

A detailed 3D rendering of Actinomycetes, showing a complex network of thick, white, fibrous structures. At the ends of these structures are numerous small, spherical, pinkish-white clusters, resembling microscopic fungi or bacteria. The background is a soft, light blue gradient.

# Asian Journal of Tropical Biotechnology

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### Thesis, Dissertation:

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# Lipase activity and identification of lipolytic bacteria from leather industrial liquid waste in Magetan Small Industrial Area, Indonesia

JEDIDI'AH ABEL MEHOLA, RATNA SETYANINGSIH<sup>✉</sup>, ARI SUSILOWATI

Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret, Jl. Ir. Sutami 36A Surakarta 57126, Central Java, Indonesia. Tel./fax.: +62-271-663375, ✉email: ratna\_s@staff.uns.ac.id

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**Abstract.** Mehola JA, Setyaningsih R, Susilowati A. 2024. Lipase activity and identification of lipolytic bacteria from leather industrial liquid waste in Magetan Small Industrial Area, Indonesia. *Asian J Trop Biotechnol* 21: 61-67. Lipolytic bacteria can produce the lipase enzyme, potentially a lipid biodegradation agent. The bacteria can be found in places that contain lots of fat, one of which is the leather industry wastewater. This research aimed to obtain lipolytic bacteria that have the potential as biodegradation agents and observe the lipase enzyme activity from small industrial lather wastewater in Magetan, Indonesia. This research was conducted in several steps, including isolation, screening, lipase enzyme activity assay, and identification of lipolytic bacteria. Isolation of lipolytic bacteria using minimal media enriched with olive oil. The screening of lipolytic bacteria was carried out using Rhodamine B agar, a method that has proven effective in such studies. The enzyme activity was determined using a spectrophotometric method, with p-nitrophenyl palmitate (p-NPP) as the substrate at  $\lambda = 410$  nm. The lipolytic bacteria that exhibited high enzyme activity in each treatment were identified based on the 16S rRNA gene encoding sequence using the Basic Local Alignment Search Tool (BLAST) on the NCBI website <https://www.ncbi.nlm.nih.gov/>. The research showed significant findings; four isolates of lipolytic bacteria were discovered, each with unique characteristics. The highest lipase activity was observed in the form of crude enzyme. Further precipitation with ammonium sulfate revealed that isolate Q exhibited the highest enzyme activity among the other isolates in the 30-45% fraction. The 16S rRNA gene sequence analysis indicated that isolate Q shared a 100% similarity with *Stenotrophomonas maltophilia*.

**Keywords:** Identification, leather industrial wastewater, lipase, lipid biodegradation, lipolytic bacteria

## INTRODUCTION

Magetan District, East Java, Indonesia is famous for its leather craft products. The leather tanning industry is in the Magetan Small Industrial Area (SIA). At Technical Implementation Unit SIA Magetan, there are 35 tannery industries, all of which depend on the Same Wastewater Treatment Plant (WWTP). The leather industry uses large amounts of chemicals and water. The leather industry's operational process produces liquid, solid, and gas wastes; the three wastes, liquid waste, are the most generated. The tanning industry grew rapidly; however, the Magetan leather industry activities' negative impact is the Gandong River's environmental pollution (Perdana 2018). The leather tanning industry can potentially cause physical and chemical environmental changes. Environmental pollution from this type of waste can be identified quickly from changes in odor, color, clarity, and taste. This pollution ultimately threatens the living things and the environment around the industrial area (Azwar 1996). The remaining fat will cover the water's surface and prevent light from entering the water, inhibiting biological processes. One effort that can be made to overcome waste problems is with environmentally sound wastewater treatment technology through a biodegradation process by bacteria.

Furthermore, to obtain lipid biodegrading bacteria naturally, this can be done by isolating them from the waste (Fidiastuti and Suarsini 2017). Bacteria that can degrade oil

and fat can produce lipase enzymes. Based on its physiological function, lipase plays an essential role in the hydrolysis of fats and oils into fatty acids and glycerol, which are needed in metabolic processes (Gupta et al. 2011). Lipase (Triacylglycerol acylhydrolase EC 3.1.1.3) is a hydrolase class enzyme that catalyzes the hydrolysis reaction of triglycerides into glycerol and free fatty acids on the surface of water and oil. Bacteria-producing extracellular lipase is very important for commercial purposes because mass production is relatively easy (Treichel et al. 2010). Lipolytic bacteria that produce lipase enzymes are necessary to degrade fats in wastewater. Lipolytic bacteria can eliminate the pre-treatment sewage process or at least reduce the burden on the pre-treatment process in the waste processing process (Oktavia and Wibowo 2016). Wardani et al. (2015) studied the isolation of the diversity of indigenous bacteria in Malang's leather tanning industry's wastewater treatment plant. The indigenous bacteria found in the WWTP were proven to utilize pollutants in the waste. Because they can use pollutants, these bacteria have the potential to act as biodegradation agents. However, the identification of lipolytic bacteria and lipase enzyme activity of lipolytic bacteria from leather industry waste has not yet been carried out. Several types of lipolytic bacteria include *Achromobacter*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Burkholderia*, *Chromobacterium*, and *Pseudomonas* (Gupta et al. 2004).

The promising potential of the lipolytic bacteria identified in this research is a milestone for leather industry waste management, which is necessary in the metabolic process. These bacteria, capable of thriving in waste conditions, can be introduced to the activated sludge tank at WWTP Magetan SIA. Their production of lipase enzymes can significantly enhance the waste processing process, particularly in reducing lipid levels in leather industry waste. The significance of this research lies in its potential to isolate and identify lipolytic bacteria from Magetan SIA liquid waste. By the 16S rRNA coding gene sequence, we can determine the specific species of lipolytic bacteria and test the activity of the lipase enzyme that it produces. These findings were innovations in the leather industry's waste management, offering a sustainable solution to environmental challenges.

## MATERIALS AND METHODS

### Material

The bacteria source was meticulously selected from the liquid waste of the WWTP Magetan SIA. The carefully prepared media used in this research consisted of three types: Nutrient Agar, minimal media with olive oil, and Rhodamine B agar. Morphological observations, conducted with utmost precision, used materials such as 0.85% NaCl, crystal violet, iodine, acetone alcohol, safranin, distilled water, and immersion oil.

Media for lipase production consisting of 0.5% (w/v) peptone, 0.3% (w/v) yeast extract, 0.625% (v/v) NaCl, 0.01% (v/v)  $MgSO_4 \cdot 7H_2O$ , 0.01% (v/v)  $CaCl_2 \cdot 2H_2O$  and 1% (v/v) olive oil. Materials for enzyme activity testing are ammonium sulfate ( $(NH_4)_2SO_4$ ), 4-Nitrophenol, distilled water, p-nitrophenyl palmitate (p-NPP), isopropanol, Gum Arabic, Triton X-100, and 50 mM Tris-HCl buffer pH 8.0. The molecular identification stage of lipolytic bacteria uses the FavorPrep™ Tissue Genomic DNA Extraction Mini Kit, DNA marker, loading dye, TAE buffer (Tris base, Acetic acid, and EDTA), DNA polymerase, agarose, PCR mix, forward primer 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3'), reverse primer 1387r (5'-GGG CGG WGT GTA CAA GGC-3'), GelRed, and nuclease-free water.

### Instruments used

**Waste collection:** Measurement cups, Duran bottles, pH meters, and thermometers are the tools used for sampling liquid waste from the leather industry.

**Isolation and screening of lipolytic bacteria:** The tools used at this stage, including the versatile Biology Safety Cabinet (BSC), Petri dishes, test tubes, beakers, Erlenmeyer, measuring cups, Duran bottles, spatulas, stirring rods, Drigalsky rods, inoculation needles, vortexes, analytical balances, Bunsen burners, UV lamp, micropipette (P2, P20, P200, P1000), and incubator, are designed to maximize efficiency and accuracy in our procedures.

**Lipase enzyme activity test:** The tools used include an incubator shaker, photometer, spectrophotometer, centrifuge, Eppendorf tube, analytical balance, cuvette, Erlenmeyer, ice pack, magnetic stirrer, and freezer.

**Identification of lipolytic bacteria:** This stage uses a stereo microscope, binocular microscope, glass slide, cover glass, spin column, micropipette, PCR tube, gel electrophoresis, gel documentation, incubator, microcentrifuge, thermal cycler, chiller, and freezer.

### Procedures

#### Sample preparation

Waste is collected using the grab sampling method, and one bag of waste is collected directly from the homogenization tank at a time. Temperature and pH parameters were measured.

#### Isolation and screening of lipolytic bacteria

A total of 1 mL of waste sample was dissolved in 9 mL of saline solution (0.85% NaCl), then homogenized until mixed, and a 10<sup>-1</sup> dilution was obtained. Dilution was carried out in a 10<sup>-5</sup> dilution series. Microorganisms were grown (plated) on nutrient agar media using the spread plate method by adding 0.1 mL of microorganism source to each Petri dish and then leveling using a Drigalsky. Isolates were incubated for 24-48 hours at 37°C (Susanty et al. 2013). Each colony that grows and has a different morphology is then streaked back onto a minimal agar medium enriched with olive oil using the streak plate method. Isolates were incubated for 48 hours at 37°C. According to Kumar et al. (2012), screening was done by taking one isolate and then streaking it on Rhodamine B agar media enriched with olive oil. The bacterial isolate was then incubated at 37°C for 48 hours. Positive results can be seen by the hydrolysis of olive oil into fatty acids, which then interact with Rhodamine B so that pink or orange colonies appear, which fluoresce when illuminated using a UV lamp with a wavelength of 365 nm.

#### Morphological observations of lipolytic bacteria

Pure colonies grown on Nutrient Agar media were observed using a stereo microscope. Macroscopic morphological observations are carried out by looking at bacterial colonies' shape, edges, elevation, and color at appropriate magnification. The gram staining method was used for these observations. The preparations were observed from the smallest to the largest magnification under a microscope. Gram-positive bacteria will be blue or purple, and negative bacteria will be red (Yulvizar 2013).

#### Lipase enzyme activity test

According to Saadatullah et al. (2018), there are several stages in measuring lipase enzyme activity, namely:

**Creating of lipolytic bacterial growth curves:** One mL of bacterial culture in an enzyme production medium was put into the cuvet. The absorbance value was measured using a spectrophotometer with a wavelength of 600 nm. Absorbance was measured every three hours during 48 hours of observation to obtain a growth curve for lipolytic bacteria (Oktavia and Wibowo 2016).

**Lipase enzyme production:** The media composition for lipase production is 0.5% peptone, 0.3% yeast extract, 0.625% NaCl, 0.01%  $MgSO_4 \cdot 7H_2O$ , 0.01%  $CaCl_2 \cdot 2H_2O$ , and 1% olive oil at pH 7. A total of 5 mL Overnight culture

in NB media was added to 50 mL of lipase production media and incubated in an incubator shaker at a speed of 120 rpm and a temperature of 37°C. After reaching the optimal incubation time, the culture was centrifuged at 4,500 rpm for 20 minutes at 4°C, and the supernatant was used as a source of extracellular lipase enzyme (crude enzyme).

**Precipitation of lipase enzyme with ammonium sulfate:** Duong-Ly and Gabelli (2014) stated that the lipase enzyme was precipitated using the enzyme precipitation method by adding ammonium sulfate (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. The crude extract of the lipase enzyme was precipitated in stages using ammonium sulfate with precipitation fractions of 0-30%, 30%-45%, and 45%-60%. For 0-30% precipitation, 2.46 g of ammonium sulfate was added to 15 mL of crude lipase extract to obtain supernatant I and pellet I. For 30-45% precipitation, 1.118 g of ammonium sulfate was added to supernatant I to obtain supernatant II and pellet II. Then, for 45-60% precipitation, 1.125 g of ammonium sulfate was added to supernatant II to obtain supernatant III and pellet III. Ammonium sulfate was added slowly and then homogenized using a magnetic stirrer. The mixture was left overnight in a 4°C freezer. The protein precipitate was separated by centrifugation at 4,500 rpm at a temperature of 4°C for 20 minutes. Each pellet in each fraction was resuspended in 1 mL 50 mM Tris HCl buffer pH 8 and used for enzyme activity assays. Lipase activity was measured using p-nitrophenyl palmitate (p-NPP) as a substrate. Substrate preparation using solution A (30 mg p-NPP dissolved in 10 mL isopropanol) was added to 90 mL solution B (0.1-gram Gum Arabic and 0.4 mL Triton X-100 dissolved in 50 mM Tris-HCl buffer pH 8.0), all ingredients were stirred until wholly dissolved with a final volume of 100 mL (Ertuğrul et al. 2007). Enzyme activity measurements were performed by mixing 1.8 mL of substrate solution and 0.2 mL of crude enzyme or pellet resulting from precipitation and incubating at 30°C for 15 minutes. Absorbance was measured using a UV-Vis spectrophotometer with a wavelength of  $\lambda = 410$  nm.

#### *Identification of lipolytic bacteria*

Lipolytic bacteria with the most stable lipase enzyme activity will be identified molecularly. Lipolytic bacterial genomic DNA extracted from the screening process was extracted using the FavorPrep™ Tissue Genomic DNA Extraction Mini Kit. Amplification of the 16S rRNA gene encoding lipolytic bacteria using primers, namely 63 forward primers (63f: 5'CAGGCCTAACA CATGCAAGTC-3') and 1387 reverse primer (1387r: 5'-GGGCGGAWGTGTACAAGGC-3') (Marchesi et al. 1998). The PCR method was carried out by mixing 1 µL 63 forward primer with a concentration of 10 pmol, 1 µL 1387 reverse primer with a concentration of 10 pmol, 25 µL MyTaq™ HS Red Mix 2x, and 2 µL DNA template, and 21 µL ddH<sub>2</sub>O. Pre-denaturation was carried out at 95°C for 1 minute. One PCR cycle of 35 cycles consists of denaturation at 95°C for 15 seconds, annealing at 55°C for 15 seconds, elongation at 72°C for 15 seconds, and finalizing at 72°C for 7 minutes. Next, PCR was stopped, and the PCR amplification product was stored at 4°C. PCR amplification products were observed using gel electrophoresis. Sequencing of PCR amplification results

of the 16S rRNA gene encoding lipolytic bacteria was carried out at 1st Base Apical Scientific Malaysia. The 16S rRNA sequence obtained was analyzed using the Nucleotide BLAST device on the NCBI website ([www.blast.ncbi.nlm.nih.gov/blast.cgi](http://www.blast.ncbi.nlm.nih.gov/blast.cgi)) (Waturangi et al. 2008).

