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The Effectiveness of the Candle Bush (*Cassia alata* L.) Leaf Extract in Preventing the Growth of Disease-Causing Gram-Negative Bacteria in Aquaculture

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ABSTRACT

This study aimed to evaluate the antibacterial potential of candle bush (*Cassia alata* L.) leaf extract against *Aeromonas hydrophila*, *Enterobacter aerogenes*, and *Edwardsiella tarda in vitro*, and determine which species was most effectively inhibited. The experiment followed a split-plot design, with main plots consisting of bacterial species: *A. hydrophila* (A1), *E. aerogenes* (A2), and *E. tarda* (A3), and subplots comprising treatments of distilled water (K1) as the negative control, ethanol (K2) as the solvent control, and tetracycline (K3) as the positive control. The tested treatments included 50% (P1) and 100% (P2) concentrations of *C. alata* leaf extract, each with three replications. The results indicated that *C. alata* leaf extract was effective in inhibiting the growth of *A. hydrophila* and *E. tarda*. For *A. hydrophila*, the mean inhibition zone diameters were 9mm at 100% concentration and 7.7 mm at 50%, both categorized as moderate inhibition. For *E. tarda*, the inhibition zones measured 11mm at 100% (strong inhibition) and 7.3mm at 50% (moderate inhibition). In contrast, the extract did not inhibit the growth of *E. aerogenes* at either concentration. Among the tested bacteria, *Edwardsiella tarda* was the most effectively inhibited by *C. alata* leaf extract.

INTRODUCTION

The success of fish farming is influenced by multiple factors, including the quality and quantity of fish seed, aquaculture systems, environmental conditions, infrastructure, market access, human resources, and disease control strategies. Fish diseases do not occur spontaneously; they emerge due to imbalances between the fish, as susceptible hosts, and their surrounding environment **(Iqbal, 2016)**. Environmental changes can disrupt the fish's immune system, making them vulnerable to pathogens that may develop in the culture medium. Among the pathogenic bacteria known to infect fish are *Aeromonas hydrophila*, *Enterobacter aerogenes*, and *Edwardsiella tarda*. Indonesia has experienced four major fish disease outbreaks, one of which was caused by bacterial infections **(Subasinghe *et al*., 2001; Ratnawati *et al*., 2013; Assefa *et al*., 2018)**.

This situation underscores the need for fish farmers to enhance their knowledge and skills in disease prevention and management **(Sari, 2015)**. The use of chemical treatments, including antibiotics at appropriate dosages, can be effective in controlling infections. However, improper use may lead to bacterial resistance and leave residues that negatively impact the environment and pose risks to human health through consumption **(Diana, 2009; St-Hilaire *et al*., 2023)**.

As a safer alternative, the use of natural materials is increasingly recommended for the prevention and treatment of fish diseases. Natural remedies offer the advantage of leaving no harmful residues in the environment and producing fish safe for consumption. Indonesia, with its vast tropical forests, holds significant potential for the development of herbal medicines **(Inayah & Ernayenti, 2007; Bera *et al*., 2020; Algammal *et al*., 2022)**.

One such medicinal plant is the candle bush (*Cassia alata* L.), which is widely available and has long been used in traditional human medicine, though it remains underutilized in aquaculture. In addition to its therapeutic properties, *C. alata* has high economic value and is exported for use in pharmaceuticals and traditional health products. The plant is rich in bioactive compounds such as alkaloids, anthraquinones, flavonoids, saponins, tannins, and steroids/triterpenoids **(Egra *et al*., 2019)**. A dicotyledonous plant belonging to the division Magnoliophyta, *C. alata* typically grows wild or is cultivated in humid environments **(Saputra, 2014)**.

This study aimed to evaluate the *in vitro* antibacterial activity of *C. alata* leaf extract against *A. hydrophila*, *E. aerogenes*, and *E. tarda* and to identify which of these bacterial species is most effectively inhibited by the extract.

MATERIALS AND METHODS

Study location and duration

The study was conducted over a 32-day period, from January to February 2023, at the Freshwater Fish Hatchery in Mandiangin, Karang Intan District, Banjar Regency, South Kalimantan, Indonesia.

