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Effect of bacteriophages and chamber bitter (*Phyllanthus amarus*) in combination on *Vibrio parahaemolyticus*

Truong Thi Bich Van[1]*, Do Tan Khang[1], Le Viet Dung[2], Le Hoang Bao Ngoc[1], Nguyen Pham Anh Thi[1], Tran Thanh Men[3], Vo Ngoc Tram Anh[1], Nguyen Kim Thoa[1]

[1]Institute of Food and Biotechnology Research and Development Institute, Can Tho University, Can Tho, Vietnam.
[2]College of Agriculture, Can Tho University, Can Tho, Vietnam.
[3]College of Natural Sciences, Can Tho University, Can Tho, Vietnam.

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ABSTRACT

Vibrio parahaemolyticus is one of the causes of acute hepatopancreatic necrosis disease or early mortality syndrome in shrimps. Frequent and repeated application of antibiotics leads to generating resistant strains. Bacteriophages and plant extracts are considered potential biological agents in precaution and treatment of bacterial diseases in aquaculture. The study aimed to investigate the effects of bacteriophages and *Phyllanthus amarus* extract on strains of *V. parahaemolyticus* bacteria that were isolated from white leg shrimps (*Litopenaeus vannamei*). In this study, two strains of *V. parahaemolyticus* were used: B4XT4 (pure strain) and B4X0T2.2 (isolated strain) for preparing four treatments consisting of combining the bacteria with *P. amarus* extracts (8 mg/mL), bacteriophage (10^6 PFU/mL), a combination of *P. amarus* and bacteriophage, and a control treatment. The experiment was conducted in triplicate. The results showed that only plant extracts could inhibit bacterial growth. The colonial morphology of bacteria was changed when bacteriophages and *P. amarus* extract were added.

1. INTRODUCTION

Vibrio parahaemolyticus has been one of the causes of acute hepatopancreatic necrosis disease or early mortality syndrome in shrimps in recent years. Using antibiotics or chemicals to inhibit bacteria has been the most popular course of treatment due to the initial benefits they bring. However, the long-term use of antibiotics leads to antibiotic resistance in bacteria, which is still a challenge in the aquaculture industry and the field of shrimp farming. The research on the inhibition of *V. parahaemolyticus* using biological therapy is being considered. The method of using phages against aquatic pathogenic bacteria was first used in Japan [1] and quickly became the subject of scientific interest. In recent years, there have been a number of studies using phages in the prevention and treatment of plant and animal diseases [2-4]. Besides, there are also many studies on plant extracts with high antimicrobial efficiency [5], especially *Phyllanthus amarus* [6,7]. Bacteriophages and *P. amarus* are considered potential biological agents in the treatment of bacterial diseases in aquatic animals. At present, research and potential applications of these agents in practice are underway, but studies in our country that involve their combined use are still limited. Therefore, the study of the ability of the phage in combination with the extract of this plant to inhibit the *V. parahaemolyticus* bacteria serves as a scientific basis for herbal applications in aquaculture in general and shrimp farming. The objective of this study was to investigate the interaction of phage and *P. amarus* extract and their effectiveness in inhibiting the spread of the pathogenic *V. parahaemolyticus* on white leg shrimp.

2. MATERIALS AND METHODS

2.1. Materials

All chemicals, equipment, and bacteriophages were supplied by the Molecular Biology Laboratory, Biotechnology Research and Development Institute, Cantho University. The methanolic extract of *P. amarus* (all the body) was obtained by the College of Natural Sciences, Cantho University. Bacteria strains were isolated from diseased shrimps, pond water, and mud in the Mekong Delta, Viet Nam.

