JPPIPA 9(5) (2023)

Jurnal Penelitian Pendidikan IPA

*Journal of Research in Science Education*

http://jppipa.unram.ac.id/index.php/jppipa/index

# Effects of Keji Beling (*Strobilanthes crispus)* Crude Leaves Extract Against *Aeromonas hydrophila* Bacterial Infection in Vitro

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Received: March 27, 2023
Revised: May 26, 2023
Accepted: May 29, 2023
Published: May 31, 2023

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DOI: 10.29303/jppipa.v9i5.3502

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**Abstract:** Keji beling leaves *(Strobilanthes crispus)* can be the solution to cure the fish that infected by *Aeromonas hydrophila* because it contained active compounds. The purpose of this study was to determine the active compounds contained in the crude extract of keji beling leaves (*S. crispus*) and their ability to inhibit the growth of *A. hydrophila*. The tests which are used in this study were phytochemical test, MIC test, disk diffusion test, and SEM test. Phytochemicals analysis showed the presence of active compounds are flavonoid, alkaloid, tanin, and triterpenoid. The result of MIC test showed that concentration of 100 mg/l with a value of 0.389 was the lowest absorbance of the keji beling *(S. crispus)* crude leaves extract. The inhibition test used disk diffusin test with five concentrations of keji beling *(S. crispus)* crude leaves extract with three replications: 50 mg/l, 100 mg/l, 150 mg/l, 200 mg/l, and 250 mg/l, with the observation time of 24 hours post-incubation and 48 hours post-incubation. The concentration of 250 mg/l had the highest inhibition zone. The result of SEM analysis showed that keji beling *(S. crispus)* crude leaves extract can damage the cell walls of *A. hydrophila* and lysis the bacterial cells.

**Keywords:** Antibacterial; Inhibition Test; Phytochemicals; SEM; *Strobilanthes crispus*

## Introduction

*Aeromonas hydrophila* is a pathogenic bacterium capable of infecting cultivated commodity fish of various sizes. *A. hydrophila* can attack various types of freshwater fish such as carp, catfish, and gourami. This bacterium is capable of causing a mortality rate of up to 80%-100% within 2 weeks. In aquatic organisms, these bacteria can infect the gills, skin, liver, kidneys and digestive tract (Amatulloh et al., 2021). Yustiati et al. (2019) explained that *A. hydrophila* secretes an exotoxin or ECP (extracelluller product). The exotoxins produced by *A. hydrophila* include hemolysin, protease, enteroxin, lecithinase, and leucocidine. The bacteria secrete lecithinase which plays a role in trying to enter the bloodstream. The content of hemolysin and lechitinase derived from *A. hydrophila* is able to lyse red blood cells and destroy various tissue cells. Ruan et al. (2022) also stated that as a cause of *Motile Aeromonas Septicaemia*, *A. hydrophila* is an opportunistic pathogen that produces several virulence factors, especially haemolysin and aerolysin which are the main sources of infection. Aquatic organisms infected by these bacteria will show a variety of clinical symptoms such as anemia, hemorrhagic, ascites, ulcers, and abscesses.

Antibiotics are still one of the solutions that cultivators rely on to treat diseases caused by *A. hydrophila*, because antibiotics work specifically and play a very good role in inhibiting or killing bacteria. The use of antibiotics can have various side effects on pathogens or farmed fish. The continuous application of antibiotics can make pathogens resistant so that the use of antimicrobials becomes ineffective and residues derived from antibiotics can contaminate the aquatic environment (Maryani et al., 2018).

