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IN-VITRO INHIBITION TEST OF KEJI BELING (STROBILANTHES CRISPUS) CRUDE EXTRACT ON EDWARDSIELLA TARDA

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ABSTRACT
Bacterial infections, such as *Edwardsiella tarda*, can cause fish diseases. The onset of the disease is the least expected by the cultivators because it can result in economic losses. Antibiotics are commonly used by cultivators to treat fish diseases caused by *E. tarda* infection. However, there are some undesirable consequences due to the use of antibiotics. As an alternative to antibiotics, a natural treatment containing antibacterial properties, such as *keji beling* (*S. crispus*) leaves, can be used. The research method employed in this study was an experimental procedure with a completely randomized design that included 5 treatments, 2 controls, and 3 replications. The treatments were Treatment A (30 ppm), B (60 ppm), C (90 ppm), D (120 ppm), E (150 ppm), positive control (*chloramphenicol* antibiotics), and negative control (without treatment). Then, observations were conducted for 24 and 48 hours. The results showed that the crude extract of keji beling leaves inhibited *E. tarda* bacteria, with the largest average diameter of the inhibition zone in treatment E (150 ppm) of 7.701 0.26 mm and the lowest in treatment A (30 ppm) of 6.207 0.30mm. The relationship between the treatments formed a linear pattern with the equation y = 0.0126x + 5.979 and the coefficient of determination (R2) was 0.6268.

KEY WORDS
Fish diseases, bacterial infections, keji beling leaves, *Edwardsiella tarda*, disk method test, inhibition.

Bacteria that can infect fish are activated by various circumstances, including an increase in turbidity, salinity, pH, temperature, and a lack of dissolved oxygen. In general, there are 13 genera of bacteria that cause diseases in fish ponds, one of which *is Edwardsiella*. *E. tarda*, which causes *Edwardsiellosis*, was found to be a secondary infectious agent in catfish cultured in Tanzania (Mzula et al., 2021).

E. tarda is a gram-negative bacterium having a wide variety of attacks on animals and humans. It is a form of facultative intracellular pathogenic bacterium that may infect both phagocytes and non-phagocytic hosts. *E. tarda* is capable of living and reproducing in various hosts, including freshwater and marine fish. As a result, fish farmers face significant financial losses (Han et al., 2021). Anorexia, bleeding in body parts, and loss of body color pigmentation are common symptoms of *Edwardsiellosis* in fish (Ratnawati et al., 2013). Early symptoms of infection include lack of appetite, bleeding in the eyes, damaged scales, and gradual loss of color pigmentation in infected angelfish. Internal examination reveals an enlarged spleen, a pale liver, and a thin intestinal wall (Turgay, 2020).

Fish farmers or cultivators typically use synthetic antibiotics such as ampicillin, chloramphenicol, tetracycline, and others when dealing with sick fish. However, continuous application of antibiotics with disproportionate doses can increase bacterial resistance. Antibiotic residues in fish bodies can be harmful to the health of fish and humans who consume them and damage the environment (Lukistyowati, 2012). Given the unfavorable consequences, an alternative, including the use of natural bioactive, is required to address these issues, one of which is the crude extract of the keji beling leaves.

Keji beling leaves are herbal plants that contain antioxidants, antibacterial, anti-inflammatory, and anticancer properties (Suproborini et al., 2020). The use of natural ingredients as antibiotics has numerous advantages, including the ease with which the

ingredients can be obtained from the surrounding environment, their environmental friendliness, and their low cost (Syawal et al., 2017).

The presence of phenolic substances, flavonoids, saponins, polyphenols, alkaloids, potassium, sodium, calcium, and silicic acid in keji beling leaves contributes to their antibacterial effect (Suproborini et al., 2020). According to Nurraihana and Hanoon (2013), Keji beling leaves contain various chemicals, including polyphenols, catechins, tannins, alkaloids, caffeine, vitamins, and minerals. According to Lukistyowati (2012), phenol-derived compounds can act as antimicrobials against pathogens like *E. tarda*.

Since this evidence suggests that keji beling leaves (*S. crispus*) may be an effective antibacterial agent against the *E. tarda* bacteria, a more in-vitro examination into the inhibitory efficacy of the leaves' crude extract is necessary.

MATERIALS AND METHODS OF RESEARCH

The research was carried out from December 2021 to January 2022 at the Central Laboratory of Life Sciences at Universitas Brawijaya in Malang.