2.2. Isolation of *Vibrio* spp.

Bacteria were isolated from diseased shrimps, pond water, and mud in the Vinh Thuan district, Kien Giang, Bac Lieu Provinces. Diseased shrimps were collected and dissected for hepatopancreas and intestines and were homogenized in a tryptic soy broth (TSB) medium

*Corresponding Author:
Truong Thi Bich Van,
Institute of Food and Biotechnology Research and Development Institute, Can Tho University, Can Tho, Vietnam.
E-mail: ttbvan @ ctu.edu.vn

supplemented with 1.5% NaCl and spread on a thiosulfate citrate bile salt sucrose (TCBS) medium. Water and mud samples were added to a TSB medium supplemented with 1.5% NaCl, incubated for 24 h, and spread on a TCBS medium. Green colonies were selected for further study.

2.3. Identification of *V. parahaemolyticus* by PCR

V. parahaemolyticus was identified using two sets of primers. The amplification of the 16S rRNA gene with 700 bp amplicons using primer F (5'-CAGGCCTAACACATGCAAGTC-3') and primer R (5'-GCATCTGAGTGTCAGTATCTGTCC-3') was described by Mohamed *et al*. (2017) for identifying *Vibrio* spp. Strains that possessed the needed bands were then tested for their virulence properties using the ToxR gene F (5'- GTCTTCTGACGCAATCGTTG -3') and R (5'- ATACGAGTGGTTGCTGTCATG -3') which is specific for *V. parahaemolyticus* (Kim *et al*., 1999). The ToxR gene was amplified with 368 bp amplicons.

2.4. Double Layer Agar Technique

The test used the Double Agar Layer Technique. Two agar layers were prepared for 2 host tests: soft agar (0.3% agar, low agar concentration) was mixed with bacteria which was incubated in 24 h and overlaid on a hard agar layer (1.5% agar). The medium used in this study was King B supplemented with 0.5% NaCl. Afterward, 1 µL of bacteriophage was inoculated and incubated for 24 h to observe the plaques. The experiment was repeated once.

2.5. Plating Method

The experiment was conducted with four treatments where the extract and bacteriophage concentrations were used for testing, and they were 8 µg/mL and 20% of 10^6 PFU/mL, respectively, (treatment A consisted of 100 µL of bacteria; treatment B consisted of 100 µL of bacteria and 2 µL of extract; treatment C consisted of 100 µL of bacteria and 2 µL of bacteriophages; and treatment D consisted of 100 µL of bacteria, 2 µL of extract and 2 µL of bacteriophages).

The number and the morphology of colonies were recorded after 1 h, 3 h, and 24 h. The experiment was replicated 3 times, and the Stagraphic Centurion XVIII was used to analyze the data of a number of colonies. The size of colonies was observed using the CountPHICS software.

3. RESULTS

3.1. Isolation and Identification of *V. parahaemolyticus*

There were 48 bacterial strains that could grow on the TCBS media. All strains of bacteria were isolated from diseased shrimp with a morphological characterization similar to that of *Vibrio* spp. The colonies of these bacteria appeared circular, convex, and glossy. They were green in color and exhibited a diameter of about 1–2 mm on the TCBS medium after 24 h of incubation [8-10] [Figure 1]. For further confirmation, all the isolates were screened using 16S rRNA [Figure 2] and ToxR [Figure 3] primers, and were at 700 bp and 368 bp, respectively [11]. This study isolated 45 strains of *Vibrio* sp., including 8 strains of *V. parahaemolyticus* from diseased shrimp, pond water, and mud. B4XT4 and B4X0T2.2 strains were selected for the following experiments.

3.2. Evaluation of Host Range

The infectiveness of 5 bacteriophages against 2 strains of *V. parahaemolyticus* B4XT4 and *V. parahaemolyticus* B4X0T2.2 is noted in Table 1. It is noteworthy that phage VD3 and phage VD4 displayed the possibility of infecting both strains of *V. parahaemolyticus*, but strains phage VD1 and phage VD5 only infected *V. parahaemolyticus* B4X0T2.2. On the other hand, the strain phage VD2 could infect neither *V. parahaemolyticus* B4XT4 nor *V. parahaemolyticus* B4X0T2.2.

