destroyed. At 0.25MIC concentration, from 0 to 2 hours the number of living cells decreased from 5 to 3,523 log (cfu/ml), at this time the bacteria were not suitable for the environment. From 2 to 12 hours, the bacterial density was approximately the same, it can be said that the log value (cfu/ml) did not change, showing that the extract had the ability to inhibit the growth of *V. parahaemolyticus*. After 12 to 24 hours, the bacterial density began to increase again because the inhibitory effect of the extract began to decrease, at 24 hours it reached 6,463 log (cfu/ml) lower than the bacterial density in the environment supplemented with tetracycline (7,362 log (cfu/ml)). These results showed that red Areca nut extract has the ability to inhibit the growth of *V. parahaemolyticus* better than tetracycline antibiotic at MIC and 0.25MIC concentrations.

The results showed that red Areca nut extract at MIC concentration completely killed *V. parahaemolyticus* after 2 hours of culture and at 0.25MIC concentration inhibited the growth of *V. parahaemolyticus* for 12 hours.

Growth curve of Vibrio vulnificus: Figure 4 and table 5 displayed the *V. vulnificus* growth curves in TSA medium with red Areca nut extract added at 0.25MIC and MIC concentrations.

For the negative control sample, from 0 to 4 hours, the number of living cells decreased, the bacteria were in the latent phase. From 4 to 12 hours, the bacterial density increased from 4.391 to 8.042 log (cfu/ml), *V. vulnificus* entered the exponential phase. From 12 to 24 hours, it can be seen that the bacteria were in the steady state phase, making the bacterial density at 24 hours 8.534 log (cfu/ml) with no significant difference compared to 12 hours.

For the positive control sample, *V. vulnificus* showed signs of growth inhibition even when the medium contained tetracycline. Specifically, at 12 hours, the density of live cells decreased compared to 0 hours, decreasing from 5 to 3.765 log (cfu/ml). At 24 hours, the number of live cells was 4.169 log (cfu/ml), which increased compared to 12 hours but not significantly, this value was still lower than at 0 hours, showing that the antibiotic was able to inhibit the growth of *V. vulnificus* within 24 hours.

For red areca nut extract, at MIC concentration, after 2 hours, the bacteria could not survive. At 0.25 MIC concentration, from 0 to 2 hours the number of viable cells began to decrease from 5 to 3.486 log (cfu/ml), this result

showed that the extract had the ability to inhibit the growth of V. vulnificus in the first 2 hours. After 2 hours - 24 hours, the bacterial density began to decrease gradually to 0 at 4 hours, the growth curve showed the complete destruction of bacteria by the extract. This result showed the ability to completely destroy *V. vulnificus* bacteria at 0.25MIC and MIC concentrations of red areca nut extract.

The results showed that red Areca nut extract at a concentration of 0.25MIC completely killed *V. vulnificus* after 2 hours of culture.

In summary, from the results of the study of bacterial growth over time (Time-kill curve test), it was found that the red Areca nut extract was able to completely kill 3 strains of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* at MIC concentration after 2 hours of culture in TSA medium. At a concentration of 0.25MIC, the inhibitory ability of red Areca nut extract against *V. cholerae* and *V. parahaemolyticus* only lasted for 6 to 12 hours. *V. vulinificus* the extract was able to kill bacteria after 4 hours. Meanwhile, tetracycline antibiotics at MIC concentration were not able to kill all 3 strains of these bacteria under the same culture conditions. The antibiotic tetracycline was only able to inhibit *V. vulnificus* within 24 hours of the survey and was not able to inhibit *V. cholerae* and *V. parahaemolyticus well* after 12 hours at MIC concentration.

In particular, red Areca nut extract has the best resistance to *V. parahaemolyticus* strains with an MIC value of 3.13 mg/ml which was able to completely destroy *V. parahaemolyticus* after 2 hours of culture in liquid TSA medium.

Besides, for *V. vulnificus* strain, with 0.25MIC value of 1.56 mg/ml, red Areca nut extract can completely kill *V. vulnificus* after 4 hours of culture in TSA medium. Therefore, this study has contributed in demonstrating the potential of using red Areca nut as a raw material to produce preparations to treat acute hepatopancreatic disease (AHPNS) and white feces disease (WFD) in shrimp caused by *Vibrio* bacteria.

Table 5
Log (cfu/ml) viable cells of *V. vulnificus* were grown in TSA medium supplemented with red Areca nut extract at concentrations of 0.25MIC and MIC.