#### **Data analysis**

The bacterial isolates and the lipase enzyme activity data for each bacterium are explained descriptively. The 16S rRNA sequences obtained were analyzed using the BLAST Nucleotide tool on the NCBI website. The higher the identity percentage, the higher the similarity of the sequence being searched for with the reference sequence in Gene Bank (Hall 2001). The criteria for species identification are a minimum of 99% sequence similarity, and the ideal percentage is  $\geq 99.5\%$  (Janda and Abbot 2007).

## **RESULTS AND DISCUSSION**

#### **Waste characteristics**

The liquid waste taken is from the homogenization tank at the Wastewater Treatment Plant Magetan SIA. Waste has a pH of 9 with a waste temperature of  $\pm 30^\circ\text{C}$ . The liquid waste looks cloudy brown; there is a layer of fat on the surface of the wastewater; there is still hair, skin, and animal flesh remaining, and it also observed fat foam clumping in several corners of the homogenization tank (Figure 1). The remaining fat in the waste will appear on the water's surface. This layer of fat can prevent sunlight from entering the water and the environment becomes aerobic and inhibits water biological processes.

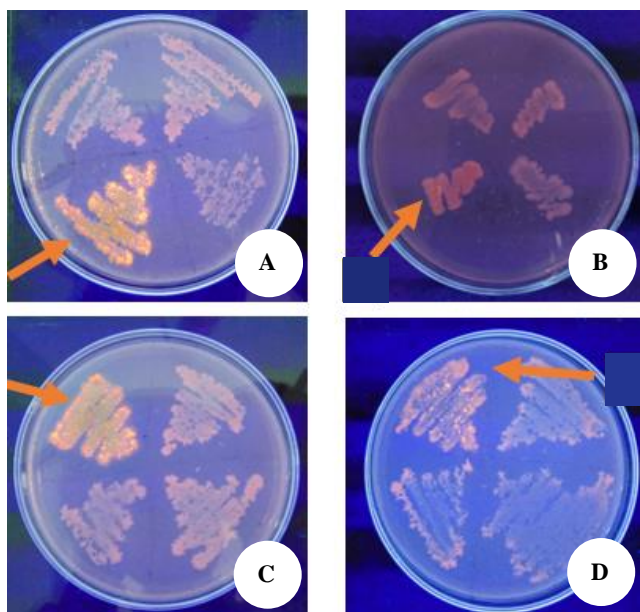
#### **Lipolytic bacteria isolate**

The process of isolating the lipolytic bacteria was conducted with scientific rigor. In the initial stage, 44 bacterial isolates were obtained from the 10<sup>-5</sup> serial dilution and then inoculated onto minimal agar media. This meticulous process yielded 22 bacterial isolates that demonstrated growth. Each isolate was carefully labeled, resulting in the isolates' identification A, B, C, D, E, F, G, H, I, J, K, L, M, N, O, P, Q, R, S, T, U, and V. Olive oil was selected as the substrate for the minimal medium due to its easy availability and digestibility by bacteria (Susilowati et al. 2018).

Four positive lipolytic bacterial isolates were isolate D, isolate H, isolate M, and isolate Q. Positive lipolytic activity was characterized by the appearance of isolates that glowed brightly and were orange or pink when exposed to UV light with a wavelength of 350 nm (Figure 2). Rhodamine B Agar was chosen because it is a sensitive fluorescent indicator for free fatty acids. Olive oil was used as a lipase substrate, and rhodamine B indicated lipase activity. The lipase enzyme is different from other hydrolytic enzymes because it can work on interfacial substrates. Lipase works actively on substrates that cannot be dissolved in water, such as triglycerides, composed of long-chain fatty acids. The interaction of rhodamine B with fatty acids released during triglyceride hydrolysis causes fluorescence around the colony under ultraviolet radiation (Rahayu 2018).

**Table 1.** Macroscopic and microscopic morphological characteristics of lipolytic bacterial isolates

Isolate name	Colony morphology				Cell morphology	
	Shape	Color	Elevation	Edges	Grams	Cell shape
D	Circular	Purple	Umbonate	Scalloped	+	Bacillus
H	Irregular	White	Flat	Undulate	+	Coccus
M	Circular	Purple	Raised	Entire	+	Coccus
Q	Punctiform	Cream	Convex	Entire	-	Bacillus

**Figure 1.** Wastewater treatment plant in a small industrial area in Magetan, East Java, Indonesia. A. Homogenization/equalization tank; B. Homogenization tank wastewater**Figure 2.** Lipolytic bacterial colonies on Rhodamine B Agar Media under a 350 nm UV lamp exposure. A. Isolate D; B. Isolate H; C. Isolate M; D. Isolate Q

According to Kasipah et al. (2013), the hydrolysis reaction of olive oil by lipase produces free fatty acids. The complex bond between rhodamine and free fatty acids creates a pink glow when irradiated under UV light. According to Sugiharni (2010), one of the reasons why non-lipolytic isolates can grow on rhodamine B agar media is the possibility that these bacteria produce esterase. An

orange glow will not form when esterase is detected in the rhodamine B medium.

The morphological characteristics of the four bacterial isolates with lipolytic activity were observed for colony and cell morphology. Colony characteristics include shape, color, elevation, and edges; the cell characters include gram characteristics and shape. The macroscopic form of each isolate was very diverse (Table 1).

Observing the morphological characteristics of bacterial colonies makes identifying the type of bacteria easier. However, further tests, namely molecular identification, need to be carried out to obtain accurate identification.

#### Sigmoid growth curve of lipolytic bacteria

Our research has led us to optimize the bacterial growth time, allowing us to determine the precise time for lipase enzyme production. The data obtained from this optimization process has revealed that each bacteria has a unique optimal growth time, as demonstrated in the lipolytic bacterial growth curve. The peak of bacterial growth, or the exponential phase, has been identified as an excellent time to produce enzymes due to bacteria's rapid binary division phase, leading to an abundance of bacterial cells. The turbidity level in the enzyme production medium increases with the incubation period. The turbidity of the medium during the growth of lipolytic bacteria indicates cell density during development, which increases the OD absorbance value (Mira et al. 2022). Bacteria can grow until the nutrients available in the medium have been used up.

The sigmoid growth curve (Figure 3) depicts the increase in lipolytic bacterial cells starting from the

adaptation phase (lag) and rapid growth in the logarithmic (exponential), stationary, and cell death phases.

The time required to divide from one cell into two perfect cells is called generation time, and the ability of each bacterium to reproduce varies from one bacteria to another. Isolate Q was the isolate that reached the fastest exponential peak at 12 hours. Even though they are under the same treatment conditions, differences in generation time occur due to genetic factors in each bacterium. Genetics is an intrinsic factor in a microorganism that is not changed (Sariadji et al. 2015). Lipolytic activity is part of the primary bacterial metabolite activity following the microbial growth phase. It increases optimally at the end of the log (exponential) phase or the beginning of the stationary phase, then decreases with microbial activity and decreasing substrate nutrients (Bestari and Suharjono 2015).

### **Lipase enzyme activity**

Lipase enzyme activity was measured when each isolate peaked its exponential phase grown in enzyme production media. Each isolate has four variations of lipase enzyme treatment: crude enzyme, 0-30%, 30-45%, and 45-60% precipitation with ammonium sulfate (Su'i and Suprihana 2013). Lipase activity was measured using a UV-VIS spectrophotometer  $\lambda$  410 nm and p-nitrophenyl palmitate (p-NPP) substrates. Lipase enzyme activity can be observed in Figure 4; the highest enzyme activity was produced from crude enzyme lipase treatment, with the highest activity from isolate H, namely 705.3 Units/mL. Meanwhile, from the enzyme precipitation, isolate D had the highest enzyme activity of 167.7 units/mL in the 45-60% ammonium sulfate precipitation. The enzyme activity of isolate H when precipitating 45-60% ammonium sulfate had the highest value, 224.5 Units/mL. Isolate M had the highest enzyme activity value at 30-45% precipitation of 344.8 Units/mL. Meanwhile, isolate Q had the highest enzyme activity value of 384.7 units/mL when 30-45% ammonium sulfate was deposited. Apart from that, the enzyme precipitation from isolate Q always had the highest value in each precipitation compared to other isolates. In this study, precipitation with ammonium sulfate did not seem to increase the concentration and activity of the lipase enzyme compared to the crude extract.

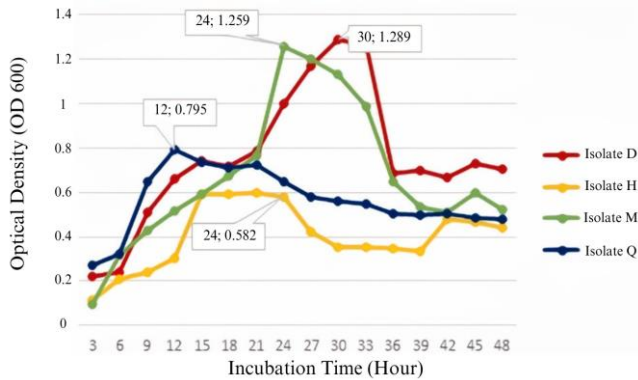
Moreover, adding ammonium sulfate salt, called the salting-out process, causes hydrophobic interactions between proteins and reduces protein solubility so that proteins precipitate, including enzymes (Duong-Ly and Gabelli 2014). According to Lehninger (2004), salting-out is an effort to produce enzymes with better specific activity than crude extract and high enzyme yields. The crude enzyme has higher activity than the precipitated lipase enzyme, and the lipase activity after precipitation decreased drastically compared to the lipase activity of the crude extract. This shows that most of the enzymes are necessary to be appropriately deposited. During precipitation, the ammonium sulfate temperature plays a significant role in enzyme stability. The time the enzyme is stored due to multilevel precipitation with ammonium sulfate also affects the enzyme activity value. The study revealed that the

enzyme activity resulting from multilevel precipitation with ammonium sulfate decreased because the enzyme was stored in the freezer before its activity was measured (Figure 4). Enzyme activity will decrease with the length of storage time (Ardian et al. 2014). Each protein will precipitate at a different salt concentration. Su'i and Suprihana (2013) states hydrophobic proteins will precipitate at low salt concentrations. Meanwhile, hydrophilic proteins require a high salt concentration to precipitate them. The higher activity of the crude enzyme compared to the precipitated lipase enzyme can be attributed to several factors: Protein Conformation and Activity: The crude enzyme preparation typically contains the enzyme in its native, active conformation. During precipitation, the enzyme may undergo conformational changes or partial denaturation, leading to activity loss. Presence of Cofactors and Stabilizers: Crude enzyme extracts often contain various cofactors, stabilizers, and other proteins that help maintain enzyme stability and activity. These may be lost or removed during precipitation, resulting in lower enzyme activity. Enzyme Inhibitors: The precipitation process might introduce conditions (such as changes in pH, ionic strength, or the presence of precipitating agents) that can inhibit the enzyme's activity. In contrast, the crude extract might maintain a more favorable environment for the enzyme. Enzyme Concentration and Aggregation: Precipitation can lead to enzyme aggregation, reducing the availability of active sites for substrate binding. In the crude extract, enzymes are more likely to be in a dispersed, active form. Loss of Enzyme During Precipitation: Some enzyme activity may be lost during precipitation due to incomplete recovery or losses during washing and handling steps.

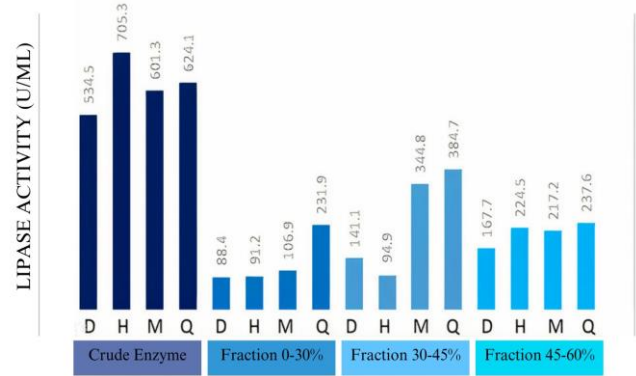
Furthermore, the ammonium sulfate salt ion will affect protein solubility. At low concentrations, salt ions surround protein molecules and prevent protein molecules from joining together. Meanwhile, at high salt concentrations, the electrical charge around the protein increases, attracting water molecules from the protein colloid and causing hydrophobic interactions between proteins, which is called salting out (Scopes 1994). The enzymes from salting out in each fraction are free from non-protein contaminants but still contain non-enzyme proteins (Putri et al. 2013).

### **Lipolytic bacterial species based on molecular identification**

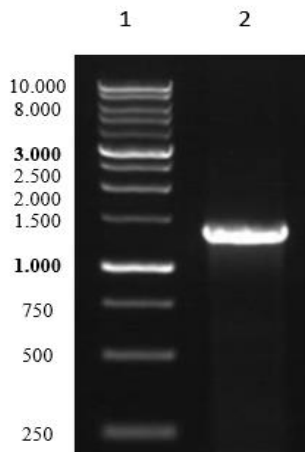
This study identified one isolate of lipolytic bacteria with a unique characteristic of stable lipase enzyme activity. This characteristic, whether in the form of a crude enzyme extract or after going through the sedimentation stage, was most pronounced in isolate Q. This discovery opens up exciting possibilities for further research with significant potential for industrial application improvements. The total enzyme activity value from the sedimentation of isolate Q consistently surpassed other isolates at each deposition level, suggesting its suitability for various industrial processes. The electrophorogram of the PCR product, which provides further evidence of these findings, can be seen in Figure 5.



**Figure 3.** Sigmoid growth curve of lipolytic bacteria in lipase enzyme production media



**Figure 4.** Histogram of lipase enzyme activity from each isolate D, H, M, Q (crude enzyme, ammonium sulfate fraction 0-30%, ammonium sulfate fraction 30-45%, and ammonium sulfate fraction 45-60%)



**Figure 5.** Electrophoregram of PCR products of lipolytic bacteria. Note: 1. 1kb ladder marker, 2. PCR product of isolate Q's 16S rRNA gene

The electrophoregram shows a DNA band measuring approximately 1300 bp. The actual base sequence was 765 nucleotide bases because the sequence was obtained from partial (single-pass) sequencing. These sequences were analyzed using the BLASTN program on the NCBI website and then matched with the Gene Bank database to get the names of the species with the closest relationship and a description of the similarity percentage. Based on the results of BLASTN analysis, isolate Q has a max score of 1411, a total score of 1411, a query cover of 100%, an E value of 0.0, and a percent identity or similarity percentage of 100% with *Stenotrophomonas maltophilia*. A 16S rRNA sequence similarity  $\leq 99\%$  and  $\geq 97\%$  allowed the strain and species identification at the genus level (Drancourt et al. 2000). Isolate Q is Gram-negative and has a bacilli cell shape, creamy white, and small round colonies. This is aligned with the literature stating that *S. maltophilia* is an aerobic Gram-negative bacterium. These bacteria are found widely in aqueous environments, in soil, and on plants and have been used in biotechnological applications (Berg et al. 1999).

