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Research Article

Benefits of Tiwai Onion (*Eleutherine americana*) Extract as Phytopharmaceutical Plant to Inhibit the Growth of *Vibrio harveyi* Through *in-Vitro* and *in-Vivo*

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Abstract

The use of vibriosis antibiotics in tiger shrimp was often not controlled, so the results obtained were not effective. The addition of antibiotics would cause resistance to *V. harveyi*. *Eleutherine americana* is a plant that is known to contain antibacterial alkaloids, steroids, phenols, and flavonoids. This study aimed to determine the effectiveness of inhibitory effects of *E. americana* extract against *V. harveyi* through *in-vitro* and *in-vivo* tests on tiger shrimp larvae. *In-vitro* testing consisted of 7 treatments and 3 replications, namely treatments A (0.1%), B (0.2%), C (0.3%), D (0.4%), E (0.5%), F ethanol 70% (K-), and G chloramphenicol 0.01% (K+) treatment. The largest inhibition zone diameter of *E. americana* extract was shown in treatment C (0.3%), with an average value of the inhibition zone produced of 7.5 mm. Challenge test with *V. harveyi* concentration of 107 CFU / ml in the *in-vivo* test consisted of 5 treatments and four replications namely; A treatment without *E. americana* extract, B extract 6 ppm, C extract 12 ppm, D extract 18 ppm, and treatment E without extract and *V. harveyi*. The results of the challenge test with *V. harveyi* bacteria were significantly different in control (chloramphenicol 0.01%), where the highest survival rate was in the treatment of 12 ppm extract (43.34%). *E. americana* extract could inhibit the development of *V. harveyi* bacteria both through *in-vitro* and *in-vivo* tests on tiger shrimp (*Penaeus monodon*).

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1. Introduction

Shrimp diseases could be caused by bacteria, fungi, viruses, and parasites found in water. Diseases caused by bacteria, in addition to causing mass death, also disrupt the quality of shrimp by reducing the quality of infected shrimp meat, lowering the price. A common cause that became an obstacle and disadvantage in conducting tiger shrimp farming in ponds was infectious diseases caused by viruses and bacteria, especially *Vibrio harveyi*. The attacks of vibriosis often occur in the nauplius stage, zoea stage, mysis stage, and sometimes in post larvae and during maintenance in ponds until around the age of 1-1.5 months. *V. harveyi* are opportunistic pathogens, which were organisms that normally exist in aquatic environments and developed from saprophytic to pathogenic when environmental and host conditions deteriorate. This condition would cause the shrimps to experience stress, which resulted in a decreased immune system that has the potential for an attack of vibriosis.

In tackling this disease, nursery managers generally used antibiotics to fight glowing bacteria (*V. harveyi*), but in their use, they were often uncontrolled so that the results obtained were not effective and the application of antibiotics continuously into pond waters would cause immunity (Saptiani and Hartini, 2008). Also, the antibiotic content of shrimp commodities caused the collapse of shrimp prices in the international market; in addition to that the exporting country would be subject to strict sanctions in the form of rejection of the shrimp market because it endangers consumers.

The utilization of medicinal plants as phytopharmaceutical plants was an alternative in handling the problem of vibriosis and could be a bioenrichment in the feed (Cahyadi et al., 2018). Research on efforts to inhibit and prevent the attack of *V. harveyi* bacteria in recent years has been carried out including using active ingredients of gotu kola leaves and mangrove fruit pedada (Satriani et al., 2017; Cahyadi et al., 2017).

Tiwai onion (*Eleutherine americana*) is a plant known to contain alkaloid compounds, steroids, and other active ingredients (Kusuma et al., 2010). The material could be categorized as an antibiotic if it was bacteriostatic, which is able to inhibit bacterial or bactericidal growth if it was able to kill bacteria (Mayer, 2011). Plants that contained components of phenolic compounds, such as alkaloids and flavonoids, were antibacterial (Wostmann and Liebezeit, 2008). For generations, the *tiwai* onion plant was believed to have a variety of medicinal properties ranging from minor illnesses to severe illnesses, including diseases caused by microorganisms.