The method used in this study was an experimental method with a completely randomized design (CRD), consisting of 5 treatment doses, 3 replications, and 2 controls. The treatment doses performed were A (30 ppm), B (60 ppm), C (90 ppm), D (120 ppm), and E. (150 ppm). In addition, positive control (chloramphenicol 30 ppm) and negative control were also used in this study (without treatment)

The tools used in this study were as follows: an autoclave, a beaker glass, a biological safety cabinet, a blender, a film bottle, a Bunsen, a petri dish, a colony counter, a funnel, a destructor, an Erlenmeyer, a measuring cup, scissors, a hotplate, an incubator, a calliper, an inoculation needle, a lighter, a refrigerator, a 100-1000 µl micropipette, a microscope, a tray, object glasses, tweezers, pipette filler, pipette volume, test tube rack, rotary evaporator, spatula, sprayer, test tube, scale, glass jar, triangle, vortex mixer and washing bottle. The materials needed in the study were distilled water, 70% alcohol, aluminum foil, antibiotics, *E. tarda* bacteria, keji beling leaves, 10% DMSO, 70% ethanol, cotton, rubber bands, waste paper, disc paper, label paper, filter paper, iodine solution, crystal violet solution, Mc Farland solution, safranin solution, NaCl, plastic wrap, spirits, tissue, TSA and TSB.

Furthermore, the maceration process is used for extraction. Maceration is the most commonly used method because it is simple and can be applied on a small to large scale (Mukhriani, 2014). The researchers begin the process by washing and drying 1000 grams of fresh keji beling leaves to yield 818 grams. Two hundred grams of dried leaves were then crushed to produce a powder and macerated for 3 days in a glass jar with 70 per cent ethanol (2 litres) at a ratio of 1:10, stirring occasionally until homogenous, and stored in a position not exposed to direct sunlight. To maximize the compound binding process, extraction was performed by re-maceration with the same solvent every day. Then, to obtain a thick extract, the maceration results were filtered and concentrated using a rotating vacuum evaporator set to a temperature of 60–70°C (Djamil et al., 2020). A total of 8 grams of the thick extract was then calculated and diluted with 10% DMSO to create the treatment dosages.

Bacterial rejuvenation was performed using a TSA agar slant, which weighed 0.56 grams and was stored in an Erlenmeyer tube with 14 ml of distilled water and homogenized. Next, the media was poured into a 7 ml test tube, which was then wrapped in cotton, aluminum foil, and plastic wrap. The media were sterilized for 15 minutes at 121°C and 1 atm pressure in an autoclave. The sterile media was positioned at a 30° angle and allowed to harden. *E. tarda* pure cultures were streaked zigzag across the surface of the media and incubated at 32°C for 1 x 24 hours.

Bacterial culture was performed in TSB liquid media, which was weighed up to 0.6 grams and homogenized with 20 ml of distilled water. The media was put into a 10 ml test tube, sealed in aluminum foil and plastic wrap, and sterilized in an autoclave for 15 minutes at 121°C and 1 atm pressure. The rejuvenated bacterial colonies were homogenized after

being transferred to liquid TSB media as much as 1 ose. At 32°C, the bacteria-containing medium was incubated for 1 x 24 hours.

The inhibition test was performed using solid TSA media that weighed 5.6 grams and was homogenized in an Erlenmeyer after adding 140 ml of distilled water. The media was sterilized by autoclaving at 121°C for 15 minutes at 1 atm, and 20 mL of sterile medium was placed into Petri dishes and allowed to set. A total of 100 µl of bacterial isolates from TSB culture media were dripped onto a plate and levelled at a 10^7 CFU/ml density. The paper discs were placed on the surface of the media and incubated for 24-48 hours at 32°C after being soaked in the leaf extract of Keji beling plant according to the dose, positive control, and negative control.

With a 95% confidence level, the data were assessed for variance using the F test (ANOVA), the Least Significant Difference Test (LST), and the Polynomial Orthogonal test.

RESULTS AND DISCUSSION

The efficacy of adding a crude extract of keji beling (*S. crispus*) leaves against *E. tarda* bacteria was indicated by the emergence of a clean region surrounding the disc that had been previously soaked with various extract doses. After 24 hours of incubation, the inhibition test results were obtained (Figure 1).