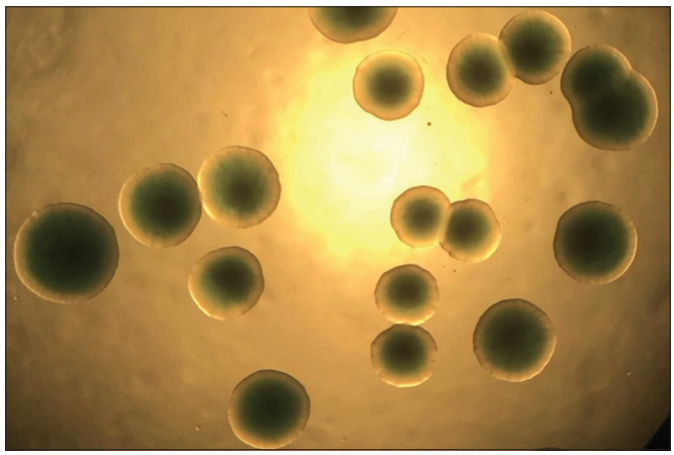

Figure 1: *Vibrio parahaemolyticus* colony morphology on TCBS agar.

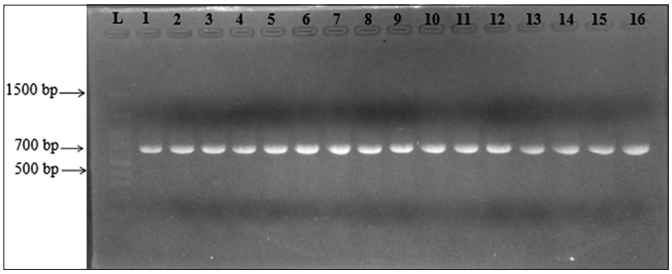

Figure 2: Gel electrophoresis of PCR products of 16S rRNA gene. (L: 1500bp ladder; 1: B4X0T2.1; 2: B4X0T2.2; 3: B4X0T2.3; 4: V4.1; 5: B4XCT1.1; 6: B4XCT1.2; 7: V4.2; 8: B4XCT1.2; 9: B4XCT1.5; 10: V4.3; 11: B3XD2; 12: V4.6; 13: B3DX4; 14: V4.7; 15: B3XD1; 16: V4.10).

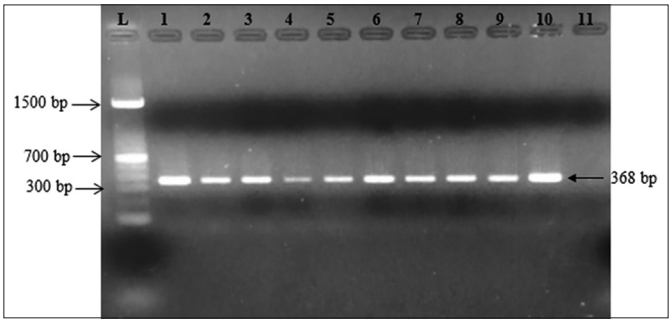

Figure 3: Gel electrophoresis of PCR products of toxR gene (L: 1500 bp ladder; 1: positive control; 2: B4X0T2.2; 3: B4X0T2.3; 4: B3XD2; 5: V4.1; 6: V4.2; 7: V4.3; 8: V4.6; 9: V4.6; 10: V4.10; 11: negative control).