However, until now there has been no specific research that showed the truth of the efficacy of these plants, both with preclinical and clinical trials. Based on this information, it is necessary to investigate the potential of *tiwai* onion extract as an inhibitor of *V. harveyi* growth in-vitro and in-vivo procedures.

The purpose of this study was to determine the effectiveness of inhibition of *tiwai* onion extract (*E. americana*) against *V. harveyi* bacteria in-vitro and to find out the benefits of *tiwai* onion extract in inhibiting the growth of *V. harveyi* bacteria in tiger shrimp larvae through an in-vivo test.

2. Materials and Methods

2.1 Sample Preparation

Tiwai onion was cut into pieces, and air-dried for 5-7 days to dry, then blended smoothly and sifted.

2.2 Extraction

The manufactured extract was used for preliminary tests in vitro. The extraction stage has begun with weighing each plant material as much as 200 g and it took 200 ml of 70% ethanol solution as extract solvent through the process of maceration (immersion) and stirring using hot plates with stirrer each 1 hour for 3 days at 45°C, it aimed to make the active ingredients contained could be dissolved optimally. The results of the immersion were then filtered using filter paper. After that, evaporation was carried out using an evaporator for 1-2 hours at 60°C until the extract solution becomes concentrated. Concentrated extract solution that has been obtained, dissolved with 70% ethanol solvent with serial concentration of treatment A (0.1%), treatment B (0.2%), treatment C (0.3%), treatment D (0.4%), treatment E (0.5%), ethanol treatment 70% (K-), and G chloramphenicol 0.01% (K+) treatment. Each treatment was repeated three times. Chloramphenicol, as synthetic antibacterial was used as a benchmark to determine the ability of extracts to inhibit bacteria. If the value of the inhibitory zone produced approached or exceeded the positive control value, the extract has the potential to be antibacterial. The extract concentration which has the best ability to inhibit *V. harveyi* bacteria would be used as the final concentration in the in-vivo test.

2.3 Phytopharmaca Testing

Phytopharmaca testing referred to several methods that have often been carried out, including Hydroquinone and Flavonoid Alkaloid Test, Saponin (Harbone, 1987).

2.4 Bacterial Culture

The culture of *V. harveyi* bacteria was carried

out using Thiosulfate Citrate Bile Salt Sucrose (TCBS) media. Bacterial inoculation carried out by taking bacteria using an inoculum needle and then smeared on the surface of the media then incubated for 24 hours at 37°C.

2.5 Antibacterial Activity Test

Antibacterial activity testing was carried out using the disc paper method through the modified antibacterial activity test using the Harlis (2010) method, by implanting several points on the agar media or cultured media. The prepared TCBS media was poured slowly on each Petri dish and allowed to condense until it formed jelly. Bacteria that have been cultured were taken using inoculum needles that have been sterilized with Bunsen burner, then etched evenly on each agar medium and labeled on the sides of each cup as markers on various extracts and controls. After that, planting paper discs that have been soaked at each extract and control concentration then incubated for 24 hours at 37°C.

2.6 Bacterial Growth Inhibition Zones

The ability of the extract as an antibacterial was shown by the inhibition (clear zone) around the disc paper. To get the value of the inhibitory zone produced, measurements were made using calipers. Inhibition zones that are formed as the main parameters were measured from vertical and horizontal inhibition zones then averaged (Paliling *et al.*, 2016).

Supporting parameters for the effectiveness of inhibition of various plant extracts from antibiotics were calculated based on the equation (Arora and Bhardwaj, 1997), namely:

$$E = \left(\frac{D}{Da} \right) \times 100\%$$

Description:

E: Inhibitory effectiveness (%)

D: Diameter of inhibition zone of an extract of plant material (mm)

Da: Diameter of antibiotic inhibition zone (mm)

2.7 Container Preparation

The preparation phase started with cleaning all aquariums of size 50 x 40 x 40 cm (PxLxT) and equipment with soap. Then, rinsed with clean water and dried for 24 hours. After drying, the aquarium was placed on a wooden shelf and filled with seawater with a volume of 20 liters. Test animals were PL5 tiger shrimp larvae with a density of 10 fish/l, which have been adapted for three days, then aerated.