Antibacterial assay method

The antibacterial assay was carried out using the standard disc diffusion method, following the guidelines established by the Clinical and Laboratory Standards Institute **(CLSI, 2012)** and **Balouiri *et al*. (2016)**. Laboratory equipment used included disc papers, Petri dishes, Erlenmeyer flasks, an oven, a laminar flow cabinet, an inoculating loop (ose needle), a Bunsen burner, an incubator, a caliper, a micropipette, a Vortex blender, and a McFarland 0.5 turbidity standard **(NCCLS, 2003; CLSI, 2012)**.

Materials

Biological materials included dried candle bush (*Cassia alata* L.) leaves and three species of bacterial pathogens—*Aeromonas hydrophila*, *Enterobacter aerogenes*, and *Edwardsiella tarda*—which are commonly associated with freshwater fish diseases

(Harikrishnan *et al*., 2011). The culture medium used was Mueller-Hinton Agar (MHA), widely accepted for antibiotic susceptibility testing **(Balouiri *et al*., 2016)**. Additional materials included sterile distilled water, tetracycline (as a positive control), 0.85% NaCl solution, 70% ethanol, and sterile cotton swabs for inoculation and surface spreading.

Research procedures

1. Preparation of Mueller-Hinton agar (MHA)

A total of 15.2g of MHA powder was weighed and dissolved in 400mL of sterile distilled water. The solution was sterilized in an autoclave at 121°C for 15 minutes. After cooling, the medium was allowed to set at room temperature for 24 hours and was stored in a refrigerator until use.

2. Preparation of candle bush (*Cassia alata* L.) leaf extract

Dried candle bush leaves were collected from Sekata Makmur, Mantangai District, Kapuas Regency, Central Kalimantan. The leaves were ground into a fine powder. A total of 100g of leaf powder was placed into an Erlenmeyer flask and mixed with 750mL of 70% ethanol. The mixture was kept away from direct sunlight and shaken occasionally over three days. The solution was filtered to obtain the first macerate. The remaining plant residue was remacerated with an additional 250mL of 70% ethanol for one day, then filtered to obtain the second macerate. Both extracts were left to settle overnight, then evaporated using an oven until a thick extract was obtained **(Puspitasari & Lean, 2016)**.

3. Preparation of test bacterial solutions

Pure cultures of *A. hydrophila*, *E. aerogenes*, and *E. tarda* were obtained from the testing laboratory of the Freshwater Fish Hatchery (BPBAT) in Mandiangin. Bacterial cultures were suspended in 5mL of 0.85% NaCl solution and homogenized. The turbidity of the suspension was adjusted to match a 0.5 McFarland standard, corresponding to an approximate bacterial concentration of 1.5×10^8 CFU/mL **(Asifa, 2014)**.

4. Effectiveness testing of candle bush extract

The antibacterial effectiveness of the *C. alata* leaf extract was tested using the Kirby-Bauer disc diffusion method. The prepared bacterial suspension was evenly spread across the surface of MHA plates using sterile cotton swabs. Sterile paper discs were soaked in the *C. alata* extract for 10 minutes, then placed on the inoculated agar using sterile forceps. Plates were incubated at 28°C for 24 hours.

After the incubation period, the diameter of the inhibition zones around the discs was measured using a caliper and recorded in millimeters (mm). The inhibition zone was measured from the edge of the disc to the outer margin of the clear zone. The antibacterial activity was categorized based on the classification system provided by **Susanto *et al*. (2012)**.

Table 1. Bacterial inhibition zone category

Diameter	Inhibition zone category
≤ 5 mm	Weak
6-10 mm	Moderate
11-20 mm	Strong
≥ 21 mm	Very Strong

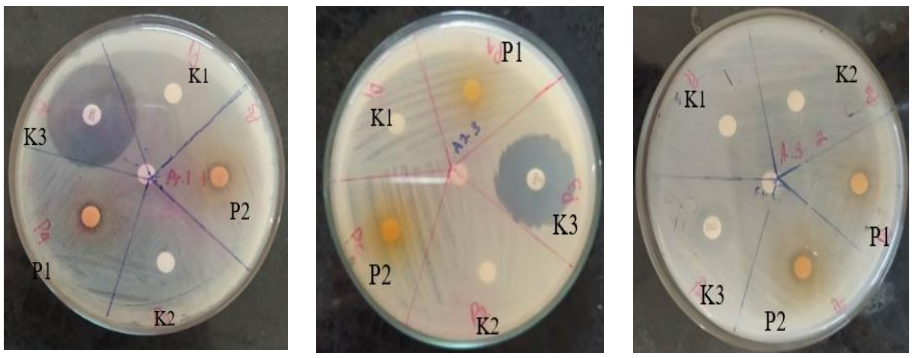
Research design

The study employed a split-plot experimental design. The main plots consisted of three bacterial species: *Aeromonas hydrophila* (A1), *Enterobacter aerogenes* (A2), and *Edwardsiella tarda* (A3). The subplots included the following treatments: distilled water as a negative control (K1), ethanol as a solvent control (K2), and tetracycline as a positive control (K3). In addition, two concentrations of *Cassia alata* (candle bush) leaf extract were tested: 50% (P1) and 100% (P2). Each treatment was replicated three times.