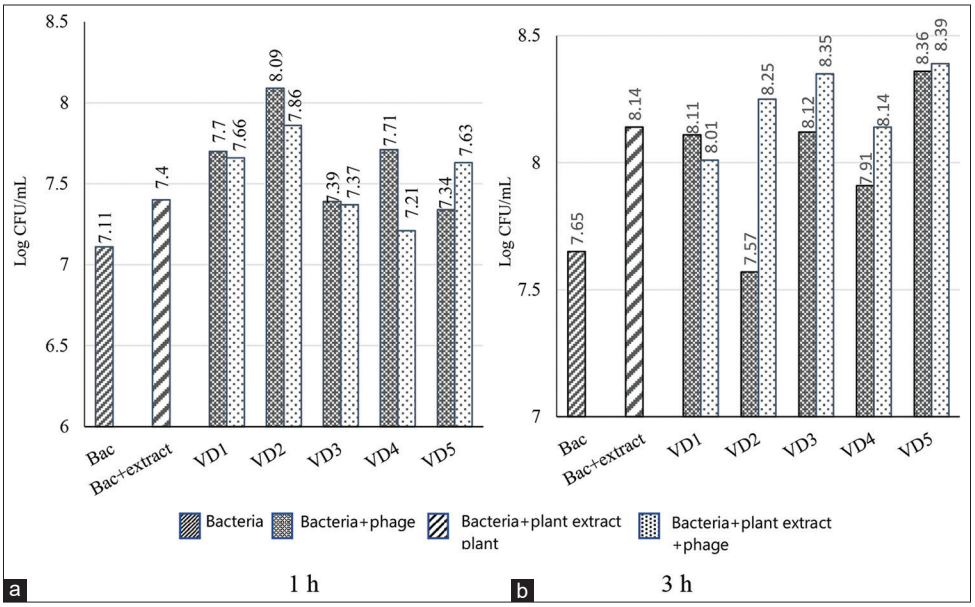

Figure 4: Interaction between extract and phage on bacterial population (a) affecting *Vibrio parahaemolyticus* B4XT4. (b) Affecting *Vibrio parahaemolyticus* B4X0T2.2.

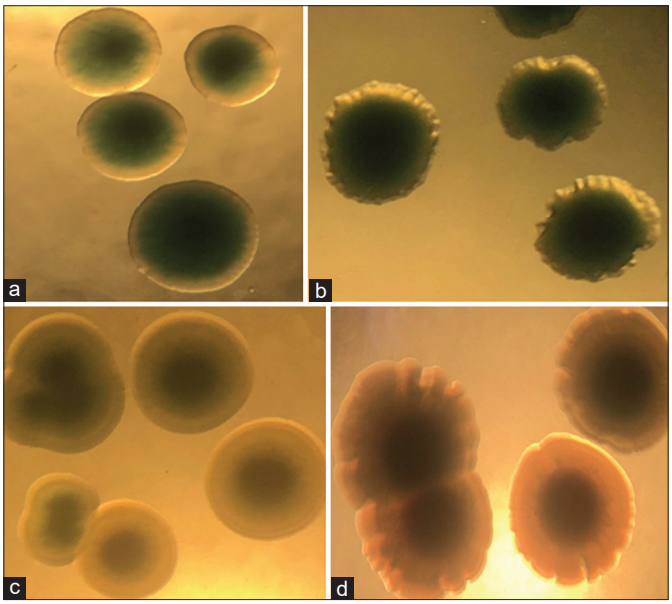

Figure 5: Effect of plant extract on colonies of *Vibrio parahaemolyticus* B4XT4 and B4X0T2.2. (a) B4XT4; (b) B4XT4+ plant extract; (c) B4X0T2.2; (d) B4X0T2.2+ plant extract.

3.3. Number of Colony Formation

The ability of the extract and phage to inhibit the growth of *V. parahaemolyticus* B4XT4 and *V. parahaemolyticus* B4X0T2.2 was different [Figure 4] after four treatments incubated at 1 h and 3 h. The number of colonies when adding extracts or phages both increased the number of bacteria compared to certificates. For *V. parahaemolyticus* B4XT4, when combining the phage and extract, the inhibitory effect on bacterial growth was more obvious with only the extract (the value decreased from 7.71 to 7.21 Log CFU/mL). For *V. parahaemolyticus* B4X0T2.2, it was the opposite, as adding the extract activated its growth. The study which was combined phage and *P. amarus* extract to inhibit the growth of *V. parahaemolytiucs*; however, initially there was no effect.