2.8 LC50 test of tiwai onion extract on tiger prawns

48-hour LC50 test directly tested the concentration of *tiwai* onion extract in PL5 tiger shrimp kept in a jar container containing two liters of water. This stage consisted of five treatments with three replications, namely:

Treatment A. without *tiwai* onion extract (control), Treatment B on 100 ppm onion extract, treatment C. 150 ppm onion extract, treatment D on 200 ppm *tiwai* extract, treatment E on 250 ppm onion extract.

2.9 Tiger Shrimp Challenge

The test was carried out by inserting PL5 stadia larvae post larvae in an aquarium, which was filled with 20 liters of sea water and *V. harveyi* bacteria with a concentration of 107 CFU / ml for 60 minutes and then transferred to rearing tiger shrimp (*P. monodon*) media. Maintenance of tiger shrimp larvae was carried out for 15 days and every 6 hours observed and counted dead. At the end of the experiment, the survival of shrimp larvae was calculated and compared with the control, ie, treatment without challenge test with *V. harveyi*. This study uses a completely randomized design (CRD) method consisting of 5 treatments four replications, namely:

- Treatment A: challenge test with *V. harveyi* 107 CFU / ml bacteria without *tiwai* onion extract.
- Treatment B: administration of *tiwai* onion extract with a concentration of 16 ppm and *V. harveyi* bacteria with a density of 107 CFU / ml
- Treatment C: administration of *tiwai* onion extract concentration of 22 ppm / l and *V. harveyi* with 107 CFU / ml
- Treatment D: administration of 28 ppm *tiwai* onion extract and *V. harveyi* bacteria with 107 CFU / ml
- Treatment E: control (without the administration of onion extracts and *V. harveyi*)

2.10 Observed parameters

The main parameter observed in this study was survival rate (SR).

2.11 Data analysis

Data obtained in the form of inhibition zone diameter of each treatment and survival rate were analyzed descriptively by displaying a table and figure regarding the emphasis of the effect of each treatment on *V. harveyi* bacteria. The inhibition zone and SR diameter data were then statistically analyzed using analysis of variance (One-Way ANOVA) to determine the significance of the average difference with a 95% confidence level. The program used to analyze the data uses SPSS 21.0 software.

3. Results and Discussion

3.1 Phytochemical Test

Based on phytochemical test results qualitatively, showed that *Artemia salina* bioenrichment given immersion treatment of tiwai extract showed positive results containing alkaloid compounds, flavonoids, saponins, steroids and phenols and tannins. Qualitative phytochemical tests (alkaloids, flavonoids, saponins, phenols, steroids, and tannins) on the results of bioenrichment (Table 1).

Table 1. Phytochemical screening and evaluation of Tiwai Onion (*Eleutherine americana*) Extract as antimicrobial agents

Parameter	Tiwai onion extract	Bioenrichment	Indicator
Alkoloid	+++	+++	The formation of Brown sediment (Harbone,1987)
Fenol hidrokuinon	+++	+++	The formation of Brown sediment (Harbone,1987)
Flavonoid	+++	+++	The formation of bubbles (Harbone, 1987)
Saponin	+++	+++	The formation of orange color (Harbone,1987)
Steroid	+++	++	The formation of blue color (Indarto, 2011)
Tanin	+++	+++	The formation of black blue, blue and green color (Indarto, 2011)

Noted : +++ (very strong), ++ (Strong), + (less strong), - (not contained)

Some compounds have free radical scavenging properties and can work as antioxidants in the body and have the ability to inhibit bacterial growth as in alkaloid compounds have inhibitory mechanisms by disrupting the constituent components of peptidoglycan on bacterial cells so that the cell wall layer was not kept intact and caused the death of these cells.