RESULTS AND DISCUSSION

This section presents the results obtained from the research, structured systematically in accordance with the study's objectives and methodology.

Fig. (1) illustrates the inhibition zones produced by candle bush (*Cassia alata* L.) leaf extract against the bacterial growth of *Aeromonas hydrophila*, *Enterobacter aerogenes*, and *Edwardsiella tarda* under various treatments. These treatments included distilled water as a negative control (K1), ethanol as a solvent control (K2), tetracycline as a positive control (K3), candle bush leaf extract at 50% concentration (P1), and candle bush leaf extract at 100% concentration (P2).



(a) (b) (c)

Fig. 1. The inhibition zone of the candle bush leaf (*C. alata* L.) extract against the bacterial growth: (a) *A. hydrophila,* (b) *E. aerogenes,* and (c) *E. tarda*

Fig. (1) displays the inhibition zones formed by control treatments and candle bush (*Cassia alata* L.) leaf extract at concentrations of 50 and 100% against the growth of *Aeromonas hydrophila*, *Enterobacter aerogenes*, and *Edwardsiella tarda*. The corresponding inhibition zone diameters are presented in Table (1).

The candle bush leaf extract produced an inhibition zone of 9mm at the 100% concentration and 7.7mm at the 50% concentration against *A. hydrophila*. For *E. tarda*, inhibition zones of 11mm (100%) and 7.3mm (50%) were recorded. However, the extract showed no inhibitory effect against *E. aerogenes* at either concentration, as no inhibition zone was observed.

Among the positive control treatments, tetracycline exhibited the highest antibacterial activity, with inhibition zones measuring 31mm against *A. hydrophila*, 25.3 mm against *E. aerogenes*, and 13.7mm against *E. tarda*. The negative controls—distilled water and ethanol—did not produce any inhibition zones for any of the tested bacteria.

Table 1. Mean diameter of the inhibition zone against *A. hydrophila*, *E. aerogenes*, and *E. tarda*

Bacteria	Concentration	Diameter (mm)			Mean Diameter (mm)	Category
		1	2	3		
Aeromonas hydrophila	*Tetracycline* control	32	30	31	31	very strong
	Distilled water control	0	0	0	0	weak
	Ethanol control	0	0	0	0	weak
	Candle bush leaf extract of 50%	8	7	8	7.7	moderate
	Candle bush leaf extract of 100%	9	8	10	9	moderate
Enterobacter aerogenes	*Tetracycline* control	25	25	26	25.3	very strong
	Distilled water control	0	0	0	0	weak
	Ethanol control	0	0	0	0	weak
	Candle bush leaf extract of 50%	0	0	0	0	weak
	Candle bush leaf extract of 100%	0	0	0	0	weak
Edwardsiella tarda	*Tetracycline* control	14	14	13	13.7	strong
	Distilled water control	0	0	0	0	weak
	Ethanol control	0	0	0	0	weak
	Candle bush leaf extract of 50%	7	8	7	7.3	moderate
	Candle bush leaf extract of 100%	12	10	11	11	strong

According to the classification proposed by **Susanto *et al.* (2012)**, the inhibition zone diameters of candle bush (*Cassia alata* L.) leaf extract against the growth of *Aeromonas hydrophila* at both 50 and 100% concentrations are categorized as moderate. The inhibition zones produced by tetracycline, used as a positive control, fall into the very strong category, while those of the distilled water and ethanol controls are classified as weak. Against *Edwardsiella tarda*, the 100% concentration of *C. alata* leaf extract produced a strong inhibition zone, while the 50% concentration produced a moderate effect. In contrast, the extract showed weak or no inhibitory activity against *Enterobacter aerogenes* at both concentrations.