Based on Thomas et al. (2014), lipase activity was detected in *S. maltophilia* growth media. The extracellular lipase produced by *S. maltophilia* helps it develop in an environment with limited carbohydrates, making lipids the only carbon source. Research by Larik et al. (2018) also stated that *S. maltophilia* is a lipolytic bacterium that can be used in biotechnology. However, our research went further by isolating and characterizing lipolytic bacteria from a specific environment, making a unique contribution that *Stenotrophomonas* has many important environmental roles. These species in the *Stenotrophomonas* genus have an important ecological role as natural elements in biocontrol and bioremediation agent cycles. The *S. maltophilia* bacteria that secrete solvent were organic-resistant lipases isolated from oil-contaminated samples (Li et al. 2013).

Lipase produced by *S. maltophilia* showed stability in environments containing high hydrophobic solvents and pure hydrophilic solvents for seven days. Lipase activity is high at low temperatures and in the alkaline pH range; this characterizes lipase from *S. maltophilia* for industrial applications involving organic solvents such as organic synthesis (Li et al. 2013). Several researchers studied enzyme activity in processing domestic waste using an activated sludge system. Lipase and protease enzyme extraction in activated sludge evaluate the activity of the lipase enzyme in terms of its degradation of organic compounds in wastewater (Kasipah et al. 2013). Based on the references above, lipase from *S. Maltophilia* isolates obtained from this research is expected to be helpful as a waste biodegradation agent. These practical implications of our research significantly contribute to environmental science and biotechnology.

In conclusion, a total of 4 isolates of lipolytic bacteria were successfully isolated and selected from the homogenization tank waste from WWTP SIA Magetan and were proven to have lipase enzyme activity, namely isolate D, isolate H, isolate M, and isolate Q. The highest lipase enzyme activity was produced in the form of crude enzyme, and from the multilevel enzyme precipitation showed that isolate Q had the most stable and highest enzyme activity among the other isolates; the enzyme

activity of isolate Q was optimal at 30–45% ammonium sulfate precipitation. The 16S rRNA gene sequence from isolate Q has a similarity percentage of 100% with *S. maltophilia*.

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# Hepatoprotective effect of *Ocimum basilicum* aqueous extract against doxorubicin-induced hepatic damage in albino rats

ISLAM BOULAARES<sup>1,2</sup>, SAMIR DEROUICHE<sup>1,2,\*</sup>, IMANE YOUSRA GUEMARI<sup>1</sup>

<sup>1</sup>Department of Cellular and Molecular Biology, Faculty of the Sciences of Nature and Life, El-Oued University. El-Oued 39000, Algeria.  
Tel.: +213-552285234, \*email: dersamebio@gmail.com

<sup>2</sup>Laboratory of Biodiversity and Application of Biotechnology in the Agricultural Field, Faculty of the Sciences of Nature and Life, University of El Oued. El-Oued 39000, Algeria

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**Abstract.** Boulaares I, Derouiche S, Guemari IY. 2024. Hepatoprotective effect of *Ocimum basilicum* aqueous extract against doxorubicin-induced hepatic damage in albino rats. *Asian J Trop Biotechnol* 21: 68-74. This investigation aimed to evaluate the effect of *Ocimum basilicum* L. aqueous extract (ObE) on hepatotoxicity induced by doxorubicin in rats. Standard procedures were used to extract bioactive compounds and to analyze phytochemical compounds quantitatively and qualitatively. The in-vivo study was performed using 15 female albino rats that were grouped into 3 groups (n=5): a control group, a doxorubicin-treated group (DOX), and a group co-treated with doxorubicin and *O. basilicum* aqueous extract (DOX + ObE). Various biochemical, enzymatic, and liver oxidative stress markers were analyzed. Phytochemical results demonstrated that the ObE contained highly phenolic compounds. The results of the in vivo study showed that the treatment of doxorubicin caused a significant decrease of WBC (P<0.01), lymphocyte (P<0.05), also a significant increase in GOT (p<0.05), CPK (p<0.001) and LDH (p<0.05) activities compared to control group/normal rats. In addition, doxorubicin-treated rats resulted in a significant decrease in GSH (p<0.01) and an increase in MDA (p<0.01), SOD (p<0.001), and GST (p<0.01) levels in liver cells compared to the control group. Treatment with ObE partially reversed all of the previously mentioned parameters. This study indicated that basil aqueous extract's antioxidant activity could protect the liver from the harmful effects of doxorubicin or the destructive effects of various liver diseases such as hepatitis or other drug hepatotoxicity.

**Keywords:** Doxorubicin, hepatotoxicity, *Ocimum basilicum*, oxidative stress, rat

**Abbreviations:** DOX: Doxorubicin, GSH: Reduced glutathione, MDA: Malondialdehyde, ObE: *Ocimum basilicum* extract, ROS: Reactive Oxygen Species, TFC: Total Flavonoids Contents, TPC: Total Phenolic Contents, WBC: White Blood Cells

## INTRODUCTION

Cancer is a group of diseases that cause abnormal cell growth and proliferate beyond control (Golemis et al. 2018). It is a major public health problem worldwide (Siegel et al. 2020) and the second leading cause of death after cardiovascular diseases (Lódi et al. 2019). There are many cancer treatments, the most important of which is chemotherapy, considered the most effective and extensively used for most types of cancer (Ibrahim et al. 2022). Doxorubicin is an anthracycline glycoside antitumor antibiotic used as a first-line drug in a mixture of several chemotherapy drugs for various types of cancer (Choi et al. 2020). However, the use and effectiveness of doxorubicin can be compromised by its hazardous side effects on many organs (Lei et al. 2020), especially the liver. These free radicals could harm the hepatic membranes by phospholipid activation and lipid peroxidation, which increase intracellular Ca<sup>2+</sup>, produce ALT, and ultimately lead to apoptotic cell death (Boulaares et al. 2024a).

An imbalance in the ratio of pro-oxidants to antioxidants, emphasizing antioxidants, is called oxidative stress (Guemari et al. 2024). Chronic hepatotoxicity is the primary adverse effects that limit the therapeutic use of anthracycline medications (Boulaares et al. 2024b). Several

studies have shown a relationship between increased Reactive Oxygen Species (ROS) and oxidative stress and doxorubicin-induced toxicity. The three primary ROS that cause toxicity are superoxide radical (O<sub>2</sub><sup>-</sup>), hydroxyl free radical (HO•), and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (Gasser et al. 2019). Antioxidants are the primary defense against molecular oxidative damage (Acila et al. 2024).

Inflammation is another mechanism to induce hepatotoxicity by doxorubicin; the quantities of specific inflammatory chemokines and cytokines, including TNF- $\alpha$ , IL-1 $\beta$ , IL-6, COX-2, and CCL2/MCP-1, are significantly increased by doxorubicin (Guo et al. 2013). Cytokines are essential mediator proteins that facilitate the networking and communication of the immune system. Monocytes or lymphocytes can produce both pro- and anti-inflammatory cytokines. Cytokines with chemotactic action are called chemokines. It is thought that the balance between pro-inflammatory cytokines (IL-1 $\beta$ , IL-2, TNF $\alpha$ , IL-6, IL-8, IFN- $\gamma$ ...) and anti-inflammatory cytokines (IL-10, IL-4, TGF $\beta$ ) is crucial for immune response homeostasis and inflammation, which are the root causes of many disorders (Chetehouna et al. 2024a).

The liver, an essential organ for digestion and detoxification, is the first and most susceptible to toxins (Zhao et al. (2022). It is a vital organ for metabolism in the

body that secretes bile and converts various nutrients into proteins (Aruna and Gayathiri 2018). The liver transforms, neutralizes, and eliminates toxins through hepatocyte-mediated enzymatic detoxification mechanisms, which is the primary mechanism of tissue detoxification (Allameh et al. 2023).

The plants are traditionally used to treat diseases, and the confirmation of their biological effects has stimulated their therapeutic use. Their active ingredients may cure diseases and relieve symptoms (Belhouala et al. 2021). Nevertheless, research on these plants' bioactive components showed that these compounds are used in traditional and modern medicine (Salmerón-Manzano et al. 2020). *Ocimum basilicum* L., locally known as sweet basil, is a popular medicinal plant. Since ancient times, several species in the genus *Ocimum* have been used to treat different disorders and diseases (Boulaares et al. 2024c). The *O. basilicum* is an important species of the genus *Ocimum* because of its many therapeutic uses (Boulaares et al. 2024d). The *O. basilicum* has shown antioxidant activities due to its bioactive compounds (Derouiche et al. 2020). The antioxidant activity of *O. basilicum* aqueous extracts showed potent free radical scavenging activity (Boulaares et al. 2024b). The objective of the present study is to assess the hepatotoxic effects of doxorubicin and to study the hepato-protective properties of therapeutic systems based on *O. basilicum* in rats.

## MATERIALS AND METHODS

### Plant materials

The *O. basilicum* was harvested from the region of EL-OUED (Guemar) in August. The leaves were cleaned and dried out by direct sunlight at room temperature. A mechanical grinder powdered the dry leaves until a fine powder was obtained. The *O. basilicum* powder was stored at room temperature in airtight containers until the experiment was performed.

### Method of aqueous extract preparation

Aqueous extract was prepared by boiling 10 g of dried leaves powder of *O. basilicum* with 100 mL of distilled water over low heat (50°C) for 2 hours. The extract was then cooled and macerated for 24 hours at room temperature and filtered through Whatman filter paper. Then, it was evaporated using a rotary evaporator and dried using an oven (Chetehouna et al. 2024b).

### Phytochemical analysis

Qualitative phytochemical analysis was applied to the aqueous extracts using qualitative characteristics techniques according to the standard method.

### Total Phenolic Contents (TPC)

The following protocol carried out the quantitative analysis of total phenols: for 125  $\mu$ L of the plant extract, add 500  $\mu$ L of distilled water, then add 125  $\mu$ L of Folin-Ciocalteu reagent (FCR) and homogenized. After 5 min, 1250  $\mu$ L of sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) at a concentration

of 7.5 g/L is added to trigger the oxidation-reduction reaction and completed by adding distilled water to 3 mL. The reaction mixture was stirred and kept in the dark for 2 h at room temperature. The absorbance of each solution is determined at 765 nm using a UV-VIS spectrophotometer (Boizot and Charpentier 2006).

A standard calibration curve was obtained from gallic acid solutions of different concentrations (50 -100 - 150 - 200 $\mu$ g/mL) using the same dosage; all measurements were repeated three times.

### Total Flavonoid Contents (TFC)

One mL of each sample and standard (prepared in methanol) is added to 1 mL of the  $\text{AlCl}_3$  solution (2% dissolved in methanol). After 10 minutes, absorbance was measured relative to the reagent blank prepared at  $\lambda_{\text{max}} = 430$  nanometers (Ahn et al. 2004). The concentration of flavonoids is calculated using a calibration curve of quercetin, ranging from (0.01, 0.03, 0.05, 0.07, 0.09 mg/mL). All the measurements are repeated three times; the results are expressed in mg equivalent to quercetin/g of extract.

### Acute toxicity test

The acute toxicity test was performed on albino Wistar rats aged 8 weeks and divided into three groups of five rats each ( $n=5$ ). Animals were fasting for 12 hours before dosing plant extract (each group of rats was treated with extract doses of 2000 and 5000 mg/kgBW. After administering the extract, animals are observed individually after dosing at least once during the first 30 minutes, periodically during the first 24 h, with particular attention given during the first 4 h, and daily after that, for 14 days. The observed parameters were behavioral and physiological, including skin, fur, eyes, tremors, diarrhea, coma, and sleep. The protocol of the acute toxicity based on the OECD guideline for testing of chemicals ( $n^\circ$  423): Acute Oral Toxicity–Acute Toxic Class Method. 1-14 (2001) with the approval from Islam Boulaares no. 03EC/AM/EU2024, February 12, 2024.

### Animal care and experimental design

Thirty female Wistar rats from the Pasteur Institute of Algiers, aged 8 weeks, weighed  $184.84 \pm 8.48$  g. The rats were kept in identical conditions: room temperature, a 15-day adaption period, and a 12-hour light/12-hour dark photoperiod at Echahid University Hamma Lakhdar-El-Oued's Department of Cellular and Molecular Biology. The rats are housed in plastic cages and have free access to water and food by a standard diet. The experiment was conducted for 4 weeks.

Following a time of adaption, the animals were split up into three groups of five for each experiment as follows: (i) Group 1 (Control): were normal rats inoculated with physiological saline (one dose/week, 1.5 mL/kg) for 4 weeks. (ii) Group 2 (DOX): Drug control (Dox) was injected once a week at 1.5 mL/kg for 4 weeks. (iii) Group 3 (DOX + ObE): Rats were injected with Doxo (once a week, 1.5 mL/kg) and supplemented with ObE (200mg/kg)

for 4 weeks. Doxorubicin dose-induced hepatotoxicity used in this study follows AlQahtani et al. (2019).

### Sacrifice, blood sampling, and tissue collection

After 16 h of fasting, the experimental animals were sacrificed under slight anesthesia by chloroform (94%) inhalation. Blood samples were collected into dry tubes at the end of the experiment. Blood samples were centrifugated at 4,000 rpm for 10 min to obtain the serum for enzymatic activity analysis. The liver was carefully sampled, washed with normal saline (NaCl), then weighed and stored at -20°C for oxidative stress analysis.

### Enzymatic activities and hematological markers analysis

Commercial kits (Spinreact) were used to assess the enzymatic activity in the serum, and the hematology autoanalyzer (Sysmex) was used for the hematological analysis (FNS).