The various impacts of the use of antibiotics encourage the search for alternatives in the form of natural ingredients that are environmentally friendly but have the ability to inhibit or kill a pathogenic

**How to Cite:**
Gerrine, G., Prajitno, A., Fadjar, M., & Kenitasari, R.E. (2023). Effects of Keji Beling (Strobilanthes crispus) Crude Leaves Extract Against Aeromonas hydrophila Bacterial Infection in Vitro. *Jurnal Penelitian Pendidikan IPA*, *9*(5), 3849–3855. https://doi.org/10.29303/jppipa.v9i5.3502

bacterium. According to Stratev et al. (2018) medicinal plants can be used as an alternative in replacing the role of antibiotics. Medicinal plants can be used not only as healing treatment, but also as growth promoters, for the prevention of stress and infectious diseases. Several parts of medicinal plants can be used to extract the active substance, but the most widely used part is the leaves.One of the natural ingredients that can be used is keji beling leaves (*Strobilanthes crispus*). According to Ng *et al.* (2021), keji beling leaves contain active compounds in the form of 3.98% of flavonoids, 3.2% of alkaloids, 1% of tannins, and 1.18% of catechins. The results of research conducted by Wantenia et al. (2020) showed that the use of keji beling (*S. crispus*) crude leaves extract was effective as an antibacterial against *Aggregatibacter actinomycetemcomitans* and *Fusobacterium nucleatum.* Based on this explanation, the aim of this study was to determine the active compounds contained in the crude extract of keji beling (*S. crispus*) and their ability to inhibit the growth *of A. hydrophila*.

## Method

*Location and Time*

This research was conducted from November 2022 to December 2022 at Laboratory of Fish Disease and Health Division, Faculty of Fisheries and Marine Sciences, Brawijaya University.

*Research Design*

This research used experimental method. The research design in this study was completely randomized design (CRD). This research used five treatments with different doses, positive control, negative control, and also with three repetitions.

*Preparation of Keji Beling Crude Leaves Extract*

The preparation of keji beling (*S. crispus*) crude leaves extract refers to the method of Djamil et al. (2020) with slight modifications. The leaves are sorted and then rinsed using water, then dried the leaves. Next, the leaves are crushed to form a powder with a blender, then the powder will be made into an extract through maceration with a ratio of 1:10. Put 200 grams of powder into a glass jar, add 2 liters of 96% ethanol, then stir. The solution was soaked for 3 days with occasional stirring and filtered every 24 hours using Whatman filter paper no. 42. The filtering process is repeated using the same type and amount of solvent, so that macerate will be produced on the first day, second day, and third day, then all macerate will be combined and then process it using rotary vacuum evaporator until extract is produced in the form of a paste.

*Preparation of A. hydrophila*

Isolate of *A. hydrophila* were obtained from Balai Besar Perikanan Budidaya Air Payau (BBPBAP) Jepara, Jawa Tengah. The bacteria were stored in Trypticase Soy Agar (TSA) and sub-culture in Trypticase Soy Broth (TSB) overnight before use.

*Minimum Inhibitory Concentration (MIC) Test*

The MIC test refers to the method used by (Fera et al., 2021). First, prepare 7 sterile test tubes. Tubes 1-7 are filled with 4.5 ml of TSB medium. Each test tube is labeled 1-7, tube 6 is labeled K(-) which is a negative control, namely treatment without extract administration. Tube 7 is labeled K(+) as a positive control, which is a tube containing the synthetic antibacterial chloramphenicol 50 ppm given as much as 0.5 ml. Next, 0.5 ml of extract was added to tubes 1-5 with each predetermined concentration. In the next step, 0.1 ml of *A. hydrophila* bacterial isolate with a density of CFU/ml $10^7$ was added to all tubes, incubated at 30˚C for 1 x 24 hours. After incubation, the media was checked for turbidity and the absorbance was measured using spectrophotometer, then compared them to the K(+) absorbance value. The dose that has the lowest absorbance value or close to K(+), this is what shows the minimum inhibitory concentration.