Table 1 – Mean of Inhibition Zone Measurement Results after 24 Hours of Incubation

Table	Replication (mm)			Total (mm)	Mean ± STDEV (mm)
	1	2	3		
A (30 ppm)	6.040	6.550	6.030	18.620	6.207 ± 0.30
B (60 ppm)	7.070	6.540	7.100	20.710	6.903 ± 0.32
C (90 ppm)	7.560	6.080	7.550	21.190	7.063 ± 0.85
D (120 ppm)	7.070	8.010	8.020	23.100	7.700 ± 0.55
E (150 ppm)	8.005	7.555	7.545	23.105	7.701 ± 0.26
Total				106.725	

The highest mean of inhibition zone measurement was obtained in treatment E at a dose of 150 ppm, whereas the lowest result was obtained in treatment A at 30 ppm (Table 1). Barrier response classification is divided into several categories. The first category is without an inhibition zone, which is shown by the absence of a clear zone surrounding the disc. The categorization is weak if the inhibitory zone formed is less than 5 mm. The classification is medium when the observable inhibitory zone ranges between 5 and 10 mm.

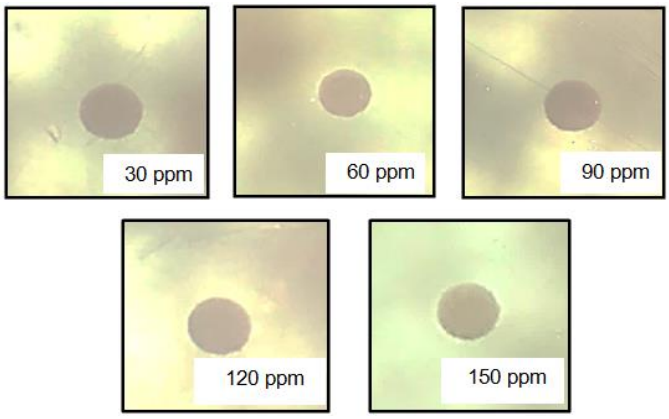

Figure 1 – Inhibitory Zone Diameter of Crude Extract of Keji Beling Leaves against E. tarda Bacteria

If the zone of inhibition emerges in the 11–20 mm range, it is considered strong. As suggested by Kaawoan et al., (2016), when the measurement findings exceed 20 mm, the inhibition zone is very strong. Based on the categorization, it was found that treatment A – E was classified as a medium resistance response.

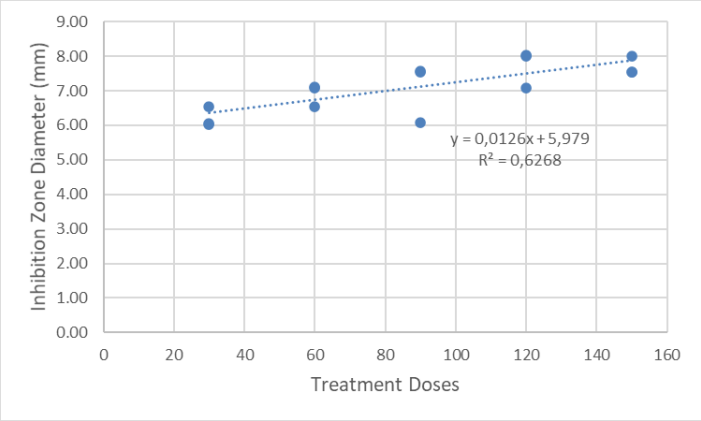

Figure 1 – Graph of Orthogonal Polynomial Test Results

The use of crude extract of keji beling (*S. cripus*) leaves at different doses to the inhibitory zone formed by *E. tarda* bacteria revealed a linear regression pattern with the equation y = 0.0126x + 5.979 and coefficient R^2 = 0.6268. The R^2 coefficient value obtained indicates that 63 percent of the use of keji beling (*S. crispus*) leaves extract at a dose affects the inhibition produced. The graph illustrates a rise in the size of the inhibition zone from 30 ppm to 150 ppm, with the lowest point in treatment A (30 ppm dose) and the greatest point in treatment E. (150 ppm dose).

The results indicated that the crude extract of keji beling (*S. crispus*) leaves was efficient against *E. tarda*. The area of inhibition generated appears to increase in proportion to the treatment dose given. The higher the dose of crude extract of keji beling (*S. crispus*) leaves utilized, the higher the concentration of active compounds. As a result, the inhibition zone formed is greater. Laoi et al. (2020) found that higher concentrations of the antimicrobial agent resulted in faster diffusion rates. Thus, the generated antibacterial activity increases as the diameter of the ensuing inhibition zone develops. Because of the high concentration of antibacterial used, the bacteria will die more quickly.