3.4. Effect of Plant Extract on the Colony of *V. parahaemolyticus*

Both strains of *V. parahaemolyticus* (B4XT4 and B4X0T2.2) were affected by the extract *P. amarus*, which changed the colony's phenotype. The extract affected the shape of colonies — it caused the edge of *V. parahaemolyticus* B4X0T2.2 shrink and dentate with sharp and small peaks, compared to the round colonies of the control [Figure 5a and b]. The cover of the colonies was toothed, and lobate margins arose for B4XT4 [Figure 5c and d]. With regard to the colonies of *V. parahaemolyticus* B4X0T2.2, the size is smaller but insignificant. The most significant change in colony size was observed during a sharp decrease of 4 mm^2 and 4.5 mm^2, which was a drop of 1.3 times and 3.0 times, respectively [Figure 6a and b]. The decline in colony size apparently occurred in *V. parahaemolyticus* B4XT4; indeed, a size of 6 mm^2 và 6.5 mm^2 is notable, with a drop of 1.2 times and 1.1 times, respectively [Figure 6c and d].

3.5. Changes in Colonial Morphology in the Presence of Bacteriophages

The colonial morphology of *V. parahaaemolyticus* B4XT4 and *V. parahaaemolyticus* B4X0T2.2, when infected with a phage, changed compared with the control. PhageVD1 infected *V. parahaemolyticus* B4XT4 and *V. parahaaemolyticus* B4X0T2.2, causing the colony to shrink and change its serration compared to the control [Figure 7].

3.6. Impact of Plant Extract and Phages on *V. parahaemolyticus*

A comparison of the colony sizes in treatment between phage VD1, that could infect both strains *V. parahaemolyticus,* and phage VD3, which could only infect B4XT4, was conducted. As expected, the colony sizes of the former experienced a remarkable decline [Figure 8], though the latter made *V. parahaemolyticus* B4X0T2.2 remain the size of the colony [Figure 9].

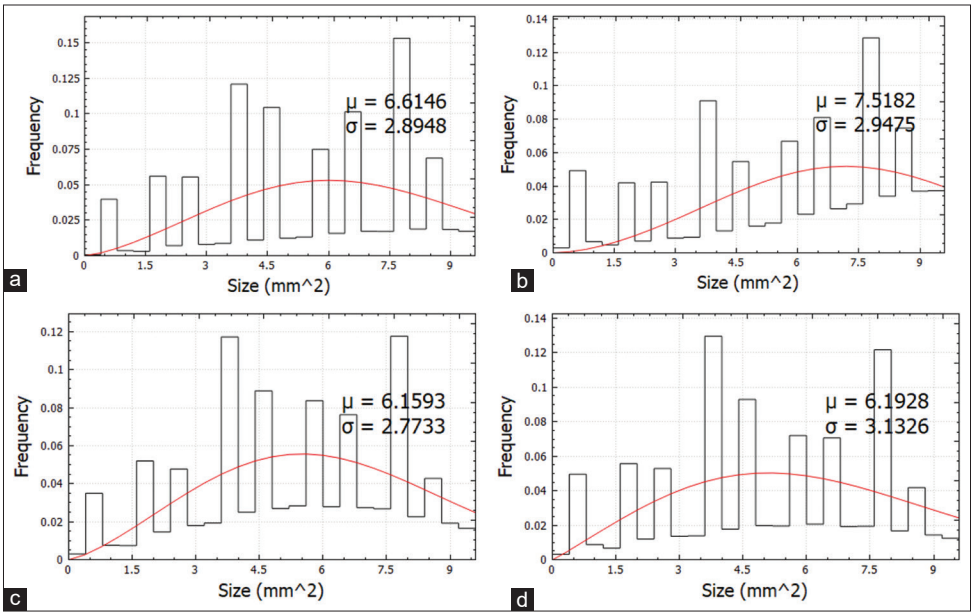

Figure 6: Colony size changes of *Vibrio parahaemolyticus* B4X0T2.2 and B4XT4 in the presence of *Phyllanthus amarus* extract. (a) B4X0T2.2; (b) B4X0T2.2+ plant extract; (c) B4XT4; (d) B4XT4+ plant extract.