*Inhibition Test – Disk Diffusion*

Testing the ability of a natural substance as an antimicrobial was carried out using the paper disc diffusion method with reference to Behbahani et al. (2019). Paper discs were soaked for 15 minutes in the crude extract of keji beling leaves (*S. crispus*) at a dose determined based on the MIC test results, starting from 50 mg/l, 100 mg/l, 150 mg/l, 200 mg/l, and 250 mg /l, as well as the positive control (chloramphenicol 5 mg/l) and negative control (no treatment). Next, the paper discs were placed on TSA media in a petri dish that already contained *A. hydrophila* bacteria, then incubated for 24 hours at 30°C. After incubation, the clear zone was measured with a caliper.

*Scanning Electron Microscope (SEM) Test*

Characterization using SEM aims to show the morphological description of the particles (Prasetiowati et al., 2018). In this study, two preparations were used, namely the first treatment was normal and the second treatment was by giving *A. hydrophila* bacteria with crude extract of keji beling leaves (*S. crispus*) at a dose of 250 mg/l. Observations focused on the damage to the cell wall of *A. hydrophila.*

*Data Analysis*

Data were analyzed using the SPSS 25.0 for Windows software application. The results obtained will be caried out ANOVA test (0,05) to determine effects of

keji beling (*S. crispus*) crude leaves extract on *A. hydrophila*.

## Result and Discussion

*Phytochemical Screening*

Phytochemical screening was carried out on the crude extract of keji beling leaves (*S. crispus*) using 96% ethanol as a solvent. Phytochemical screening was carried out to identify the active compounds contained in the extract. The results of the phytochemical screening of the keji beling (*S. crispus*) crude leaves extract is presented in Table 1.

**Table 1.** Phytochemical screening results of keji beling crude leaves extract

| Identification of Compound | Characteristics | Results |
|---|---|---|
| Flavonoid | Orange, Brick Red, Pink, Dark Red | (+) Positive |
| Alkaloid | | |
| Meyer | White Precipitate | (-) Negative |
| Dragendrof | Orange Precipitate | (-) Negative |
| Bouchardat | Brown Precipitate | (+) Positive |
| Tanin/Fenol | Blackish Brown, Blackish Blue | (+) Positive |
| Terpenoid | | |
| Steroid | Bluish Green | (-) Negative |
| Triterpenoid | Orange, Browish Orange | (+) Positive |
| Saponin | Permanen Foam | (-) Negative |

Based on the results of the phytochemical test, the crude extract of keji beling leaves (*S. crispus*) contains active compounds such as flavonoids, alkaloids, tannins and triterpenoids. These results are supported by Angelina et al. (2019) which stated that keji beling (*S. crispus*) crude leaves extract contains active compounds in the form of alkaloids, flavonoids, saponins, triterpenoids, and tannins. In the phytochemical test conducted by Fardiyah et al. (2020), it is known that there are alkaloids, saponins, terpenoids, tannins, and flavonoids in keji beling (*S. crispus*) crude leaves extract. Differences of the active compounds contained in keji beling (*S. crispus*) crude leaves extract can be influenced by several factors, starting from the quality of the extract used and the conditions at the location where the sample was taken. Egra et al. (2019) explained that the quality of an extract can be influenced by biological and chemical factors. Biological factors are related to the natural materials used, such as the type of plant, the age of the plant, the part used, and the environmental conditions the plant comes from which includes water, temperature, light, and soil.

*Minimum Inhibitory Concentration (MIC)*

*Minimum Inhibition Concentration* Test aims to determine the minimum concentration of keji beling (*S. crispus*) crude leaves extract in inhibiting the growth of *A. hydrophila*. Maftuch et al. (2018) explained that the MIC test was carried out to find out the smallest dose to inhibit bacterial growth, which can be seen from the absorbance value and the color change that occurs in the media. The absorbance value of each concentration was obtained using a spectrophotometer. The results of the MIC test with keji beling (*S. crispus*) crude leaves extract can be seen in Table 2.