The mechanism of phenol compounds as an antibacterial at low concentrations was by damaging the cytoplasmic membrane; causing leakage from the cell nucleus. In contrast, at high concentrations the phenol compound is accumulated with cellular proteins so that the activity was very effective when the bacteria are in the cleavage stage where the phospholipid layer around the cell was under conditions so thin that phenol compounds could easily damage cell contents (Volk and Wheller, 1990). According to Sabir (2003), flavonoid compounds have a mechanism of action as an antibacterial that could damage microbial cell permeability, namely the ability to bind to cell functional proteins and

DNA to inhibit microbial growth.

Steroid compounds were found in nature as a lipid fraction from plants or animals. This substance was very important as a regulator of biological activity in living organisms. Steroids were compounds found in the leaf and fruit wax layers that function as protectors from insects and microbial invasion.

3.2 Antibacterial Activity

Antibacterial activity was measured by paper disc

diffusion method (Kirby et al., 1996). The inhibition zone observations produced from each treatment have different diameters and irregular shapes. Therefore, observations were made by measuring the diameter of the inhibition zone formed vertically and horizontally from the zone formed around the paper disk.

The results of testing the effectiveness of inhibition of tiwai onion extract on the growth of *V. harveyi* bacteria in vitro, it was known that the tiwai onion extract was able to inhibit the growth of *V. harveyi* bacteria. Generally, the inhibition zone diameter tends to increase in proportion to the increasing concentration of the extract. However, this test did not occur as usual, and the resulting inhibition zone was unstable at some concentrations. According to Stout and Davis (1971), the diameter of the inhibition zone formed did not always increase in proportion to the increase in extract concentration, this was due to differences in the diffusion rate of antibacterial compounds on jelly media and the

types and concentrations of different antibacterial compounds also gave different inhibition zone diameters at a certain time. In addition, the difference in the diameter of inhibition zones in each treatment was influenced by several factors. These factors include the concentration of antibacterial compounds, the number of bacteria, types of bacteria, and temperature (Pelczar and Chan, 1986).

and probiotics, which are quite expensive; causing high production costs, making it less efficient for small-scale (traditional) farmers.

Inhibition zone test resulted by *tiwai* onion extract on the growth of *V. harveyi* bacteria in-vitro, showed the inhibition zone of the extract against the test bacteria. The mean diameter of inhibition zone produced by *tiwai* onion extract also showed a difference (Figure 1).

Table 2. The results of the test of the effectiveness of inhibition of Tiwai onion extract on the growth of *V. harveyi* in vitro.

Treatment(%)	Average inhibitory value (mm)	Inhibitory Effectiveness (%)	Responses of inhibition
A = 0,1	3,5	9.9	Weak
B = 0,2	5,3	15	Average
C = 0,3	7,5	21.2	Strong
D = 0,4	7,0	19.8	Average
E = 0,5	7,2	20.3	Strong
F = K(-)	0	0	-
G = (K+)	35,4		Strong

In various treatments, the highest average diameter of inhibition zone of *tiwai* extract was shown in treatment C (0.3%) with an average inhibition zone value of 7.5 mm, then successively by treatment E (0, 5%) of 7.2 mm; D (0.4%) of 7.0 mm; treatment B (0.2%) at 5.3 mm and treatment A (0.1%) at 3.5 mm. An ingredient was said to have antibacterial activity if the diameter of the formed barrier is greater or equal to 6 mm (Bell and Carde, 1984).

The test results showed that the inhibition zone formed by the onion extract of *tiwai* on the growth of *V. harveyi* bacteria was included in the weak to strong category. Based on the results of inhibition zone testing, no inhibition zone occurred in the negative control, because the solution used in this case was 70% ethanol which did not have antibacterial compounds. While positive control of diameter showed a difference with an average value of 35.4 mm, this was because chloramphenicol is a bacteriostatic antibiotic chemical (Mayer, 2011). According to Figure (2005), the use of antibiotics for long-term uncontrolled and incorrect dosages could cause negative effects that were feared to cause resistant bacterial strains that can be dangerous for aquaculture animals. In addition, the price of antibiotics, vitamins,

The resulting inhibitory activity was indicated by the presence of inhibition zones around paper discs. Inhibition zones around disc paper indicated antibacterial activity. Allegedly, the antibacterial content of *tiwai* onion extract contained bioactive compounds that could inhibit the growth of *V. harveyi* bacteria. In line with the opinion of Azis (2017), the inhibitory zone around the disc paper was an area of diffusion of extracts, which indicates the presence of antibacterial activity that influences bacterial growth.