Overall, the inhibition zones produced by *C. alata* extract were greater than those observed for distilled water and ethanol controls, but smaller than those of tetracycline. This is likely due to the fact that tetracycline is a broad-spectrum antibiotic capable of inhibiting or killing both Gram-negative and Gram-positive bacteria **(Pangestika, 2017)**.

Fig. (1) suggests that the antibacterial activity observed against *A. hydrophila* and *E. tarda* is attributable to the bioactive secondary metabolites present in the candle bush leaf extract, notably flavonoids, saponins, and alkaloids. According to **Ikechukwu *et al.* (2013)**, the phytochemical content of *C. alata* leaf includes alkaloids (1.14mg/ mL), flavonoids (0.36mg/ mL), saponins (1.14mg/ mL), phenols (0.28mg/ mL), oxalates (0.26mg/ mL), and phytates (0.34mg/ mL).

Flavonoids are distributed in the leaf, flower, and stem of *C. alata* **(Kuati, 2013)** and are known for their antibacterial, antioxidant, and anti-inflammatory properties. The plant also contains anthraquinones and carbohydrates **(Egra *et al.*, 2019)**. Further analysis by **Lumbessya *et al.* (2013)** identified 26.86mg/ mL of flavonoids in *C. alata* leaves, primarily consisting of 3,5,7,4′-tetrahydroxy flavone and 2,5,7,4′-tetrahydroxy isoflavone. UV-VIS spectral analysis has confirmed the presence of aromatic groups (benzene rings) at 220 nm, with peak total flavonoid content observed at 330nm **(Lumbessya *et al.*, 2013; Putra, 2019)**.

C. alata possesses strong therapeutic potential and has been traditionally used to treat infectious diseases such as syphilis, bronchitis, tinea versicolor, ringworm, eczema, and malaria **(Egra *et al.*, 2019)**. It also exhibits anti-hyperglycemic activity and has been shown to reduce pancreatic damage and blood glucose levels **(Lumbessya *et al.*, 2013; Naowaboot & Piyabhan, 2016)**.

The mechanism of antibacterial action by these secondary metabolites is well documented. Flavonoids inhibit the formation of protein complexes on the bacterial cell membrane, disrupting essential functions. Alkaloids interfere with cell wall synthesis, altering membrane permeability and impairing protein transport and structural integrity, which ultimately leads to cell lysis **(Pelczar & Chan, 1988; Rahmawati *et al.*, 2015; Nugraha *et al.*, 2017; Nik Mohamad *et al.*, 2022)**. Saponins interact with phospholipid bilayers, compromising cell membrane integrity and causing morphological changes that result in bacterial cell death **(Oktavia *et al.*, 2017; Egra *et al.*, 2019; Roy *et al.*, 2022)**.

The lack of inhibition zones against *E. aerogenes* may be attributed to the bacterium's capacity to develop self-defense mechanisms against the bioactive compounds in *C. alata*. **Sanders and Sanders (1997)** reported that *Enterobacter* species can neutralize antibiotics through enzyme inactivation (e.g., proteases, amylases, cellulases), modification of drug targets, and limited drug uptake. Additionally, **Halim (2003)** and **Huda (2016)** noted that *E. aerogenes* can develop antibiotic resistance through the production of degrading enzymes and the presence of resistance genes located on plasmids and chromosomes, which regulate efflux pumps and alter metabolic pathways.

CONCLUSION

The candle bush (*Cassia alata* L.) leaf extract demonstrated the ability to inhibit the growth of *Aeromonas hydrophila* and *Edwardsiella tarda*. At a concentration of 100%, the extract produced a mean inhibition zone of 9mm against *A. hydrophila* and 7.7mm at 50%, both of which fall under the moderate inhibition category. Against *E. tarda*, the extract showed greater efficacy, with a mean inhibition zone of 11mm at 100% concentration (strong inhibition) and 7.3mm at 50% (moderate inhibition). However, the extract showed no inhibitory effect on *Enterobacter aerogenes* at either concentration.

Among the tested bacteria, *Edwardsiella tarda* was the most effectively inhibited by the *C. alata* leaf extract. These findings support the potential application of candle bush leaf extract as a natural antibacterial agent for fish disease management, particularly against *E. tarda*.

Nevertheless, further research is needed to explore the broader antibacterial potential of *C. alata* leaf extract, including testing against other fish pathogens and understanding the stability and durability of the extract under various environmental and storage conditions.

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