### Oxidative stress parameters

About 1g of liver was homogenized in 9 mL of Tris buffer saline solution (pH=7.4). Homogenates were centrifuged at 4,000 rpm for 20 min + 4°C, the obtained supernatant was used to determine antioxidant activity. MDA was measured using the method described by Sastre et al. (2000). The level of reduced glutathione was determined according to Weckbecker and Cory (1988). The SOD activity testing method uses NBT with superoxide anion (O<sub>2</sub>), which is used to detect the presence of SOD using spectrophotometry at 560 nm (Beauchamp and Fridovich 1971). The GSTs were measured according to Habig et al. (1974).

### Statistical analysis

The data were expressed as a percentage or an average ± SD (standard deviation), and the Student's t-test of independent samples was used. All data in this study were analyzed using Minitab 13.0 software. P<0.05 indicates a statistically significant difference.

## RESULTS AND DISCUSSION

### Qualitative and quantitative phytochemical analysis of *Ocimum basilicum*

The phytochemical assays of the aqueous extract of *O. basilicum* showed several chemical compounds, such as flavonoids, steroids, phenols, catechic tannin, saponoside,

carbohydrates, and alkaloids, but did not contain steroid derivatives. (Table 1). The Total Phenolic and flavonoid compounds were expressed in terms of Gallic acid equivalents (mg of GAE/g sample) and of Quercetin equivalents (mg of QE/g sample), respectively, using the following equation based on the calibration curve:  $Y = 0.0045x + 0.009$ ;  $R^2 = 0.995$  for phenolic compounds and  $Y = 0.0096x + 0.0521$ ,  $R^2 = 0.994$  for flavonoids compounds. The total phenolic and flavonoid contents of *O. basilicum* aqueous extract are  $77.66 \pm 3.27$  mg GAE/g and  $16.52 \pm 0.06$  mg of QE/g, respectively (Table 1).

### Acute toxicity assays of *O. basilicum* aqueous extract

The results showed no mortality after 24 hours of administration, suggesting the extracts were non-toxic. The other physiological parameters of the rats, i.e., sleep, diarrhea, tremors, and convulsions, were also determined during the experimental period and showed that treatment of aqueous extract of *O. basilicum* caused no adverse effects on the experimental animals during the treatment period (Table 2)

### Enzymatic activities

As shown in Table 3, the DOX group increased Glutamate Pyruvate Transaminase (GPT), lactate dehydrogenase (LDH), and creatinine phosphokinase (CPK) significantly; however, Glutamate oxaloacetate transaminase (GOT) was decreased significantly ( $p < 0.01$ ) in the DOX than in the control group. Additionally, a significant decrease ( $p < 0.001$ ) in the GPT ( $p < 0.01$ ), GOT ( $p < 0.01$ ), and CPK ( $p < 0.001$ ) was observed in the ObE group as compared to the DOX group.

**Table 1.** Qualitative and quantitative phytochemical compounds of *Ocimum basilicum* aqueous extract

Phytochemical compound	The presence/absent
Flavonoids	+
Unsaturated steroids	+++
Steroids derivatives	---
Phenols	+++
Catechic Tannin	+++
Saponoside	+++
Carbohydrate	+++
Alkaloid	+++
Total phenols (mg GAE/g extract)	$77.66 \pm 3.27$
Total flavonoids (mg QE/g extract)	$16.52 \pm 0.06$

Note: +: Present, -: Absent

**Table 2.** Effect of *Ocimum basilicum* aqueous extract on physiological parameters

Parameters	0 mg/kg					2000 mg/kg					5000 mg/kg				
	0h	3h	7h	14h	24h	0h	3h	7h	14h	24h	0h	3h	7h	14h	24h
Death rats	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Eyes	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Sleep	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Diarrhea	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N

Note: N: Normal

**Table 3.** Enzyme activities in control and experimental groups

Parameters	Control	DOX	DOX+ObE
GPT (UI/l)	47.2±7.68	60±24.70*	42.55±3.92 <sup>b</sup>
GOT (UI/l)	146±15.20	132.5±3.51**	121.7±11.23
CPK (UI/l)	182.5±1.51	1337±60.38***	220.12±31.32 <sup>c</sup>
LDH (UI/l)	1133±137.06	1419±140.37*	1320±55.42

Note: Data are expressed as mean ± SD (n=6). \*p<0.05, \*\*p<0.01, \*\*\*p<0.001: Significantly different from control group, <sup>a</sup>p<0.05, <sup>b</sup>p<0.01, <sup>c</sup>p<0.001: Significantly different from DOX group

**Table 4.** Leukocyte count in control and experimental groups

Parameters	Control	DOX	DOX+ObE
WBC (10 <sup>3</sup> /ul)	3.43±0.27	2.04±0.44**	4.248±0.396 <sup>c</sup>
Neutrophil (10 <sup>3</sup> /ul)	0.78±0.11	0.55±0.09*	1.358±0.16** <sup>c</sup>
Lymphocyte (10 <sup>3</sup> /ul)	1.98±0.18	1.03±0.14*	2.52±0.262 <sup>c</sup>
Monocyte (10 <sup>3</sup> /ul)	0.04±0.00	0.18±0.06***	0.056±0.0116 <sup>c</sup>
Eosinophil (10 <sup>3</sup> /ul)	0.10±0.00	0.06±0.009**	0.0625±0.014*
Basophil (10 <sup>3</sup> /ul)	0.20±0.02	0.04±0.003***	0.22±0.0653 <sup>a</sup>
Platelet (10 <sup>3</sup> /ul)	896.8±67.4	1232±35.5*	1362.5±48.3*** <sup>a</sup>

Note: Data are expressed as mean ± SD (n=6). \*p<0.05, \*\*p<0.01, \*\*\*p<0.001: Significantly different from control group, <sup>a</sup>p<0.05, <sup>b</sup>p<0.01, <sup>c</sup>p<0.001: Significantly different from DOX group

**Table 5.** Markers of liver oxidative stress in control and experimental groups

Parameters	Control	DOX	DOX+ObE
GSH (µmol/g tissue)	6.21±1.5	5.733±0.82**	2.72±0.36*** <sup>c</sup>
MDA (µmol/g tissue)	16.1±1.32	31.5±5.72**	27.41±2.23** <sup>a</sup>
SOD (UI/mg of prot)	0.39±0.06	1.52±0.039***	0.72±0.001* <sup>c</sup>
GST (nmol/min/mg prot)	0.5±0.003	4.5±0.06**	0.21±0.0032*** <sup>c</sup>

Note: Data are expressed as mean ± SD (n=6). \*p<0.05, \*\*p<0.01, \*\*\*p<0.001: Significantly different from control group. <sup>a</sup>p<0.05, <sup>b</sup>p<0.01, <sup>c</sup>p<0.001: Significantly different from DOX group

### Hematological markers

The results of the hematological markers (Table 4) showed a significant decrease in WBC (P<0.01), lymphocyte (P<0.05), eosinophil (P<0.01), basophil (P<0.001), neutrophil (P<0.05), PLT (P<0.05), and a significant increase in monocyte (P<0.01). The findings also demonstrated a significant amelioration in the level of WBC, neutrophil, lymphocyte, monocyte, and basophil in the treatment group compared to the DOX group. This study's findings demonstrated a reduction of monocytes in ObE groups (p<0.001, p<0.01), respectively. Furthermore, no significant (P>0.05) variation in the eosinophil and platelet parameters exists.

### Oxidative stress parameters

Our results (Table 5) showed a significant decrease (p<0.01) in the GSH level of the liver in the DOX group compared to the control. However, the results showed a very significant diminution (p<0.001) of GSH in the ObE group compared to the DOX group. The malondialdehyde (MDA) levels were increased very significantly (p<0.001) in the DOX group compared to the control. The MDA level of the liver treated with ObE was decreased significantly compared to the DOX group. SOD and GST showed a highly significant increase (p<0.01) in the DOX group compared to the control. However, treatment with ObE

presented a highly significant decrease (p<0.01) in the SOD and GST activities compared to the DOX group.

### Discussion

As an anthracycline chemotherapeutic drug, doxorubicin (DOX) (Adriamycin) is used alone or in combination with other treatments/medications to treat various cancer types (Ajzashokouhi et al. 2019). As such, doxorubicin (DOX) is a broad-spectrum anticancer drug with high efficacy in numerous hematological malignancies and solid tumors, but its clinical use is restricted due to its cardiotoxicity (Abboud et al. 2018)

The result of phytochemical screening confirmed the presence of flavonoids, unsaturated steroids, phenols, catechic tannin, saponoside, carbohydrates, and alkaloids in aqueous extracts of the leaves of *O. basilicum* is considered a high source of secondary metabolites, such as glycosides, tannins, phenolic compounds, saponins, flavonoids, and terpenes (Tariq et al. 2016). Terpenes exhibit anti-inflammatory activity (Chetehouna et al. 2024b). Flavonoids exert anti-oxidative effects as free radical scavengers and possess therapeutic potential for osteoporosis and cancer. Moreover, consuming phenolic compounds reduces the risk of liver disease (Rahman et al. 2021). The *O. basilicum* has shown antioxidant activities through its bioactive compounds like flavonoids and

phenolic compounds (Derouiche et al. 2020). The antioxidant activity of *O. basilicum* aqueous extracts showed very strong free radical scavenging activity, and it is a widely used medicinal plant (Boulaares et al. 2024c). Another study by Pistelli et al. (2020) showed that basil contains phenolic antioxidant compounds.

In the present study, doxo-induced hepato-toxicity increases enzymes LDH, CPK, and GPT activities, known as liver function markers. DOX induces immunosuppression by reducing leukocyte levels (WBC, lymphocyte, eosinophil, and basophil). Bioaccumulation of doxorubicin generates free radicals that trigger membrane degradation and liver disruption, leading to elevations of LDH, CPK, ALT, and AST (Boulaares et al. 2020). Doxorubicin, as a chemotherapy treatment, possesses several side effects, including cardiotoxic and hepatotoxic. It suppresses the immune system by decreasing the expression of IL-2, production of the  $\gamma$ -interferon, Natural Killer (NK) cells, proliferation of lymphocytes, and ratio CD4+/CD8+ (Shaty et al. 2019). The levels of pro-inflammatory cytokines, such as IL-1 and TNF $\alpha$ , were significantly increased following doxorubicin administration. Doxo-induced hepatotoxicity is also related to increases in oxidative stress and production of ROS (Gasser et al. 2019). The primary types of cardiotoxicity ROS are superoxide radical (O $_2^-$ ), hydrogen peroxide (H $_2$ O $_2$ ), and hydroxyl free radical (HO) (Hajare et al. 2016).

The treatment of the aqueous extract of *O. basilicum* has resulted in decreased LDH, CPK, and GPT activities compared to the doxorubicin-treated group. The bioactive phytochemical compounds (flavonoids, tannins, and phenolic compounds) may explain their efficacy in cardioprotective capacity (Zhakipbekov et al. 2024). Flavonoids have effectively reduced the increase of CPK activity induced by doxo. The combination of flavonoids with doxorubicin has been proven to reduce the toxicity of doxorubicin (Syahputra et al. 2022). Flavonoids have a high antioxidant capacity, allowing them to mitigate oxidative stress's adverse effects (Derouiche et al. 2022). These results could be due to bioactive compounds that have liver-protective effects. Flavonoids reduced the high level of CPK activity caused by doxorubicin. Therefore, it has been demonstrated that combining flavonoids with doxo could lower doxo cardiotoxicity (Sadzuka et al. 1997). The ability of almost all flavonoid groups as antioxidants has the potential to be applied in various fields. Our phytochemical analysis of *O. basilicum* extract revealed high phenolic and flavonoid concentrations, suggesting potential health benefits. Flavonoids, with their anti-inflammatory and antioxidant properties, could be utilized for developing new drugs or supplements to scavenge free radicals and improve human health through beneficial pharmacological effects (Hozayen and Seif 2011; Derouiche 2020).

In our study, the level of reduced glutathione (GSH) in the liver of the group treated with doxorubicin was significantly decreased compared to the control. These findings align with the study of Antonucci et al. (2021), who stated that Doxo generates free radicals and reduces

the ability to detoxify ROS. Also, our results indicate an increase of MDA, SOD, and GST in the liver of the group treated with doxorubicin. Doxorubicin reduces nicotinamide adenine dinucleotide phosphate (NADPH) cytochrome P-450 reductase enzyme by one electron, which raises malonaldehyde levels (Anber 2018). MDA was the end product of lipid peroxidation (Acila et al. 2024). Doxo-induced oxidative stress is confirmed by the elevation of oxidized lipids (MDA) (Shosha et al. 2023); the pathway involves the formation of a semiquinone free radical by the action of several NADPH-dependent reductases that produce a one-electron reduction of the doxo to the corresponding doxo semiquinone. In the presence of oxygen, redox cycling of doxo-derived quinone-semiquinone yields superoxide radicals (Boulaares et al. 2024a). The application of ObE in the doxorubicin treatment group resulted in a high decrease of MDA in liver homogenate compared to the doxo group. The plant extract decreases the MDA due to the content of phenolic and flavonoid compounds with high antioxidant activities. Flavonoids suppress doxo-induced lipid peroxidation in the heart and liver (Hasnat et al. 2024). The results of our previous phytochemical analysis of *O. basilicum* show the presence of high phenols and flavonoids. It has been demonstrated that the polyphenolic flavonoid protects the liver from various cardiovascular illnesses. Its antioxidant and anti-inflammatory properties, which may control several cellular signaling pathways, are known to be associated with its function (Ma et al. 2017). Furthermore, one of the primary phenolic compounds in *O. basilicum*, Rosmarinic Acid (RA), possesses pharmacological and antioxidant qualities. It was shown that rosmarinic acid inhibits these apoptotic traits by restoring the potential of the mitochondrial membrane and lowering the production of intracellular Reactive Oxygen Species (ROS) (Ferreira et al. 2014).

In conclusion, *O. basilicum* is highly efficient in limiting the risk of doxorubicin in healthy tissues and thus reducing its toxicity, especially in the liver. Treating *O. basilicum* aqueous extract improves enzymatic and hematological markers and reduces oxidative stress. It confirms the effectiveness of these compounds in protecting the body's organs from the side effects of doxorubicin, especially the liver.