Hour	Negative control	Tetracycline	MIC	0.25MIC
0	5.000 ± 0.000	5.000 ± 0.000	5.000 ± 0.000	5.000 ± 0.000
2	3.924 ± 0.644	3.724 ± 0.106	0.000 ± 0.000	3.486 ± 0.016
4	4.391 ± 0.279	3.443 ± 0.276	0.000 ± 0.000	0.000 ± 0.000
6	6.649 ± 0.503	3.611 ± 0.227	0.000, ± 0.000	0.000 ± 0.000
12	8.042 ± 0.512	3.765 ± 0.227	0.000 ± 0.000	0.000 ± 0.000
24	8.534 ± 0.594	4.169 ± 0.402	0.000 ± 0.000	0.000 ± 0.000

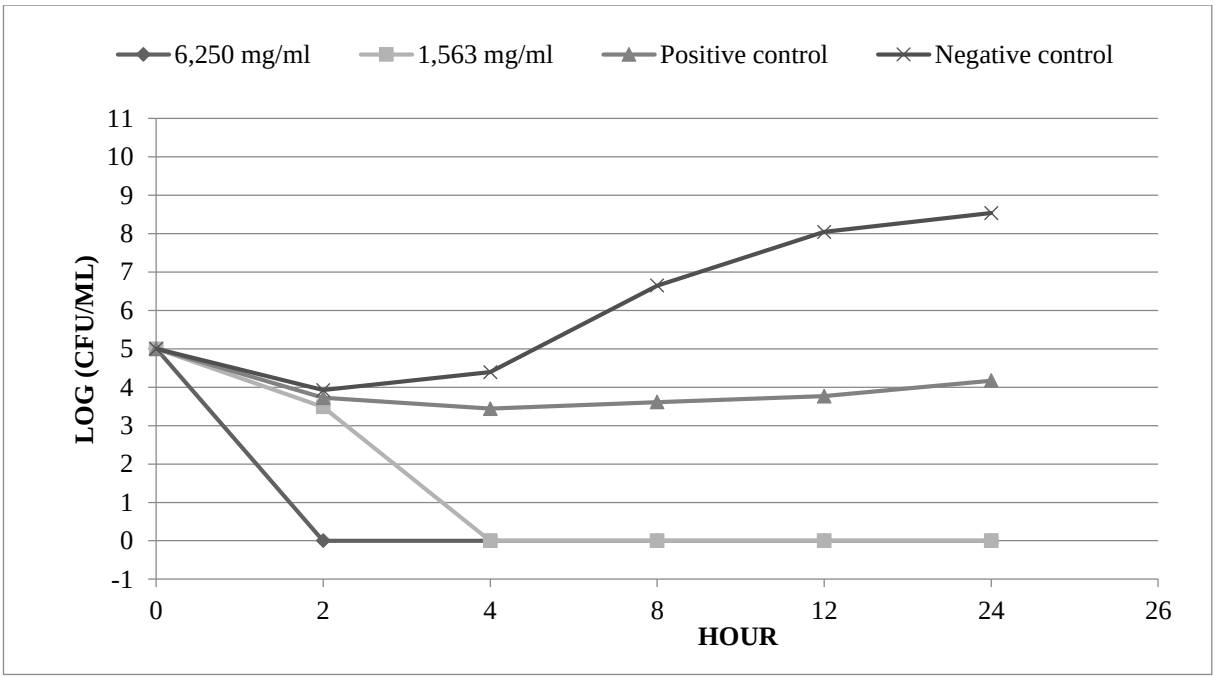

Figure 4: Growth curve of *Vibrio vulnificus* in TSA medium supplemented with test substances at concentrations of 0.25 MIC and MIC.

Conclusion

The research results showed that the red Areca nut ethanol extract contains phenolic compounds, tannins and flavonoids. This extract has the best antibacterial activity on *V. cholera*, *V. parahaemolyticus* and *V. vulnificus* with MIC concentrations of 12.5 mg/ml, 3.125 mg/ml, 6.25 mg/ml and MBC concentrations of 200 mg/ml, 16.67 mg/ml, 10.42 mg/ml respectively. From the results of the study of bacterial growth over time (Time-kill curve test), it was shown that the red Areca nut ethanol extract has good antibacterial activity against all 3 species of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus.*

The red Areca nut extract had the best resistance against *V. parahaemolyticus* (MIC was 3.13 mg/ml) and was able to completely kill *V. parahaemolyticus* after 2 hours of culture in TSA-enhanced medium. For *V. vulnificus* (0.25 MIC was 1.56 mg/ml), the red Areca nut extract was able to completely kill *V. vulnificus* after 4 hours of culture in TSA-liquid medium. Meanwhile, the positive control is tetracycline that only inhibits *V. cholera* and *V. parahaemolyticus* in the first 12 hours and *V. vulnificus* in the 24 hours of the survey. Clearly, red Areca nut is a potential raw material that can be exploited as a medicinal resource to treat diseases caused by *Vibrio*, especially in aquatic products.

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