The effectiveness of inhibition by *tiwai* onion extract was obtained by comparing the inhibitory zone values of plant material with the inhibitory zone values of positive control, namely the antibiotic solution chloramphenicol. The inhibitory effectiveness of *tiwai* onion extract with concentrations of 0.3% and 0.5% was included in the strong category, while other treatments were in the weak and moderate category. This was presumably due to differences in the concentration of antibacterial compounds in each extract in inhibiting the growth of *V. harveyi* bacteria. The higher concentration of *tiwai* onion extract did not work optimally to inhibit the development of *A. hydrophila* bacteria (Azis, 2017).

According to Jawetz *et al.* (2007), inhibited bacterial

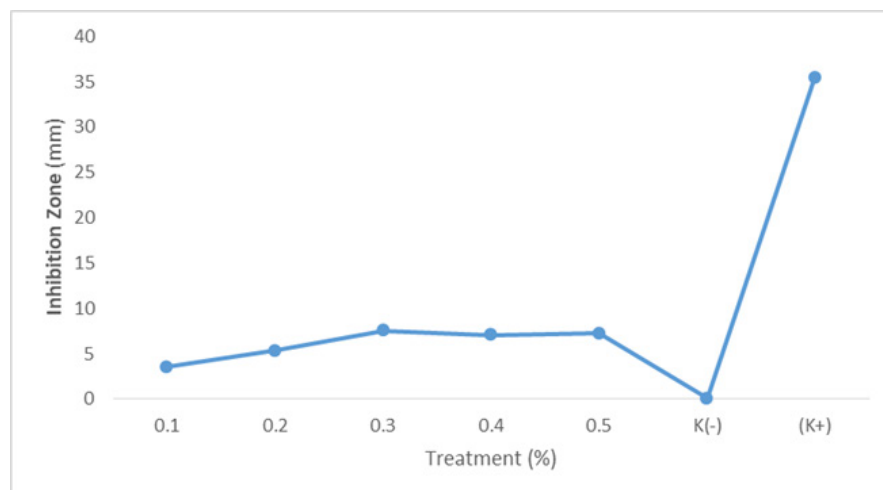


Figure 1. The average value of the diameter of inhibitory zone produced by *tiwai* onion extract at different treatment doses.

growth or bacterial death is due to inhibition of protein synthesis by bioactive compounds. The resistance of gram-negative and gram-positive bacteria to antibacterial compounds varies. The difference in sensitivity of gram-negative and gram-positive bacteria was related to the structure in the cell wall, such as the number of peptidoglycans (the presence of receptors, pores, and lipids), the nature of cross-linking and the activity of autolytic enzymes. These components were factors that determine the penetration, binding, and activity of antimicrobial compounds. The mechanism of antibacterial inhibition against bacterial growth could be in the form of cell wall damage resulting in lysis or inhibition of cell wall synthesis, changes in cytoplasmic membrane permeability so as to cause food release through the cell wall, denaturation

of cell proteins and destruction of metabolic systems in cells by inhibiting intracellular enzyme work (Pelczar *et al.*, 2008). Based on the ANOVA statistical analysis, the use of *tiwai* onion extract gave a significantly different effect (Sig. <0.05). According to the hypothesis testing criteria, the value of F count = 42.38 > F table = 3.48. on the growth of *V. harveyi* bacteria.

3.3 In-Vivo Test

Toxicity of *tiwai* onion extract was carried out to determine the toxic concentration of the extract, using the LC50 Test. The inhibitory effect of *tiwai* onion extract was applied in the 48 hour LC50 test. 48 hour LC50 test results of *tiwai* onion extract can be seen in Table 3 below.