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## Review: The potential of *wader* fish (*Puntius* spp.) as a source of food, medicine, and traditional use

HAFIZH MUHAMAD ZEN<sup>1</sup>, FURQON DARMA NURCAHYO<sup>1</sup>, HUSNA' PUTRI NUR 'AZIZAH<sup>1</sup>,  
MURNI NURWULANDARI<sup>1</sup>, DARLINA MD. NAIM<sup>2</sup>, AHMAD DWI SETYAWAN<sup>1,3,✉</sup>

<sup>1</sup>Department of Environmental Science, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret. Jl. Ir. Sutami 36A, Surakarta 57126, Central Java, Indonesia. Tel./fax.: +62-271-669371, ✉email: volatileoils@gmail.com

<sup>2</sup>School of Biological Sciences, Universiti Sains Malaysia. 11800 Penang, Malaysia

<sup>3</sup>Biodiversity Research Group, Universitas Sebelas Maret. Jl. Ir. Sutami 36A, Surakarta 57126, Central Java, Indonesia.

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**Abstract.** Zen HM, Nurcahyo FD, 'Azizah HPN, Nurwulandari M, Naim DMD, Setyawan AD. 2024. Review: The potential of *wader* fish (*Puntius* spp.) as a source of food, medicine, and traditional use. *Asian J Trop Biotechnol* 21: 75-88. Freshwater ecosystems rich in biodiversity are often inhabited by *wader* fish (*Puntius* spp.: Cyprinidae). *Wader* fish are omnivores, consuming various plankton and aquatic insects, and are typically found in river streams with relatively clear water and pH around 6.0-6.5. Its adaptive habitats, including rivers, lakes, and ponds, and widespread distribution from India to Southeast Asia make them critical elements in freshwater ecosystem sustainability. This study aims to maintain the availability of nutritious food, preserve traditional knowledge in medicine, and safeguard the population of *wader* fish for future generations. Morphological analysis is crucial for understanding *wader* fish's characteristics and phylogenetic relationships, with *wader* fish exhibiting diverse morphological traits, including unique body colors and structures. In Indonesia, *Puntius* consists of 33 species, classified into three subgenera: *Puntius*, *Poropuntius*, and *Barbodes*. Besides providing significant economic value, *wader* fish also play a crucial role in preventing community malnutrition. They are an essential protein source and offer various health benefits. The morphology of *wader* fish includes a compact body shape, flat head, and various colors and patterns on its body. As a nutritious food source, *wader* fish are rich in protein, fats, vitamins, and minerals that benefit humans. Additionally, the potential uses of *wader* fish in medicine are diverse, including antioxidant, antibacterial, anti-inflammatory, immunity, and liver health benefits. For further research, exploring the specific mechanisms by which bioactive compounds in *wader* fish, such as antimicrobial properties and antioxidant effects, contribute to human health is recommended. Understanding these mechanisms and effects can offer deeper insights into the potential therapeutic applications of *wader* fish in traditional and modern medicine.

**Keywords:** Food, medicine, potential, traditional use, *wader*

### INTRODUCTION

Freshwater ecosystems host the most extensive diversity of ecosystems but face significant threats (Roesma et al. 2016). *Wader* fish (*Puntius* spp.), a term for small fish in the family Cyprinidae, are freshwater fish classified in the *Puntius* spp. (Yang et al. 2015). Migration in fish is influenced by fitness and habitat landscape (Prasetyo and Retnoaji 2020). *Wader* fish shoal and migrate to areas with ample food sources (Sumiarsih and Eddiwan 2018). As omnivores, they consume plankton, algae, aquatic insects, and larvae. *Wader* fish are typically found in slow-flowing rivers with a pH of 6.0-6.5 (Pratami et al. 2018). Their adaptive habitat extends to lakes (Chavan and Kumbhar 2020), rivers, rice fields, irrigation channels, and fish ponds (Desrita et al. 2018). For breeding, they need clear water, moderate currents, and good oxygenation (Hertika et al. 2021). Murky waters hinder respiration, reproduction, and growth (Kjelland et al. 2015). The size and condition of the fish are crucial for reproduction rates (Kant et al. 2016). They are widely distributed in India and Southeast Asia (Patel et al. 2020).

In Indonesia, *wader* fish can be found on various islands, such as Java, Sumatra, Kalimantan, and Sulawesi (Yustian and Zulkifli 2022). The distribution of fish is greatly influenced by environmental factors, including biotic, abiotic, technological, and human activities (Putri et al. 2021). A water body's biotic and abiotic factors can also impact the structure and density of fish (Miranda and Miqueleiz 2021). Additionally, physical and chemical aspects estimate the stages of oogenesis and reproduction (Susatyo et al. 2022). The diversity of fish species in a water region affects sustainability and conservation efforts (Su et al. 2021). Hematology can be used as an indicator of fish health with measurements of erythrocytes, leukocytes, hemoglobin concentration, hematocrit, and micronucleus tests (Arfiati et al. 2020), demonstrating responses to changes related to water quality, nutrition, and disease (Fazio 2019). *Wader* fish have promising economic value (Kumar et al. 2021a) and also have a major component in tropical fish trade (Gupta et al. 2018), contributing to high protein intake (Setiyoko et al. 2022). Nutritionally, these fish are a source of protein, carbohydrates, and essential

micronutrients to help prevent malnutrition in communities (Hossain et al. 2018).

Morphological analysis is conducted to understand the phylogenetic relationship from evolutionary aspects such as morphological, behavioral, and ecological characteristics (Matsuura 2015), including fish performance, morphometrics, and meristics (Rahayu and Nugroho 2014). Knowledge of the morphology and characteristics of *wader* fish is crucial for sustainable management (Prajapati et al. 2022). *Wader* fish has great potential as a highly nutritious food source and valuable traditional medicinal ingredients; it provides a high supply of animal protein in food sources that play an important role in the health of the human body (Pratiwi et al. 2017). Regular consumption of *wader* fish can prevent various diseases and fulfill essential nutritional needs for growth and development (Fiqriani et al. 2023). Apart from being a food source, *wader* fish also plays a role in medicine with bioactive compounds that have long been used to treat various diseases and health problems. Its benefits in medicine can be in the form of external treatment, such as on open wounds, and internal treatment, such as relieving inflammation in the joints. Even though *wader* fish is a food ingredient, it is part of local culture and traditions in some regions. For example, in some traditional ceremonies or festivals, *waders* are used as a symbol of fertility or good luck. Although not fully recognized scientifically, using *wader* fish in traditional medicine is important to cultural heritage and local knowledge. Preserving traditional knowledge in medicine, such as using *wader* fish, is crucial as it not only provides effective treatments but also preserves the cultural identity of communities.

Understanding the benefits of *wader* fish is not only important for maintaining the availability of nutritious food but also important for preserving traditional knowledge in medicine. If understood more deeply, the nutritional content of *wader* fish will result in significant benefits in the development of *wader* fish utilization. It can positively impact the protection of *wader* fish populations by promoting the sustainable utilization of resources; this will ensure their availability for future generations while supporting growth in the health and economic sectors.

#### DISTRIBUTION AND HABITAT OF *Puntius* spp.

According to the 2013 Marine Life Census, there are over 23,000 species worldwide, with approximately 12% being fish species (2,760-3,000 species) (Darmanto 2019). Indonesia has approximately 1,248 freshwater species, constituting about 9% of the total freshwater species worldwide (Kristanto et al. 2014). *Puntius* spp. is a native species that used to be abundant in rivers, streams, ponds, and floodplains in South and Southeast Asian countries (Arunkumar et al. 2017). According to Roesma et al. (2018), The generic name *Puntius* spp. was introduced by Hamilton in 1822. Later, several synonym names like *Barbonymus*, *Barbus*, *Barbodes*, *Systomus*, *Capoeta*, and *Hypsibarbus* were used by researchers as generic names, although these have not been widely accepted. *Puntius* spp.

is commonly known as the silver barb or spotted barb and is one of the most important commercial fish for food and freshwater aquariums. It has the most significant number of species among the Cyprinidae family and inhabits various freshwater environments. *Barbus* is typically used in Europe, while *Puntius* is used in Asia, especially in India, where *Puntius* spp. has a high abundance and variety of species that, according to Hamilton, are very commonly found (Kapuri et al. 2020). These fish play a significant role in their native habitats, contributing to biodiversity and serving as important species in local ecosystems and fisheries. The widespread presence and ecological importance of *Puntius* spp. species underscore the need for conservation efforts to preserve their populations amidst environmental changes and anthropogenic pressures. In addition, its cultural and economic value in many local communities, where it is a highly nutritious fish that commands a high price, further underscores the importance of prioritizing the conservation and sustainable management of this species over other species that may not provide similar ecological or socioeconomic benefits (Munandar and Eurika 2016). *Puntius* spp. is known as a freshwater fish with high economic potential, and its abundant availability in nature is also one of the reasons why *Puntius* spp. should be prioritized over other fish species (Azzahra et al. 2024).

In Indonesia, the genus *Puntius* consists of 33 species distributed across various aquatic regions. Based on their scale structures, the genus *Puntius* is further classified into three main subgenera: *Puntius*, *Poropuntius*, and *Barbodes* (Haryono 2006). According to Damayanti et al. (2022), observations of the morphological characteristics of *wader* fish in Sempor Reservoir, Central Java, Indonesia revealed that the exact species could not be identified using the fish identification guide by Kottelat et al. (1993). This challenge arises due to the similarities in morphological traits among several species, including *Puntius anchisporus* (Vaillant, 1902), *Puntius microps* (Günther, 1868), and *Puntius parrah* (Day, 1865). Key characteristics for, *P. anchisporus* has a yellowish body, a Standard Length (SL) of 45 mm, dorsal fin formula D.II.10, and 5½ transverse body scales. *P. microps* is dark-colored, with an SL of 79 mm, 21-23 lateral line scales, dorsal fin formula D.I.8, and distinctive circular projections on its scales. Meanwhile, *P. parrah* is silvery-black, with an SL of 65-70 mm, 25 lateral line scales, and a dorsal fin formula of D.III.8. All three share an abdominal fin formula of V.I.8, but differences in lateral line scales and dorsal fin structure serve as the primary distinguishing traits.

Their habitats include natural lakes, flooded marshes, and rivers unaffected by strong currents and sedimentation. *Wader* fish prefers calm water habitats overflowing water. They are usually found on the riverbed, in floodplain areas, and occasionally in reservoirs (Apriliani et al. 2018). The preference for such habitats highlights the adaptability and ecological significance of *wader* fish in maintaining the biodiversity of freshwater ecosystems. These fish are commonly found in ponds, reservoirs, and rivers with clear water (Firmansyah et al. 2015). Although they have an adaptive habitat, *wader* fish require relatively cool

temperatures, clear water, moderate currents, and adequate oxygen for their reproductive processes. Vegetation also affects water quality, providing a more stable zone for *wader* fish to live and reproduce. Changes in environmental conditions related to water quality, including temperature, pH, and oxygen content, due to pollution or habitat destruction can affect their well-being and survival. The blood profiles of *wader* fish also indicate water quality changes (Hertika et al. 2021). *Wader* fish is one of the freshwater fishes that is gaining popularity as a fishery commodity in Indonesia (Apriliani et al. 2018). Its ability to adapt well makes it widespread in various freshwater habitats, where it plays an important role in maintaining ecosystem balance by controlling populations of other organisms and becoming a food source in the food chain.

### SPECIES DESCRIPTION/MORPHOLOGY OF WADER FISH (*Puntius* spp.)

#### Body

The body size and shape of *wader* fish (*Puntius* spp.) can vary depending on the species. Generally, they possess small to medium-sized bodies with a slender appearance and a well-proportioned head. The shape and size of their fins, including the caudal, dorsal, pelvic, and pectoral fins, also vary depending on the species and their environment. *Wader* fish typically have compact bodies with dorsolaterally flattened heads. Their bodies are covered in regularly shaped cycloid scales, providing additional protection and enhancing their aquatic capabilities, with a distinctive black and pearlescent coloration. A line of curved ribs runs along their sides, with approximately 22-23 scales (Damayanti et al. 2022). Common features of the *wader* fish include circuli on scales that do not curve backward (Damayanti et al. 2022). These fish have elongated slender bodies with small dorsal and anal fins. They are relatively small, typically ranging from 5 to 10 cm. The length and weight of the fish vary according to gender, gonad maturity level, season, and even time of day (feeding). This variability in size and weight indicates the adaptability and resilience of *wader* fish in diverse environmental conditions. Morphological characteristics of *wader* fish can also be seen from the mosaic structure of their scales (Lubis et al. 2021). *Wader* fish has various species with different physical characteristics commonly observed in size (Utomo et al. 2023).

#### Mouth

Their mouths are positioned subterminally and can be protracted. Each corner of the mouth has a pair of barbels, and their operculum is pearlescent. Their mouths are at the snout's tip, facing upwards, reflecting their surface feeding habits (Ardiansyah et al. 2024). This mouth positioning is well-adapted for their feeding strategy, which involves capturing prey from the water surface. The shape of the mouth of the *wader* fish can also vary depending on the species and its diet. Generally, the shape of the *wader's* mouth can be classified into several types: terminal,

superior, and inferior. The terminal mouth shape of a *wader* fish is a mouth located at the tip of the head and facing forward (Budiantoro et al. 2024). This mouth shape is often found in predatory *wader* fish species actively pursuing prey, such as *wader* fish that feed on insects or small fish. The terminal mouth allows the fish to capture and swallow prey quickly. At the same time, *wader* fish with a superior mouth has a mouth located at the top of the head and slightly jutting upwards. This mouth shape is often found in *wader* fish that forage for food on the water's surface, such as plankton or small invertebrates. The superior mouth of some *wader* fish allows them to filter food from the water's surface effectively. In contrast, species with an inferior mouth have mouths located at the bottom of their heads, jutting downward. This mouth shape is typically found in species that forage for food on the substrate, such as detritus or bottom-dwelling invertebrates. The inferior mouth enables these *wader* fish to gather food from the bottom substrate efficiently. Overall, the subterminal mouth shape in *wader* fish reflects adaptations to their specific diet and environment (Setyaningrum et al. 2020).

#### Fins

The fins of *wader* fish (*Puntius* spp.) are also important parts of their morphology. Fins on *wader* fish have different fin structures and size variations depending on the species and habitat environment of the *wader* fish. Some species of *wader* fish may have longer or shorter fins, some have thicker or thinner fins, and some have fins with different numbers or shapes within the species. Their dorsal fins are tall and erect, providing good maneuverability when swimming. These strong dorsal fins help *wader* fish adapt to various aquatic environments, from fast-flowing rivers to calm marshes. The fins of *wader* fish also adapt to the type of habitat they live in, such as rivers, lakes or swamps. For example, when *wader* fish live in fast-flowing rivers, they tend to have longer and stronger fins to help them better navigate the strong currents. *Wader* fishes that live in lakes with calm currents or slower-flowing waters tend to have shorter fins that are wider fish in size, allowing them to move efficiently in calm water. *Wader* fish also have pectoral and ventral fins on their bodies, which maintain balance and provide direction while swimming; it can be classified into species with weak backbones, no repeating backbones, simple dorsal fins, and strong dorsal fins (Plamoottil et al. 2016). These adaptations in fin structure enable *wader* fish to thrive in diverse aquatic habitats, enhancing their survival and reproductive success. Figure 1 illustrates the general body structure and distinctive features of the *wader* fish, including its compact body shape, coloration, and fin variations.