Table 2 – Mean of Inhibition Zone Measurement Results after 48 Hours of Incubation

Treatment	Replication (mm)			Total (mm)	Mean ± STDEV (mm)
	1	2	3		
A (30 ppm)	8.080	7.040	8.085	23.205	7.735 ± 0.60
B (60 ppm)	7.020	9.030	11.030	27.080	9.027 ± 2.01
C (90 ppm)	9.095	7.070	11.100	27.265	9.088 ± 2.02
D (120 ppm)	13.010	9.015	8.070	30.095	10.032 ± 2.62
E (150 ppm)	9.040	10.045	11.095	30.180	10.060 ± 1.03
Total				137.825	

After 48 hours of incubation, observational data (Table 2) indicated that the crude extract of keji beling (*S. crispus*) leaves exhibited bactericidal properties against the growth of *E. tarda* bacteria. As a result, when comparing data from observations after 48 hours of incubation, it was found that there was a greater inhibitory zone after 24 hours of incubation (Table 1). Because the inhibitory zone develops, it implies that keji beling leaves can inhibit *E. tarda* bacteria development and kill bacteria.

If there is a growth in the inhibition zone along with the addition of contact or incubation time with antibacterial substances, antibacterial chemicals exhibit selective bactericidal toxicity. The antibacterial bactericidal properties are based on the growing diameter of the inhibition zone as the duration of bacterial cells exposed to antibacterial substances increases (Iien et al., 2018).

According to Sinurat et al. (2019), antibacterial are classified as either bactericidal or bacteriostatic, depending on how they work. Antibacterial that functions as a bacteriostatic contain chemicals capable of disrupting the bacterial development. Bactericidal antibacterial, on the other hand, work by killing bacteria. The sample can be incubated for up to 48 hours

to examine the antibacterial properties. Bacteriostatic characteristics inhibit protein synthesis by temporarily binding to the organism's ribosomes. Because these bonds do not bind strongly, if the concentration and stability of the antimicrobial agent decrease, the antimicrobial agent releases ribosomes, allowing bacteria to re-grow. In contrast to the bactericidal mechanism, it forms a tight bond with the target cell and is not rereleased, resulting in the death of the microorganism cells. The antimicrobial properties produced are influenced by several parameters, including the concentration of the extract utilized, the content of antibacterial chemicals in the extract, the extract's diffusion ability, and the type of bacteria inhibited.

Phytochemical test of the leaves of keji beling (*S. crispus*) found that the crude extract contained many antibacterial components, including flavonoids, alkaloids, and tannins. Sulastri et al. (2021) did a phytochemical screening of Keji beling leaves and discovered that the leaves were positive for alkaloids, flavonoids, saponins, tannins, terpenoids, and steroids.

According to Bontjura et al. (2015), flavonoids act as antimicrobials by inhibiting nucleic acid synthesis, impairing the function of cell membranes, and limiting energy metabolism. Due to the interaction between flavonoids and bacterial DNA, flavonoids can cause the breakdown of cell wall permeability, microsomes, and lysosomes. In addition, Dwicahyani et al. (2018) also explained that alkaloids work as antibacterial by inhibiting the constituent sections of the bacterial cell peptidoglycan. When alkaloids affect the cell wall layer, it becomes damaged or incomplete, and the process of peptidoglycan formation is inhibited. Due to the lack of peptidoglycan, cell formation is inefficient and incomplete, and the cell wall consists only of cell membranes. Arlofa (2015) mentioned that tannins work as antibacterial by suffocating cell walls or cell membranes, causing cell permeability to be disrupted. Therefore, the cell is unable to carry out life processes, posing a barrier to development. This disorder has the potential to result in cell death.

CONCLUSION

The application of a crude extract of Keji beling (*S. crispus*) leaves affected *E. tarda* bacteria with the largest mean diameter of inhibition indicated at a concentration of 150 ppm, covering an area of 7.701 0.26 mm. A dosage of 150 ppm is the recommended dosage for inhibiting *E. tarda* bacteria development.