Tabel 3. Tiger Shrimp Mortality after LC₅₀ Test

The concentration of tiwai onion extract (ppm)	Total number of shrimps (n)	Total number of dead shrimps (n)	LC ₅₀ 48 hours
50	20	1	-
100	20	9	120
150	20	13	-
200	20	16	-

Tabel 4. Survival Rate of Tiger Shrimp

Treatment (ppm)	Survival Rate (%)	Standard deviation	Rating
Positive Control	0	-	C
Negaative Control	61,67	10.40	A
6	38,34	2.88	B
12	43,34	7.63	B
18	31,62	7.63	B

Probit analysis results showed the concentration of *tiwai* onion extract of 150 ppm and 200 ppm, causing the death of tiger shrimp larvae exceeding 50% within 48 hours. This showed that the higher the *Tiwai* onion extract, the higher the mortality of tiger shrimp.

Factors that cause mortality in tiger prawns were the presence of alkaloid compounds contained in *tiwai* onion extract, which was quite high; it was because the alkaloids were more active, if these compounds entered the larvae body, the digestive apparatus would be disrupted and could cause death (Harborne, 1987). According to Wibisono (1989), the value of safety (Safe concentration) for organisms from the toxicity of toxicity was 10% of the LC50 value. Therefore, the concentration of *tiwai* onion that was safe to use for tiger prawns is 10% of 100 ppm, which was the range of \pm 10 ppm.

3.4 Challenge Test

The results of observations of the *V. harveyi* bacterial challenge test with a density of 107 CPU / ml for 60 minutes in a jar, showed clinical symptoms after immersion of *tiwai* onion extract which showed that tiger prawns in treatment A (positive control) experienced death on day 1, 2, and day 3 after being infected with *V. harveyi* bacteria. This has also been reported in research by Rukyani *et al.*, 1993, that *V. harveyi* was very malignant because it could kill shrimp larvae populations that were attacked within 1 to 3 days from the initial impact.

Treatment of *tiwai* onion extract at doses of 6 ppm, 12 ppm, and 18 ppm, shrimp began to show recovery on the 3rd day after immersion and clinical symptoms were not detected again on the 6th day after immersion. At the same time, treatment E (negative control) did not experience recovery of conditions due to not being infected by *V. harveyi* bacteria.

The condition of tiger shrimp after immersion of *tiwai* onion was relatively more resistant to *V. harveyi* bacteria allegedly related to the content of active compounds in *tiwai* onion that function as antibacterial. The compounds contained in onion *tiwai* include alkaloids, glycosides, flavonoids, phenolics, triterpenoids/steroids, and anthraquinones (Galingging, 2009).

While the results of Rosidah and Afizia's (2012) research stated that the higher the concentration of the extract, the greater the antibacterial ability of inhibitory zone formed. This proved that the active compound in *tiwai* onion acted as an antibacterial that influenced the recovery of the condition of tiger shrimp after immersion of *tiwai* onion extract.

3.5 Survival Rate of Tiger Shrimp

The survival rate of tiger shrimp (*P. monodon*), in the treatment of *tiwai* onion extract, can be seen in Table

4. The results of the analysis showed that the survival rate of tiger shrimp was significantly different between treatment A (positive control) with 6 ppm, 12 ppm, 18 ppm, and E (negative control), negative control was significantly different from positive treatment and control.

The results obtained could be seen that *tiwai* onion extract could inhibit the growth of infected *V. harveyi* bacteria in tiger prawns. Because *tiwai* onions contained active compounds namely alkaloids, alkaloids have inhibitory mechanisms by disrupting the constituent components of peptidoglycan on bacterial cells, so that the wall layer cells were not formed intact and caused the death of these cells. Flavonoids could damage the cytoplasmic membrane, which could cause the leakage of important metabolites and activate the bacterial enzyme system (Volk and Wheeler 1990).

4. Conclusion

Tiwai onion extract (*E. americana*) could inhibit the development of *V. harveyi* bacteria both through in-vitro and in-vivo testing on tiger shrimp (*Penaeus monodon*).

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Authors' Contributions

Jimmy cahyadi collected the data, drafted the manuscript and designed the figures. Dr. Azis devised the main conceptual ideas and critical revision of the article. All authors discussed the results and contributed to the final manuscript.

Conflict of Interest

The authors declare that they have no competing interests

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