#### Color and pattern

One characteristic of *wader* fish (*Puntius* spp.) is their body color and pattern, ranging from color combinations (red, orange, yellow, black) to vertical lines and spots. The colors on the bodies of *wader* fish play a role in their adaptation to the surrounding environment, such as

foraging, camouflaging from predators, and attracting mates during mating seasons. Morphologically, *wader* fish has a black spot at the base of the tail (Fakhrurrozi et al. 2016). Their scales are pearlescent with black spots on the back; *wader* fish has color with attractive patterns (Roesma and Chornelia 2014). The colors and patterns of *wader* fish are pretty varied; color variations can be found in a single species, and color variations can be found depending on age, sex, environmental conditions, and genetic factors. The body color of *wader* fish is often influenced by environmental factors such as lighting, water depth, substrate type, and vegetation. It is known that *wader* fish that live in darker waters or have dark substrates tend to have a darker or neutral body color that serves to disguise and avoid predators. It is inversely proportional to *wader* fish that live in bright waters or with bright substrates and tend to have brighter or striking body colors to attract mates or outwit prey. In addition to environmental factors, some species of *wader* fish also have differences in color due to the role of genetics and natural selection that occurs in the environment. The body color of *wader* fish serves various ecological functions, including communication, sex identification, and adaptation to survive in their environment. Besides having body colors that may be pretty striking, *wader* fish also often have unique and complex patterns. Patterns can include stripes, spots, or other patterns found throughout their bodies. This patterning on the body of *wader* fish itself may serve as an evolutionary adjustment that aids *wader* fish in camouflage or same-sex recognition. These vibrant colors and patterns serve ecological functions and make *wader* fish popular for ornamental aquariums.

### Eye

The eyes of *wader* fish (*Puntius* spp.) are relatively large, allowing for good vision even in murky water, facilitating the detection of prey and predators. In ecological processes, *wader* fish assist in controlling organism populations in water and serve as a food source for other predators in the food chain, as they feed on plankton and small insects. Additionally, *wader* fish is also a primary food source for the catfish (*Kryptopterus limpok*) Bleeker, 1852) from the Cyprinidae family (Adiyanda and Yusfiati 2014). When infected, *wader* fish tend to be difficult to identify solely based on their external morphology (Patra et al. 2016). This highlights the importance of regular health monitoring and effective disease management practices in aquaculture to ensure the sustainability of *wader* fish populations.



**Figure 1.** *Wader* fish (*Puntius* spp.). Source: Masykuri (2015)

Generally, female *Puntius* spp. possess a larger abdomen size than male fish (Sentosa and Djumanto 2010). The variation in abdomen size is associated with its role, since female *wader* fish hold many eggs for reproduction, leading to a bigger abdomen. At the same time, male *wader* fish possess a more slender midsection. An adult male *wader* fish has a body length of about 7-9 cm, whereas an adult female *wader* fish measures approximately 8-10 cm (Raharjeng et al. 2022).

### FOOD SOURCE OF WADER FISH (*Puntius* spp.)

Food primarily serves as a provider of about 40 types of nutrients, including carbohydrates, proteins, fats, vitamins, minerals, and water, needed as a source of energy for growth, tissue maintenance, and metabolic regulation according to gender, age, physical activity, and body weight (Sibuea 2021). One of the food sources that can provide many nutrients for humans is fish. Fish are rich in chemical compositions such as protein, fats (especially polyunsaturated fatty acids), vitamins (including vitamin A, vitamin B2, vitamin B6, and others), minerals (such as iron, calcium, iodine, potassium, and other minerals), and carbohydrates. In many parts of the world, fish are considered a good source of nutrition (Fitri et al. 2022). Fish consumption is significant in regions with limited access to other protein sources, making *wader* fish (*Puntius* spp.) a valuable component of local diets.

One type of fish commonly consumed in Indonesia is *wader* fish. Some previous studies have shown that *wader* fish plays a significant role as a food source and an important source of micronutrients in preventing malnutrition, vitamin deficiency, and mineral deficiency in rural areas, especially for vulnerable groups such as women and impoverished children (Hasan et al. 2018). Small fish like those from the *wader* fish species contain various vitamins and minerals important for embryonic growth, bone formation, muscle and tooth formation, brain and nerve development, intelligence in children, and milk production in breastfeeding mothers (Borah 2019). These nutritional benefits underscore the importance of promoting *wader* fish as a dietary staple in communities facing nutritional challenges.

### Nutritional composition of *Puntius* spp.

The proximate composition analysis of *wader* fish (*Puntius* spp.) conducted by Mahanty et al. (2014) indicates that the fish is rich in protein (exact amount unspecified) and minerals. Amino acid profiles reveal a high level of the essential amino acid histidine (22.94±0.01%). Despite its low-fat content (5%), the fatty acid profile shows that the fish is abundant in unsaturated fatty acids, with oleic acid being the most prominent (28.63±0.02%), and it contains significant amounts of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Mineral profiles indicate that calcium is the most abundant macro mineral (9,748.2±23.96 mg kg<sup>-1</sup>), followed by potassium (2,283.7±12.75 mg kg<sup>-1</sup>) and sodium (1,610±22.34 mg kg<sup>-1</sup>). The fish is also rich in micro minerals such as iron

(115.0±8.81 mg kg<sup>-1</sup>), zinc (51.1±10.15 mg kg<sup>-1</sup>), and manganese (11.2±2.09 mg kg<sup>-1</sup>). Vitamin profiles demonstrate that the fish is rich in all fat-soluble vitamins (A, D, E, and K), with vitamin E being the most abundant (30,685.8 µg kg<sup>-1</sup>). With this comprehensive nutritional profile, *wader* fish shows potential as a highly nutritious food component.

The current review highlights the significant nutritional benefits of *wader* fish (Table 1), which includes high protein content, essential vitamins, and omega fatty acids. However, several research gaps and future directions remain to be explored. First, while general nutritional information is available, detailed quantitative profiling is lacking across various fish populations and environmental conditions. Future studies should investigate how factors such as habitat, diet, and seasonal changes impact the nutritional composition of *wader* fish. Additionally, although some research suggests potential health benefits such as anti-inflammatory and antioxidant properties, clinical evidence supporting these claims is limited. Long-term human studies are necessary to validate the therapeutic effects of consuming *wader* fish particularly about heart health, inflammation, and immune function.

Moreover, further research is needed to understand how different processing methods (such as drying, freezing, and fermenting) affect the nutritional and bioactive components of *wader* fish. This would not only have implications for the food industry but could also improve the shelf life of fish products. The potential for *wader* fish to be incorporated into functional food products, such as protein supplements or omega-3-enriched items, also warrants investigation. Research should optimize product formulations to maintain the fish's health benefits while appealing to consumers. Finally, with growing interest in the nutritional use of fish, research into sustainable harvesting practices for *wader* fish is essential. This will help prevent overfishing and ensure the long-term availability of the species without disrupting local ecosystems.

**Table 1.** Nutritional composition of *wader* fish (*Puntius spp.*)

Nutritional component	Average content	Unit	Reference
Protein	21.50	%/100 g	Sarjubala et al. (2018)
Fat	2.70	%/100 g	Sarjubala et al. (2018)
Calcium (Ca)	190	mg/100 g	Musa (2009)
Phosphorus (P)	150	mg/100 g	Mahanty et al. (2014)
Iron (Fe)	5.5	mg/100 g	Sarjubala et al. (2018)
Vitamin A	750	IU/100g	Mahanty et al. (2014)
Vitamin D	20	µg/100g	Mahanty et al. (2014)
Omega-3 (EPA+DHA)	0.75	g/100g	Mustafa et al. (2015)
Omega 6	0.10	g/100g	Mustafa et al. (2015)
Carbohydrates	1.55	%/100 g	Musa (2009)
Moisture content	75.60	%/100 g	Musa (2009)

## Carbohydrates

Carbohydrates are the primary energy source for the human body, providing 4 calories (kilojoules) of food energy per gram (Fitri and Fitriana 2020). They serve many crucial functions, such as supplying energy to the brain and nerves, regulating metabolism, and serving as the main energy source for the body. Carbohydrates are highly essential at every stage of life, especially during childhood when there is a high demand for energy for play and brain development. Inadequate carbohydrate consumption has been linked to an increased risk of stunting by up to 1.7 times (Azmy and Mundiastuti 2018). Stunting, characterized by impaired growth and development, can have long-term detrimental effects on a child's health, cognitive abilities, and overall quality of life.

Research by Mohanta et al. (2007, 2009) on the impact of the varying carbohydrate content on the growth and nutrient utilization of *wader* fish (silver barb) has shown consistent results. The first study (2007) revealed that an optimal carbohydrate content of around 260 g/kg leads to good growth and nutrient utilization, with the best performance achieved by the group with this carbohydrate level (D-2). Additionally, body protein concentration, Protein Productive Value (PPV), and Energy Productive Value (EPV) were also higher in this group. The second study (2009) reinforced these findings by showing that fish given carbohydrate content between 260 g/kg and 340 g/kg, particularly in groups R and D-5, exhibited better weight gain, feed intake, and Feed Conversion Ratio (FCR). This research also noted an increase in body protein and lipid levels with increased carbohydrates and a protein-sparing effect of carbohydrates, allowing for a reduction in protein content without reducing growth performance. Overall, these two studies demonstrate that the optimal carbohydrate content for *wader* fish ranges from 260 g/kg to 340 g/kg, which supports optimal growth and efficient nutrient utilization.

## Protein and amino acids

The human body requires approximately 45-46 grams of protein daily (Andhikawati et al. 2021). *Wader* fish (*Puntius spp.*) is rich in high-quality protein containing essential amino acids needed for human health. High levels of protein, fats, and ash are recorded in *wader* fish (Sarjubala et al. 2018). According to Mahanty et al. (2014), *wader* fish has a composition with crude protein levels of 16.2±0.14%, high crude fat levels, and ash content reaching 5.36±0.12%. This protein contains both essential and non-essential amino acids. Essential amino acids are crucial for various biochemical processes in the body. Still, the body cannot synthesize them independently, so they must be obtained from additional nutritional intake (Rahayu et al. 2019). The amino acid profile shows that histidine is dominant (22.93±0.01%), while tryptophan is 1.69±0.005 mg kg<sup>-1</sup>. Glycine is the dominant non-essential amino acid (10.39±0.07%), essential for skin tissue regeneration. This fish protein is rich in essential amino acids needed for human nutrition. High-protein foods are believed to have a greater satiating effect than high-carbohydrate or high-fat foods (Cuenca-Sánchez et al. 2015). Although *wader* fish

has high protein content, in fish storage, attention must be paid to temperature and storage time because, according to Mazrouh (2015), significant protein content decreases during frozen storage at  $-50^{\circ}\text{C}$  for 20 days in the muscles of frozen fish *Labeo rohita* (Hamilton, 1822). In *wader* fish, protein content decreases due to denaturation and loss of gelatin caused by long-term frozen storage, as well as proteolysis caused by psychotropic microbial growth. Thus, proper storage and handling are crucial to preserving the nutritional quality of *wader* fish.

Proteins are essential for body growth and tissue repair. Animal protein is the best for human growth and development because its composition is similar to human protein. This protein is beneficial as fuel for the body and provides essential amino acids needed for building tissues (Islamiati et al. 2024). The protein content in *wader* fish is relatively high, with an excellent amino acid profile. This high protein content makes *wader* fish a valuable food source for supporting muscle growth and repair in humans and other animals. This indicates that *wader* fish can be one of the choices for animal protein sources that we can consume.

#### Fat and fatty acids

Consumption of fats in foods in moderate amounts is important for human health because it provides the body with essential Fatty Acids (FAs) and fat-soluble vitamins and regulates satiety and energy balance (Pepino et al. 2014). These fish contain beneficial fats, including omega-3 and omega-6 fatty acids. According to Mustafa et al. (2015), *wader* fish (*Puntius* spp.) contains a total lipid of 10.2% in the head and 8.7% in the body. There are 21 types of fatty acids, with the head containing 64.52% Saturated Fatty Acids (SFA), 25.76% monounsaturated fatty acids (MUFA), and 8.72% Polyunsaturated Fatty Acids (PUFA). The body contains 34.84% SFA, 23.97% MUFA, and 40.99% PUFA. The omega-3 content is 4.28% in the head and 17.86% in the body, while omega-6 is 4.43% in the head and 23.12% in the body, with an omega-3/omega-6 ratio between 0.35 to 1.50. *Wader* fish is an important source of beneficial omega-3 and omega-6 fatty acids for health. According to Majumdar et al. (2017), *wader* fish belongs to the 'semi-fatty' fish group (lipid content usually ranges from 2-5% in seasonal farming). Variations in lipid content may be associated with raw fish lipid content. Increased protein and product lipid content is caused by reduced water content. These fatty acids are crucial for cardiovascular health and cognitive function.

Proteins are essential for body growth, tissue repair, and numerous metabolic functions. *Wader* fish is a rich source of protein, boasting a well-balanced amino acid profile. This makes it a precious food for supporting muscle growth and repair in humans and other animals. The proteins in *wader* fish are also crucial for various metabolic processes, including the production of enzymes that catalyze biochemical reactions and the synthesis of hormones that regulate physiological activities. Additionally, proteins play a vital role in maintaining and repairing cellular structures and ensuring the proper functioning of the immune system. Specifically, proteins and mitochondrial

DNA interact with TLR9 and FPR receptors, activating the NLRP3 inflammasome, which triggers inflammatory signaling pathways and recruits immune cells to respond to infection and tissue damage (Faas and De Vos 2020). Regular consumption of *wader* fish can significantly contribute to meeting daily protein requirements, thereby supporting overall health and well-being.