**Table 2.** MIC Test Result

| Concentration (mg/L) | Absorbance Value |
|---|---|
| Control (-) | 0.837 |
| 1 | 0.485 |
| 10 | 0.450 |
| 100 | 0.389 |
| 500 | 0.421 |
| 1.000 | 0.472 |
| Control (+) | 0.024 |

Based on the results of the MIC test (Table 2), it is known that an extract concentration of 100 mg/l has the lowest absorbance value compared to other concentrations, with an absorbance value of 0.389. The absorbance value is closest to the absorbance value of the positive control, which is equal to 0.024.

MIC test according to Najiya et al. (2022) namely the lowest value of the antimicrobial concentration that will inhibit the growth of microorganisms after 24 hours of incubation. Inhibition of bacterial growth was indicated by clear media designated as MIC. This is related to the turbidity of the media which is characterized as the presence of bacterial growth, the more turbid a media is, the more the number of bacterial cells contained in the media. Therefore, to ensure the MIC is also tested with a spectrophotometer to obtain the absorbance value. The absorbance value indicates the amount of light in the spectrophotometer that is absorbed or absorbed by the cells in the cuvette, which is directly proportional to the number of bacterial cells.

Fera et al. (2022) stated that the MIC value was determined based on the lowest concentration with the smallest optical density (OD) value. The smallest OD value indicates a decrease in the absorbance value, which means a decrease in the number of cells after incubation. The largest OD value indicates an increase in the absorbance value where there is still bacterial growth. The presence of bacterial growth indicates that the concentration of the extract cannot inhibit bacterial growth.

*Inhibition Test*

In this study, the disc test was conducted to determine the inhibitory response produced by crude leaves extract of keji beling (*S. crispus*) to inhibit the growth of *A. hydrophila*. The inhibition response was observed by measuring the clear zone formed around

the disc paper using a caliper. The inhibition response was observed at 24 hours of observation to determine the ability of the keji beling (*S. crispus*) crude leaves extract to inhibit the growth of *A. hydrophila* and 48 hours to determine the characteristic of the extract against *A. hydrophila.* The results of the inhibition test can be seen in Table 3.

**Table 3.** Inhibition Test Result

| Concentration (mg/L) | Average Diameter of Inhibition Zone (mm) | | Inhibitory Response |
|---|---|---|---|
| | 24 hours | 48 Hours | |
| 50 | $4.43 \pm 0.24^{a}$ | $4.67 \pm 0.18^{a}$ | Weak |
| 100 | $5.51 \pm 0.49^{b}$ | $5.83 \pm 0.33^{b}$ | Medium |
| 150 | $6.01 \pm 0.28^{c}$ | $6.18 \pm 0.22^{c}$ | Strong |
| 200 | $6.49 \pm 0.27^{d}$ | $6.76 \pm 0.31^{d}$ | Strong |
| 250 | $7.29 \pm 0.49^{e}$ | $7.64 \pm 0.34^{e}$ | Strong |
| Control (-) | $0.00 \pm 0.00^{f}$ | $0.00 \pm 0.00^{f}$ | Weak |
| Control (+) | $14.31 \pm 0.48^{g}$ | $14.75 \pm 0.11^{g}$ | Strong |

Based on the research that has been done, it is known that at 24 hours of observation it can be seen that the keji beling (*S. crispus*) crude leaves extract is able to inhibit the growth of *A. hydrophila*, indicated by the presence of a clear zone formed. The classification of the inhibition response refers to Saptiwi et al. (2018), if the diameter of the inhibition zone is < 3 mm, it is considered weak, 3 – 6 mm is considered moderate, and > 6 mm is considered strong. The results of the average inhibition zone measurement at a concentration of 50 mg/l was 4.43 mm, including the response of weak inhibition. At a concentration of 100 mg/l it produces an average diameter of the inhibition zone of 5.51 mm so that it is a medium inhibition response. Strong inhibitory responses were produced by concentrations of 150 mg/l, 200 mg/l, and 250 mg/l with an average diameter of the inhibition zone formed of 6.01 mm, 6.49 mm and 7.29 mm. The results of measuring the mean diameter of the inhibition zone at 24 hours of observation showed that keji beling (*S. crispus*) crude leaves extract with concentration of 250 mg/l produced the largest average diameter of the inhibition zone compared to the other four concentrations.