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REFERENCES

1. Arlofa, N. (2015). Uji kandungan senyawa fitokimia kulit durian sebagai bahan aktif pembuatan sabun. Jurnal Chemtech. 1(1), 18-22.
2. Bontjura, S., Waworuntu, O. A. & Siagian, K. V. (2015). Uji efek antibakteri ekstrak daun leilem (Clerodendrum minahassae L.) terhadap bakteri Streptococcus mutans. PHARMACON: Jurnal Ilmiah Farmasi. 4(4), 96-101.
3. Djamil, R., Pratami, D. K. & Riyantika, L. V. (2020). Pemeriksaan parameter mutu dan uji aktivitas penghambatan enzim α-Glukosidase dari ekstrak etanol 70% daun keji beling (Sericocalyx crispus (L.) Bremek). Jurnal Jamu Indonesia. 5(1), 1-8.
4. Dwicahyani, T., Sumardianto & Rianingsih, L. (2018). Uji bioaktivitas ekstrak teripang keeling (Holothuria atra) sebagai antibakteri Staphylococcus aureus dan Escherichia coli. Jurnal Pengolahan dan Bioteknologi Hasil Perikanan. 7(1), 15-24.
5. Han, H., Teng, D., Mao, R., Hao, Y., Yang, N., Wang, Z., Li, T., Wang, X. & Wang, J. (2021). Marine peptide-N6NH2 and its derivative-GUON6NH2 have potent antimicrobial

activity against intracellular Edwardsiella tarda in vitro and in vivo. Frontiers in Microbiology. 12(637427), 1-16.
6. Iien, H., Wirajagat, G. C., Ramdani, R. F. & Rasmi, D. A. C. (2018). Uji aktivitas anti bakteri ekstrak daun belimbing wuluh (Averrhoa bilimbi) dan daun sirih merah (Piper ornatum) terhadap bakteri penyebab pneumonia pada balita. Prosiding Seminar Nasional Pendidikan Biologi. 76-80.
7. Kaawoan, P. T., Abidjulu, J. & Siagian, K. V. (2016). Uji daya hambat ekstrak buah pala (Myristica fragrans Houtt) terhadap bakteri penyebab periodontitis Porphyrominas gingivalis secara in vitro. Jurnal e-Gigi. 4(2), 111-114.
8. Laoi, D., Lukstyowati, I. & Syawal, H. (2020). Pemanfaatan ekstrak etanol biji manga harumanis (Mangifera indica L.) untuk menghambat pertumbuhan bakteri Edwardsiella tarda. Jurnal RUAYA: Jurnal Penelitian dan Kajian Ilmu Perikanan dan Kelautan 8(1), 18-27.
9. Lukistyowati, I. (2012). Studi efektivitas sambiloto (Andrographis paniculate Nees.) untuk mencegah penyakit Edwardsiellosis pada ikan patin (Pangasius hypopthalmus). Berkala Perikanan Terubuk. 40(2), 56-74.
10. Mukhriani. (2014). Ekstraksi, pemisahan senyawa, dan identifikasi senyawa aktif. Jurnal Kesehatan. VII(2), 361-367.
11. Mzula, A., Wambura, P. N., Mdegela, R. H. & Shirima, G. M. (2021). Present status of aquaculture and the challenge of bacterial diseases in freshwater farmed fish in Tanzania: a call for sustainable strategies. Aquaculture and Fisheries. 6, 247-253.
12. Nurraihana, H. & Hanoon, N. N. A. (2013). Phytochemistry, pharmacology and toxicology properties of Strobilanthes crispus. International Food Research Journal. 20(5), 2045-2056.
13. Ratnawati, A., Purwaningsih, U. & Kurniasih. (2013). Histopatologis dugaan Edwardsiella tarda sebagai penyebab kematian ikan maskoki (Carrasius auratus): Postulat Koch. Jurnal Sain Veteriner. 31(1), 55-65.
14. Sinurat, A. A. P., Renta, P. P., Herliany, N. E., Negara, B. S. F. P. & Purnama, D. (2019). Uji aktivitas antibakteri ekstrak methanol rumput laut Gracilaria edulis terhadap bakteri Aeromonas hydrophila. Jurnal Enggano. 4(1), 105-114.
15. Sulastri, L., Lestari, R. M. & Simanjutak, P. (2021). Isolasi dan identifikasi senyawa kimia monoterpen dari fraksi etilasetat daun keji beling (Strobilanthes crispa (L.) Blume.) yang mempunyai daya sitotoksik. Jurnal Fitofarmaka Indonesia. 8(1), 12-17.
16. Suproborini, A., Laksana, M. S. D. & Lisniawati. (2020). Potensi ekstrak etanol daun Strobilanthes crispus sebagai antidiare. EnviroScienteae. 16(1), 12-20.
17. Syawal, H., Karnila, R., Dirta, A. & Kurniawan, R. (2017). Ekstrak daun Rhizophora sp. menghambat pertumbuhan bakteri Streptococcus agalactiae dan Edwardsiella tarda. Jurnal Veteriner. 18(4), 604-609.
18. Turgay, E. (2020). Edwardsiellosis in freshwater angelfish (Pterophyllum scalare). The Israeli Journal of Aquaculture. 72, 1-8.