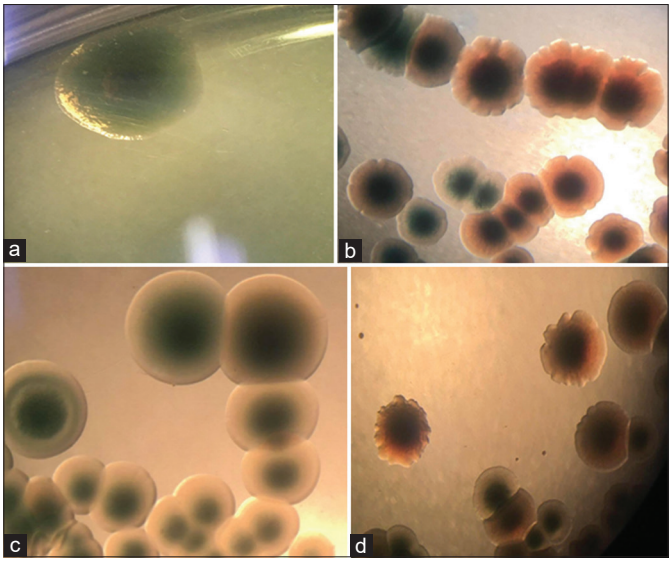

Figure 7: VD1 changes colony phenotype and size of *Vibrio parahaemolyticus* B4X0T2.2 and B4XT4. (a) B4X0T2.2; (b) B4X0T2.2+phageVD3; (c) B4XT4; (d) B4XT4+phageVD1.

Table 1: Evaluation of inhibitory activity on *V. parahaemolyticus* of bacteriophages.

Vibrio parahaelyticus	Phage VD1	Phage VD2	Phage VD3	Phage VD4	Phage VD5
B4XT4*	-	-	+	+	-
B4X0T2.2	+	-	+	+	+

+: Showed the phages capable of infecting host bacteria, - sign indicates that the phages incapable of infecting host bacteria.

4. DISCUSSION

There have been some studies utilizing bacteriophages and *P. amarus* to inhibit *V. parahaemolyticus* but it was combined in single. Even so, all those factors presented their antimicrobacterial ability [12,13]. Unfortunately, the plant extract did not show an inhibitory effect in this experiment since the number of colonies still remained. That is possibly due to the fact that the concentration and incubation time of the extract was not optimal, so we could not conclude the efficacy of the plant extract in this regard. In addition, most of the experiments involving the use of plant extracts or bacteriophages combined with plant extracts to inhibit bacteria utilized the agar plate diffusion method to test the susceptibility of bacteria. However, this method has certain limitations — for example, molecules with a higher molecular weight diffuse very slowly in the agar, making it impossible to accurately measure the diameter of the halo ring in the plate. In contrast, in this experiment, the change of colonies was observed by using the plate count method. Although the effect of the extract on *V. parahaemolyticus* has not been elucidated, the colony form changed as they were affected by bacteriophages and the extracts clearly recorded the same.