#### Vitamins

Vitamins are organic compounds that play a very important role in cell growth and function, as well as in maintaining the health and metabolism of the body to remain optimal (Ramandha and Muhsin 2023). The human body requires vitamins to function optimally, but most vitamins cannot be naturally produced, at least not in sufficient quantities to meet our daily needs (Ofoedu et al. 2021). When the body cannot meet the amount of vitamins, then vitamins must be supplied from food (Zhang et al. 2018). *Wader* fish (*Puntius* spp.) is a fish rich in vitamins, including vitamins A, D, E, and K. Vitamin E ( $30,685.8 \mu\text{g kg}^{-1}$ ) is one of the most abundant (Mahanty et al. 2014). Vitamin E, as a potent antioxidant, protects cell membranes from oxidative damage, prevents cell lysis and hemolysis, and reduces the risk of neonatal hyperbilirubinemia; these functions are supported by the regeneration of oxidized vitamin E with the help of vitamin C (Sareharto and Wijayahadi 2016). These vitamins are essential in immune function, bone health, and skin integrity.

*Wader* fish is rich in vitamins and minerals for maintaining various bodily functions and overall health. Vitamins such as A, D, and B-complex are vital for various bodily functions, including vision, bone health, and energy metabolism (El Bahgy et al. 2021). Vitamin A is crucial for maintaining healthy vision and immune function, while vitamin D is essential for calcium absorption and bone health. B-complex vitamins are important for energy metabolism and neurological function. In addition to vitamins, *wader* fish is a good source of minerals; the mineral content includes calcium, phosphorus, and iron. Calcium and phosphorus are necessary for maintaining strong and healthy bones and teeth, while iron is vital for oxygen transport in the blood and overall vitality. The presence of these minerals in *wader* fish contributes to the maintenance of bone strength, efficient oxygen transport, and the prevention of anemia. Regular consumption of *wader* fish can help meet daily vitamin and mineral requirements, supporting overall health and preventing deficiencies.

#### Minerals

Macro minerals, also known as major minerals, are minerals required in large amounts by the human body. They are an important part of the body's biological structure and play a role in various metabolic and almost all body processes (Ali 2023). The five major minerals in the human body include calcium, phosphorus, potassium, sodium, and magnesium. In addition, there are trace elements such as iodine, sulfur, zinc, iron, chlorine, cobalt, copper, manganese, molybdenum, and selenium that have specific biochemical functions in the body (Godswill et al. 2020). Fish are rich in essential minerals, including iron,

calcium, zinc, potassium, and magnesium. According to Sarojnalini and Devi (2014), Eleven essential mineral elements were found to be abundant in *wader* fish (*Puntius* spp.) with varying levels of iron, calcium, zinc, potassium, and magnesium within specific ranges, such as iron from 152.17 to 320.39 mg/100g, calcium from 902.06 to 1356.02 mg/100g, zinc from 91.07 to 138.14 mg/100g, potassium from 193.25 to 261.56 mg/100g, and magnesium from 225.06 to 229.10 mg/100g. The important function of Fe in the body includes its role as a heme component. Iron deficiency can inhibit the body's ability to bind and transport oxygen (Tasik 2022). Calcium serves as the primary messenger in all physiological functions within the body (Ahmad 2021). Humans and animals require zinc for various physiological functions, including growth, immune system, and reproductive processes (Candra 2018). Magnesium is significant in various metabolic processes, including carbohydrate, lipid, and protein metabolism, and in ATP synthesis in mitochondria (Mahardhika et al. 2019). Its role is maintaining tissue health and body functions and maintaining acid-base balance within the body (Kartika et al. 2019). Therefore, consuming *wader* fish can contribute to meeting the body's daily mineral requirements and promoting overall health.

These detailed analyses show that *wader* fish is a vital component of aquatic ecosystems and a valuable source of human nutrition. Including *wader* fish in the diet can significantly contribute to the nutritional needs of various population groups, especially in rural and underdeveloped areas where access to diverse food sources may be limited. The nutritional profile of *wader* fish indicates its potential as a valuable food source. Its high protein content, essential fatty acids, and rich vitamin and mineral composition make it an excellent choice for a balanced diet. Regular consumption of *wader* fish can support muscle growth, cardiovascular health, and overall well-being. This nutritional richness and its ecological role underscores the importance of conserving *wader* fish populations and promoting their sustainable use in aquaculture.

#### **TRADITIONAL USE OF WADER FISH (*Puntius* spp.)**

According to Altaf et al. (2020), *wader* fish (*Puntius* spp.) has been traditionally recognized for its various health benefits. These include its role in regulating blood sugar levels, maintaining chemical balance in the body, increasing hemoglobin, regulating blood balance, reducing joint pain, improving sexual function, and repairing the central nervous system. In addition to its nutritional value, *wader* fish holds significant cultural importance among various indigenous communities. Ethnozoological studies reveal that these communities rely on *wader* fish as a dietary staple and medicinal resource, citing its purported benefits in traditional healing practices for ailments such as joint pain and inflammation (Grenz 2020). Furthermore, intriguingly, several tribes have woven myths and beliefs around the *wader* fish, attributing mystical properties to its consumption. For instance, among the indigenous tribes of South Asia, a belief exists that consuming *wader* fish can

bring luck and prosperity. In contrast, others regard it as a symbol of resilience and adaptability in their folklore (Schuetz-Miller 2022). Indigenous people in South Punjab, Pakistan, have traditionally used freshwater fish like *wader* fish (*Puntius* spp.) to alleviate joint pain. This practice often involves cooking the fish with traditional herbs known for their anti-inflammatory properties, such as turmeric and ginger, which are believed to enhance its medicinal benefits, as Iqbal et al. (2023) reported.

*Wader* fish is typically not consumed in its raw form. Instead, it undergoes various culinary techniques to enhance its flavor, texture, and palatability. These techniques, such as boiling, frying, and roasting, significantly alter the taste and texture and affect its nutritional composition (Hananiah and Rahim 2022). Each cooking method has a distinct effect on the nutritional profile of *wader* fish. For instance, boiling may lead to some loss of water-soluble vitamins and minerals, while frying could result in increased fat content due to oil absorption. On the other hand, roasting may preserve more nutrients than other methods, depending on factors such as cooking temperature and duration. Choosing the right cooking technique is crucial as it can optimize the digestibility of the food while minimizing nutrient loss (Goswami and Manna 2019). This indicates that the use of *wader* fish in traditional medicine has the potential to provide effective solutions to specific health problems and is an integral part of local medical practices in various regions.

Due to its high micronutrient content, the Bangladesh community often utilizes *wader* fish for market sale. Recent market surveys have shown that the price of *wader* fish typically ranges from 100 to 150 Bangladeshi Taka/kg or equivalent to 1.18-1.77 USD/kg, depending on the season and local demand (Islam et al. 2022). Micronutrients are necessary components that humans and other living things need in different amounts at different times to coordinate various physiological processes necessary for maintaining health. Micronutrient requirements for human nutrition are typically less than 100 milligrams per day, whereas daily requirements for macronutrients are measured in grams (Godswill et al. 2020). Although the required amounts are relatively small, micronutrients are crucial in maintaining human health and bodily balance. Furthermore, traditional knowledge highlights the crucial importance of micronutrients in supporting complex brain functions. For instance, zinc is essential in metalloprotein synthesis and receptor binding, calcium contributes to signal transduction and membrane potential, and magnesium and zinc are involved in neurotransmission (Maggini et al. 2021).

Deficiency or imbalance of micronutrients, such as zinc, calcium, or magnesium, can disrupt brain function and potentially lead to various health issues, including neurological disorders. Mineral deficiencies, such as zinc, calcium, or magnesium, can profoundly affect brain function and overall health. These minerals are crucial in neurotransmitter synthesis, nerve signal transmission, and maintaining neuronal health. Zinc deficiency, for instance, has been linked to impaired cognitive function and emotional stability. Calcium is essential for

neurotransmitter release and nerve cell communication; its deficiency can lead to muscle cramps, seizures, and impaired memory. Magnesium deficiency affects synaptic function and neuroplasticity, potentially contributing to mood disorders like anxiety and depression. Without adequate levels of these minerals, the brain's ability to function optimally is compromised, potentially leading to neurological disorders such as Alzheimer's disease, Parkinson's disease, or multiple sclerosis. Thus, maintaining a balanced intake of essential minerals through diet or supplementation is crucial for supporting brain health and preventing neurological issues.

Local communities have long recognized the value of micronutrients for their traditional medicinal uses. Foods rich in zinc, calcium, and magnesium—such as certain freshwater species like *wader* fish support brain health and overall well-being. These fish are not only a dietary staple but also hold cultural significance, with traditional practices passed down through generations. To conserve these species and ensure their continued utilization, local communities often implement traditional conservation measures, such as regulating fishing seasons, promoting sustainable harvesting techniques, and advocating for the preservation of aquatic habitats. By safeguarding these practices, communities help maintain the availability of micronutrient-rich foods and contribute to the ecological balance of their environments (Dawson et al. 2021).

#### APPLICATION OF WADER FISH (*Puntius* spp.) AS MEDICINE

Fish is an inexpensive and readily available source of animal protein for human consumption, making it a primary choice in a balanced diet, alongside shrimp for antibiotics (Ghosh et al. 2021) and microalgae as a major source of nutrition, including protein, fat, vitamins, minerals, and color in aquatic animal diets (Sheikhzadeh et al. 2024). Research has shown that fish is rich in Essential Amino Acids (EAAs) and Polyunsaturated Fatty Acids (PUFAs) that play a vital role in the maintenance and development of fetuses, neonates, and infant brain development (Maulu et al. 2021). Furthermore, the presence of docosahexaenoic acid (DHA) in fish, which is a major component of many cells, especially brain nerve cells and retina cells, plays a crucial role in fetal brain development, motor skills, lipid metabolism, and cognitive function (Mohanty et al. 2016). Among the various types of consumed fish, the *wader* (*Puntius* spp.) is a small freshwater fish species commonly found in Southeast Asian waters. Its main habitats include ponds, rivers, and flowing water, making it easily accessible and important in providing nutrition. This fish belongs to the Cyprinidae family (Tiwari et al. 2021). It is considered a daily consumption choice by local communities (Sari and Dewi 2017) due to its abundant availability and delicious taste. In terms of diet and health, consuming *wader* fish can benefit from meeting daily nutritional needs and supporting overall body development and health.

*Wader* fish (*Puntius* spp.) has been widely applied and

studied in medicine. The content found in *wader* fish has been proven to have the ability to combat harmful pathogens in the human body. These pathogens can cause diseases or infections (Purbomartono et al. 2022). However, in-depth research on using *wader* fish in traditional medicine is still relatively rare. Herbal treatments are used for more than just treating diseases; this includes boosting the immune system, reducing stress, promoting growth, and enhancing disease resistance (Hodar et al. 2021). Antigen recognition is the immune system's primary function, a sophisticated network of cell connections. Viruses and bacteria that are still alive or inactive are examples of antigens. Passive immunity and active immunity are the two kinds of immunity that exist (Mulya et al. 2023). Herbal treatments are considered much safer than conventional allopathic drugs and have far fewer side effects (Modak and Chattoraj 2015). It is because herbal remedies have minimal side effects, and some often argue that herbal medicines are more effective in curing various diseases, ranging from mild to severe illnesses (Kamaluddin 2016). Nutrition experts have shown that *wader* fish has a higher calorie, protein, and fat content than snapper fish, making it an attractive choice in a healthy diet. Additionally, the high content of healthy fats and omega-3 in *wader* fish is also considered effective in reducing high cholesterol levels in the human body (Fitriana and Setiawan 2023).

Omega-3 fatty acids are essential for the brain's growth, operation, and aging since they are in many physiological processes linked to neurogenesis, neurotransmission, and neuroinflammation. Omega-3 fatty acid deficiencies in the diet have been linked to a higher chance of developing mental conditions in humans, such as autism, attention-deficit/hyperactivity disorder, bipolar disorder, schizophrenia, dementia, and depression (Lange 2020). The omega-3 fatty acids in *wader* fish significantly benefit human health. Some of its benefits include lowering blood pressure and blood lipid levels, reducing the risk of myocardial infarction, and improving the function of the human immune system and brain health. Additionally, it has been documented that omega-3 fatty acids play an important role in protecting the body from various mental disorders and the risk of cancer (Sedyaaaw et al. 2024). In addition to being rich in omega-3, *wader* fish is also abundant in omega-6. According to Mustafa et al. (2015), *wader* fish is an important source of beneficial omega-3 and omega-6 fatty acids for health. Omega-6 is a polyunsaturated fatty acid essential for human health, with various benefits such as supporting growth and development, maintaining healthy skin and hair, and strengthening the immune system. Furthermore, omega-6 plays a role in optimal brain function by supporting communication between nerve cells, which is crucial for cognition and mood. A balanced intake of omega-6 can also help reduce the risk of heart disease by lowering LDL cholesterol levels and increasing HDL cholesterol levels, as well as helping to reduce inflammation and pain in people with arthritis (Balić et al. 2020). However, it is important to maintain a balance between omega-6 and omega-3 intake to avoid the risk of excessive inflammation and chronic diseases.