The results of measuring the mean diameter of the inhibition zone at 24 hours of observation showed that the higher the concentration of the extract used, the larger the diameter of the inhibition zone formed around the disc paper. These results are in accordance with the statement of Sofidiana et al. (2022) that the higher the concentration of extracts of natural ingredients used, the diameter of the resulting inhibition zone also increases. This is due to an increase in the active ingredient content along with an increase in concentration. Antibacterial ability will decrease with decreasing concentration which can be seen from the diameter of the clear zone formed around the disc paper.

The results of measuring the mean diameter of the inhibition zone at 48 hours of observation (Table 3) show an increase in the average diameter of the inhibition zone. Based on these conditions, it can be said that keji beling (*S. crispus*) crude leaves extract has the ability to kill *A. hydrophila* bacteria so that it is classified as bactericidal. According to Sinurat et al. (2019), antibacterial properties can be distinguished according to how they work, namely bacteriostatic and bactericidal. Bacteriostatic is a substance that can only inhibit the growth of bacteria, while bactericidal is a substance that can kill bacteria. If the inhibition zone that has been formed remains clear for 48 hours, it can be interpreted that the related substance is bactericidal, whereas if the bacteria can still be growing in the inhibition zone that is formed, it can be characterized as bacteriostatic.

*Scanning Electron Microscope (SEM)*

In this study, an SEM test was carried out to determine structural changes in *A. hydrophila bacteria* before being given a treatment and after the bacteria were given keji beling (*S. crispus*) crude leaves extract. SEM test results can be seen in Figure 1.

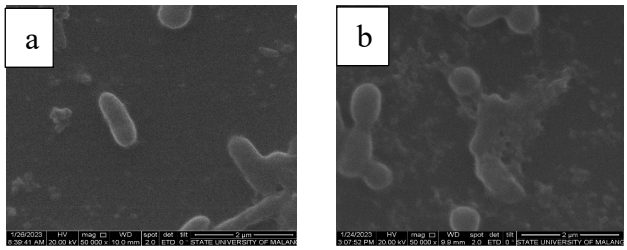


**Figure 1.** Morphology of *A. hydrophila*; (a) no treatment (b) after giving keji beling crude leaves extract

Based on the results of the SEM test (Figure 1), it can be seen that in figure (a) the condition of the *A. hydrophila bacteria* is still in good condition and without any damage, unlike the condition of *A. hydrophila* in figure (b) which has suffered damage to the structure cell walls and undergo lysis. The damage that occurs is due to the antibacterial produced by keji beling (*S. crispus*) crude leaves extract. The content of antibacterial compounds in the extract consists of flavonoids, alkaloids, tannins, and triterpenoids.

Flavonoids can cause damage to the cytoplasmic membrane and cell walls of bacteria. The mechanism of action of flavonoids in inhibiting the function of cell membranes according to Sulistiyono et al. (2018) namely by forming complex compounds with extracellular and dissolved proteins so that they can damage the bacterial cell membrane and followed by the release of intracellular compounds. Flavonoids can also inhibit energy metabolism by inhibiting the use of oxygen by bacteria. Amanda et al. (2019) also explained that the mechanism of flavonoids for damaging the cytoplasmic

membrane is by attacking the phospholipids in the cytoplasmic membrane which will have an impact on the leakage of the membrane so that various substances that serve in bacterial cell metabolism can be wasted and result in bacterial death. The mechanism of flavonoids to damage the bacterial cell wall is to form an alcohol group which will react with the amino acids and lipids contained in the bacterial cell wall so that the cell wall will be damaged. In conditions where the cell wall is damaged, flavonoids will still enter the nucleus of the bacterial cell and make contact with DNA, this will cause the lipid structure of the DNA to be damaged so that the bacteria will lyse and eventually die.