Some studies on the topic of bacteriophages combined with plant extracts showed that the results were not satisfactory, except for the separate combination of the two factors [14]. Which used two types of bacteriophages (which have been identified) and three herbs combined to inhibit *E. coli* and concluded that it is necessary to further investigate the concentration of extracts and phages as well as combine many other ingredients to ensure the best results. The phage concentrations in this study were considered low because they were diluted to 5 times less than the original concentration. That prompted the use of the compound carvacrol, which is a compound found in many herbal essential oils (marjoram essential oil, essential oil of lemon basil, etc.), to achieve a purity rate of 99%. This only acted on but not completely inhibits the spread of the bacteria. In the experiment of [15] it was also found that the number of bacteria increased over time in the presence of bacteriophages; whereas the addition of carvacrol prevented the bacteria from becoming resistant to phages. Another critical point to note is that carvacrol is a purified substance, whereas in this experiment, we used *P. amarus* extract,

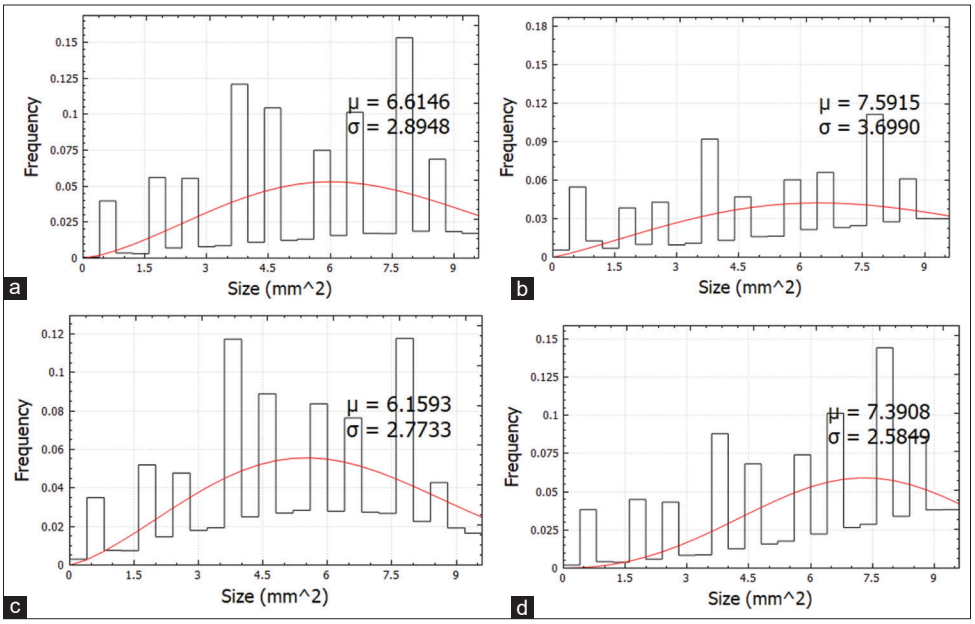

Figure 8: Colony size changes *Vibrio parahaemolyticus* B4X0T2.2 and B4XT4 in the combination of *Phyllanthus amarus* extract and bacteriophage. (a) B4X0T2.2; (b) B4X0T2.2+ plant extract+phageVD1; (c) B4XT4; (d) B4XT4+ plant extract+phageVD1.

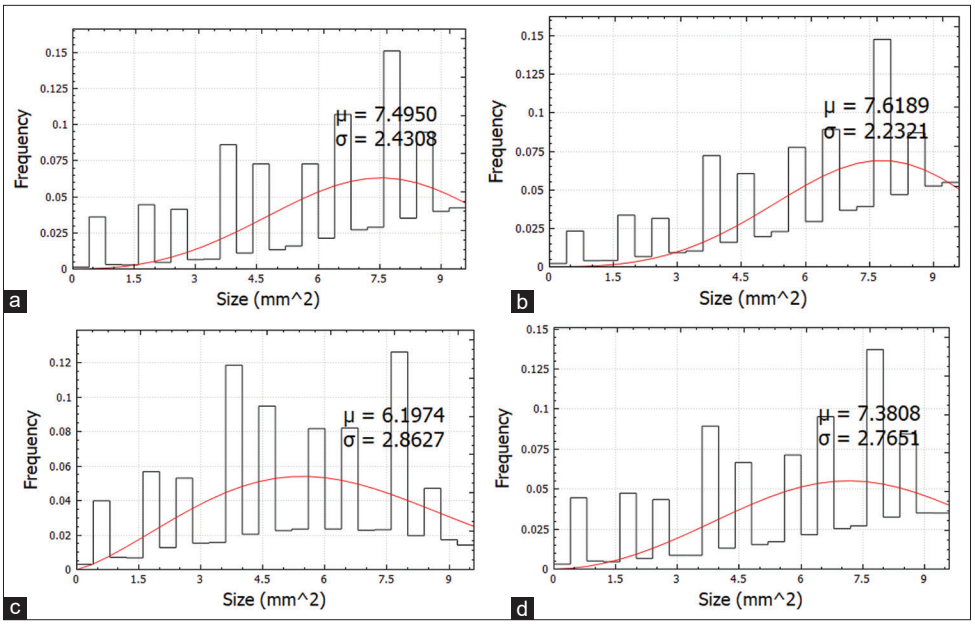

Figure 9: Colony size changes *Vibrio parahaemolyticus* B4X0T2.2 and B4XT4 in the combination of *Phyllanthus amarus* extract and bacteriophage. (a) B4X0T2.2; (b) B4X0T2.2+ plant extract+phageVD3; (c) B4XT4; and (d) B4XT4+ plant extract+phageVD3.

which contains many unknown compounds that could work against the target bacteria.

Incidentally, it can be observed that there are some differences in colony morphology when treated with phages and *P. amarus* extract. The colony shapes were not round, but were spread out like a propeller, and such a colony form was referred to as a "competition type" [16]. That meant that the bacteria were motivated against the inhibitory factors that led to formation of the competition type. It is predicted that there existed a hidden genetic variation, which caused new phenotypes, and it was proved by True and Lindquist [17] on a yeast prion. Therefore, this experiment aimed to limit the pathogenicity of *V. parahaemolyticus* by making their genome not as intact as before.

In addition, a unique feature of this study is the use of the countPHICS software to measure the diameter of bacterial colonies. This software was introduced by Brzozowska *et al*. [18] and was supposed to be more reliable than manual measurement. The μ number in the images was higher than normal, indicating that the image resolution was low. This was due to the fact that the photos on the discs were taken only at 8 MP resolution. Fortunately, the included image processing software (ImageJ) was able to correctly evaluate the colonies for processing and for retrieving colony size data [Figure 10]. The count PHICS

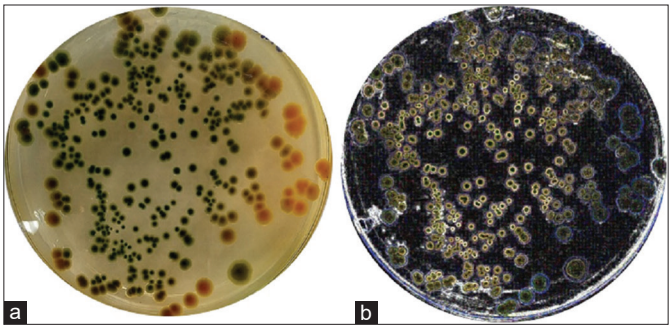

Figure 10: Images of *Vibrio parahaemolyticus B4X0T2.2* before and after undertaking the ImageJ software. (a) *Vibrio parahaemolyticus B4X0T2.2* before undertaking the ImageJ software. (b) *Vibrio parahaemolyticus B4X0T2.2* after undertaking the ImageJ software.

software was highly recommended in the colony size measurement experiments.

5. ACKNOWLEDGMENTS

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6. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

7. FUNDING

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8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects

10. DATA AVAILABILITY

All data generated and analyzed are included within this research article.

11. PUBLISHER'S NOTE

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