Alkaloids can inhibit the synthesis of the bacterial cell wall which has an impact on cell lysis. Harlita et al. (2018) explained that alkaloids can interfere with the process of forming the peptidoglycan component in bacterial cells, this results in the loss of the function of the cell wall as a protector from osmotic pressure. If the peptidoglycan component is lacking, it can result in bacterial cells becoming sensitive to osmotic pressure, so that high osmotic pressure in bacterial cells will cause bacterial cells to lyse. Dwicahyani et al. (2018) also stated that mechanism of alkaloid to inhibit the bacteria is by interfering with the constituent components of peptidoglycan in bacterial cells, so that the cell wall layer is not formed completely and interferes with peptidoglycan synthesis so that cell formation is incomplete because it does not contain peptidoglycan and the cell wall only covers the cell membrane.

Apart from flavonoids and alkaloids, tannins are also capable of causing damage to the structure of bacterial cells. According to Putri et al. (2021), the mechanism of tannin as an antibacterial is by shrinking the cell membrane or cell wall which will have an impact on disrupting the related cell permeability. This condition will cause damage to the cell wall in the form of leakage so that it will have an impact on the bacterial enzymes which become inactive.

The triterpenoids contained in keji beling (*S. crispus*) crude leaves extract is also one of the factors causing damage to the cell structure of *A. hydrophila*. Triterpenoid antibacterial mechanism according to Egra et al. (2019) namely reacting with porin (trans membrane protein) on the outer membrane of the bacterial cell wall and forming strong polymer bonds resulting in damage to the porin. Damage to the porin which is the door for entry and exit of nutrients so that the inhibitory compounds will reduce the permeability of the bacterial cell wall. The permeability of the bacterial cell wall will interfere with the entry and exit of nutrients and other compounds, so that bacterial growth is inhibited or the bacterial cell will die. Pambudi et al. (2021) explained that triterpenoid mechanism as antibacterial by involving compounds that lipophilic in destroying the cell membrane. These compounds bind to integral proteins present in the membrane bacterial cell peptidoglycan to produce solid polymer chain bonds formed from several hydrogen bonds and bonds. This will damage the porin cell walls of bacteria.

## Conclusion

Based on the results of the phytochemical test, it is known that the crude extract of the leaves of Keji Beling (*S. crispus*) contains active compounds of flavonoids, alkaloids, tannins and triterpenoids. Based on the results of the MIC test and the inhibition test, it is known that at a dose of 250 mg/l the crude extract of keji beling leaves (*S. crispus*) is able to killing *A. hydrophila* bacteria because it is bactericidal. It can also be seen from the results of the SEM test, the crude extract of keji beling leaves (*S. crispus*) was able to damage the cell walls of *A. hydrophila* and cause lysis of the bacterial cell.

**Acknowledgments**
We express our gratitude to all those who have aided in this research.

**Author Contributions**
Conceptualization, Gerrine, G., Prajitno, A., Fadjar, M. and Kenitasari, R. E.; methodology, Gerrine, G. and Kenitasari, R. E.; validation, Prajitno, A. and Fadjar, M.; formal analysis, Gerrine, G.; investigation, Gerrine, G. and Kenitasari, R.; resources, Gerrine, G., Prajitno, A., and Kenitasari, R. E.; data curation, Gerrine, G.; writing—original draft preparation, Gerrine, G.; writing—review and editing, Gerrine, G.; visualization, Gerrine, G.; supervision, Prajitno, A. and Fadjar, M.; project administration, Gerrine, G., Prajitno, A., Fadjar, M. and Kenitasari, R. E.; funding acquisition, Gerrine, G. and Prajitno, A.

**Funding**
This research received no external funding.

**Conflicts of Interest**
The authors declare no conflict of interest.

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