**Table 2.** Medicinal and health benefits of *wader* fish (*Puntius* spp.)

Medical use	Details	References
Antimicrobial	Mucus extract shows potential in inhibiting biofilm matrix structures and exhibits antibacterial activity against <i>E. coli</i> , <i>P. aeruginosa</i> , <i>B. subtilis</i> , and <i>S. aureus</i> .	(Patel et al. 2020)
Antioxidant	Contains high antioxidant activity, which helps in protecting cells from oxidative damage.	(Sarjubala et al. 2018)
Anti-inflammatory	Rich in omega-3 fatty acids, which reduce inflammation by suppressing the production of pro-inflammatory molecules such as eicosanoids and cytokines.	(Poggioli et al. 2023)
Immune system support	Omega-3 and omega-6 fatty acids, and high-quality protein and essential vitamins (A, D, E, K); support immune function.	(Mahanty et al. 2014)
Liver health	Enhances liver function by regulating typical liver enzymes (SGPT, SGOT, ALP, ACP) and supports detoxification processes.	(Kumar et al. 2021b)
Joint pain relief	Traditionally used to alleviate joint pain and inflammation, it is often combined with herbs like turmeric and ginger.	(Altaf et al. 2020)
Heart health	Omega-3 and omega-6 fatty acids in the fish help lower blood pressure and reduce the risk of heart disease.	(Sedyaaw et al. 2024)
Cognitive function	The high levels of omega-3 fatty acids contribute to improving cognitive function and brain health.	(Sedyaaw et al. 2024)
Cancer prevention	Omega-3 fatty acids have been documented to reduce the risk of certain cancers and protect the body from mental disorders.	(Sedyaaw et al. 2024)
Wound healing	Traditionally used for external wound healing and herbal treatments to enhance medicinal benefits.	(Altaf et al. 2020)

### Antioxidant

Based on Table 2, antioxidants play a critical role in maintaining health by protecting cells from oxidative damage and supporting essential bodily functions (Huang et al. 2019). *Puntius* spp. or *wader* fish, is also recognized for its rich mineral content, including magnesium, potassium, and calcium, as well as its high antioxidant activity. This suggests that consuming *wader* fish offers not only high-quality protein but also significant benefits in terms of mineral intake, antioxidant support, and monounsaturated fatty acids, all of which may contribute positively to overall human health (Sarjubala et al. 2018). Research conducted by Bora and Chutia (2023) shows that the addition of Fermented Bamboo Shoot (FBS) extract in processed fish products can function to improve the immune system, help heart problems, reduce cholesterol levels, and reduce the risk of cancer. Apart from benefits for humans, *wader* fish also have benefits for providing animal feed. Based on research conducted by Subandiyono et al. (2018), feeding *wader* fish with feed mixed with pineapple extract can increase feed efficiency (FUE), Protein Efficiency Ratio (PER), weight and length growth (RGR and RGR-L) by up to 218.06%. Furthermore, adding curcumin extract, which has benefits as an antioxidant, anti-inflammatory, antimicrobial, anticancer, and nerve protector, into fish feed can also improve fish health and nutrition (Alagawany et al. 2021).

Antioxidants, especially those derived from natural sources, have been a major focus for preventing lipid peroxidation and protecting biomolecules from damage caused by free radicals. It has been proven that proteins and peptides derived from food have significant bioactive or

physiological properties. In addition to providing essential nutrition, many peptides produced from plant or animal proteins, both in vitro and in vivo, have important regulatory roles in the human body (Majumdar et al. 2016). Colorimetric methods are commonly used to evaluate antioxidant activity in *wader* fish. These methods include testing Hydroxyl Radical Scavenging Activity (HRSA), DPPH radical scavenging, inhibition of Angiotensin-1 Converting Enzyme (ACE), and inhibition of lipid peroxidation (Chen et al. 2022). Additionally, lipids found in fish contain many Polyunsaturated Fatty Acid (PUFA) groups, making them highly susceptible to oxidation (Goswami et al. 2013). The lipids found in fish contain many Polyunsaturated Fatty Acids (PUFAs), such as omega-3 and omega-6. These fatty acids have double bonds that easily react with oxygen in the air, causing lipid oxidation. This oxidation process can change in the fish's taste, aroma, and color and decrease its nutritional quality and freshness. Moreover, lipid oxidation can also produce harmful compounds such as free radicals and aldehydes, which can damage body cells and increase the risk of chronic diseases such as cancer and heart disease. Therefore, due to the high content of PUFAs in fish lipids, they are susceptible to oxidation and need to be properly preserved to maintain their nutritional quality and freshness (Nayak et al. 2018).

### Antibacterial

Antibacterial or antibiotics are compounds that can either stop or completely eradicate bacterial development. Infections that pose a risk to human health could be brought on by these microorganisms (Hoffman 2020).

Mucus extract from *wader* fish (*Puntius* spp.) has been shown to have the potential to inhibit biofilm matrix structures and exhibit antibacterial activity against several common pathogens, such as *Escherichia coli*, *Pseudomonas aeruginosa*, and *Bacillus subtilis*, with observed additive effects on *Staphylococcus aureus* (Patel et al. 2020). The strong antimicrobial quality of this extract is crucial for probiotic bacteria in combating pathogenic microorganisms (Haider et al. 2021), especially those related to gastrointestinal infections, such as *Salmonella typhi*, *S. aureus*, and *E. coli*. Sometimes, these microorganisms become foodborne pathogens that potentially infect humans with gastroenteritis. Probiotics produce several antimicrobial compounds, including conjugated bile salts, lactic acid, hydrogen peroxide, and other organic acids, which support their antimicrobial activity (Ngasotter et al. 2021). Thus, this research provides important insights into the potential application of mucus extract from *wader* fish in controlling the growth and spread of pathogenic microorganisms in natural environments or biological systems. The histological structure of the *wader's* intestinal lining consists of the muscular tunica, serosal tunica, mucosal tunica, and submucosal tunica, which generally function for healthy digestion, nutrient absorption, and protection against infection (Nita and Retnoaji 2022).

Based on research conducted by Mahmud et al. (2019), *wader* fish powder enriched with macro and micronutrients, especially protein, has been proven safe for consumption for a minimum of three months, based on biochemical content evaluation, sensory quality, and microbiological characteristics. Analysis of the biochemical composition of *wader* fish body (male and female) indicates the presence of lipids, proteins, ash, and carbohydrates. Furthermore, several important minerals, such as iron, zinc, copper, manganese, magnesium, calcium, phosphorus, potassium, and some amino acids, are also present in the body of these fish. Although male *wader* fish have higher protein (21.50%), fat (2.70%), ash (1.90%), and carbohydrate (1.55%) content, female *wader* fish have higher water content (75.60%), as well as higher concentrations of manganese and iron minerals, in line with findings reported by Musa (2009). This research provides a deeper understanding of the nutritional composition of *wader* fish in terms of gender, which can serve as an important basis for developing quality food products beneficial to human health.

### Anti-inflammatory

The inflammatory response is a complex, multi-phase process involving a series of mediator signals and various cell types working in concert. This adaptive reaction can be triggered by dangerous signals such as tissue damage or microbial invasion, activating various inflammatory pathways (Meng et al. 2021). The primary goal of this response is to eliminate the initial cause of cell injury, clear out necrotic cells and tissues damaged from the original insult and the inflammatory process, and establish a repair process. Inflammation is intricately linked to cancer and plays a critical role in the growth, progression, and spread

of tumors. Numerous studies have established that chronic inflammation can suppress the immune system, thus hindering its ability to fight off cancer cells effectively. This suppression extends to the response to chemotherapeutic treatments, reducing their efficacy and making cancer cells more resistant to such therapies (Zappavigna et al. 2020). Furthermore, chronic inflammation creates a microenvironment conducive to cancer development by promoting tumor growth, facilitating angiogenesis (the formation of new blood vessels that supply the tumor with nutrients and oxygen), and enabling metastasis (the spread of cancer cells to other parts of the body).

*Wader* fish (*Puntius* spp.) is rich in omega-3 fatty acids, such as EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid). Omega-3 has strong anti-inflammatory properties and can reduce inflammation by suppressing the production of molecules and substances that promote inflammation, such as eicosanoids and cytokines (Poggioli et al. 2023). Eicosanoids are diverse bioactive lipid mediators from Polyunsaturated Fatty Acids (PUFAs) with 20 carbon atoms. These molecules are crucial in various physiological and pathological processes, including inflammation, immunity, and homeostasis. Eicosanoids are produced through the enzymatic oxidation of PUFAs, primarily arachidonic acid, via three major pathways: the cytochrome P450 (cytP450) pathway, the lipoxygenase (LOX) pathway, and the cyclooxygenase (COX) pathway (Calder 2016).

### Immunity and liver health

The immune system plays a critical role in protecting the body against pathogens and in the initiation and management of cancer. While the adaptive immune system's immunosurveillance can effectively prevent or limit cancer development, the innate immune system and inflammation often play dual roles in carcinogenesis and the progression of early-stage cancer (Galasso et al. 2024). The liver is the most important organ in maintaining the body's defense against various organic and inorganic toxic substances. Changes in typical liver enzyme activities, such as SGPT, SGOT, ALP, and ACP, are often used as strong markers for liver diseases (Kumar et al. 2021b). Meanwhile, the immune system's capacity to effectively recognize specific pathogens and develop immunological memory is called adaptive immunity or specific immunity. According to Shaalan et al. (2016), the use of nanoparticles in the medical field has been discovered, including drug and gene delivery, immunization, and diagnostics. The complex interaction between genes, proteins, cytokines, and cells forms the body's response when interacting with antibodies and antigens (Awad and Awaad 2017). The respiratory burst activity, which occurs when phagocytes produce Reactive Oxygen Species (ROS), is an important part of the innate immune response and antioxidants. This ROS production burst is a crucial defense mechanism in the body's immune system, contributing to the overall ability of the body to fight infections effectively (Yogeshwari et al. 2015).

*Wader* fish has several nutritional components that can enhance and maintain the immune system and overall health, including various vitamins. *Wader* fish is rich in vitamins such as A, D, E, and K (Mahanty et al. 2014). These vitamins play a crucial role in maintaining immune function and consuming foods rich in mineral fibers such as zinc and selenium is also important, as it boosts the immune system. Sarojnalini and Devi (2014) state that *wader* fish is abundant in eleven essential mineral elements. Moreover, *wader* fish is a good source of high-quality protein containing essential amino acids essential for human health (Sarjubala et al. 2018). Protein from fish sources is necessary for building and repairing immune cells. According to Mustafa et al. (2015), *wader* fish is a valuable source of beneficial omega-3 and omega-6 fatty acids for health. Omega-3 from fish helps reduce inflammation and protect cells from damage. This nutritional balance, along with the intake of complex carbohydrates and vitamin A supporting skin and mucous membrane health, is crucial for strengthening the immune system.

### CONCLUDING REMARKS

*Puntius* spp., or *wader* fish, is a highly affordable and nutrient-rich source of animal protein in Southeast Asia. It is rich in essential amino acids and Polyunsaturated Fatty Acids (PUFAs) like omega-3 and omega-6, crucial for cardiovascular and cognitive health. In addition, this fish is a valuable source of vitamins and minerals such as iron, zinc, magnesium, and calcium, which collectively support brain development and immune function and reduce the risk of heart disease. The traditional use of *wader* fish in medicine is supported by the antimicrobial properties of its mucus, which are effective against pathogens like *E. coli* and *S. aureus*. This not only highlights its nutritional value but also its significance in the cultural heritage of Southeast Asia. However, while *wader* fish has shown great promise in these areas, significant gaps in current research must be addressed. The specific mechanisms by which bioactive compounds in this fish contribute to human health, particularly its antimicrobial, antioxidant, and anti-inflammatory properties, are not yet fully understood. Longitudinal studies are necessary to explore the long-term health outcomes associated with regular consumption of *wader* fish, including its effects on cardiovascular health, cognitive function, and immune response. Additionally, research into the environmental sustainability of increasing *wader* fish cultivation would help assess its viability as a long-term food and medicinal resource. Future research should aim to provide more detailed quantitative data on the fish's nutritional composition and examine its medicinal potential in modern health care. Addressing these gaps will provide a more comprehensive understanding of *wader* fish and ensure its continued role in promoting human health while preserving cultural practices. Therefore, while *wader* fish remains a staple in the daily diet of local communities, its full potential can only be realized through further scientific inquiry.

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# Effectiveness of antibiofilm *Aspergillus niger* cell-free supernatant against *Pseudomonas aeruginosa* biofilm

TATASA DIVA CAHYAKIRANA, ARI SUSILOWATI\*, ARTINI PANGASTUTI

Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret. Jl. Ir. Sutami 36A, Surakarta 57126, Central Java, Indonesia. Tel./fax.: +62-819-46227085, \*email: arisusilowati@staff.uns.ac.id

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**Abstract.** Cahyakirana TD, Susilowati A, Pangastuti A. 2024. Effectiveness of antibiofilm *Aspergillus niger* cell-free supernatant against *Pseudomonas aeruginosa* biofilm. *Asian J Trop Biotechnol* 21: 89-95. *Pseudomonas aeruginosa* is an opportunistic gram-negative pathogenic-bacteria that can cause nosocomial infections. *Pseudomonas aeruginosa* has several virulence factors that can cause infection, including its ability to form biofilms. Biofilm-forming bacteria can limit the penetration of antibiotics toward the microbes. These limitations make it challenging to treat biofilm-producing bacterial infections. Enzymes produced by *Aspergillus niger* with anti-biofilm activity include lactonase,  $\beta$ -glucosidase, acylase, and oxidoreductase. This study aimed to test the effectiveness of cell-free supernatant (CFS) of *A. niger* against *P. aeruginosa* biofilms. The antibiofilm activity test consisted of a biofilm growth inhibition test and *P. aeruginosa* biofilm destruction test. The research method used was the microtiter plate biofilm assay. The results showed that CFS of *A. niger* had the ability to inhibit the growth and destruction of *P. aeruginosa* biofilms. In the biofilm growth inhibition test and biofilm destruction test, the minimum biofilm inhibitory concentration (MBIC<sub>50</sub>) and minimum biofilm eradication concentration (MBEC<sub>50</sub>) were 56 and 55.7% at 50% concentration, respectively. It was concluded that *A. niger* CFS had antibiofilm activity against *P. aeruginosa* biofilms.

**Keywords:** Antibiofilm, *Aspergillus niger*, cell-free supernatant, microtiter plate biofilm, *Pseudomonas aeruginosa*

## INTRODUCTION

*Pseudomonas aeruginosa* is a Gram-negative bacterium ubiquitous in diverse environments, including air, water, soil, and plant and animal tissues. It is a well-known opportunistic human pathogen. However, this seemingly harmless bacterium can become a formidable foe, causing mild infections in healthy individuals and escalating to severe, life-threatening conditions in those with weakened immune systems (Li et al. 2023; Pachori et al. 2019). *Pseudomonas aeruginosa* frequently targets individuals with weakened immune systems. The infection typically begins when the body's natural protective barriers, such as the skin or mucosal surfaces, are disrupted by factors like trauma, severe burns, surgical procedures, or the presence of medical devices. The progression of infection occurs in three distinct stages: bacterial attachment and colonization at the affected site, localized tissue invasion, and systemic dissemination, which may result in severe or widespread disease (Strateva and Mitov 2011).

*Pseudomonas aeruginosa* is often found in clinical settings and is associated with healthcare-associated infections (HAIs) or nosocomial infections (dos Santos Araújo et al. 2022). Nosocomial infections also referred to as infections acquired during the process of receiving health care that are not present during the time of admission (Isigi et al. 2023). Nosocomial infections cause 1.5 million deaths every day worldwide. In developing countries, around 40% of hospital patients are estimated to suffer from nosocomial infections. Furthermore, as many

as 8.7% of hospital patients suffer from nosocomial infections while undergoing treatment at the hospital (Sazkiah and Ismah 2022). The nosocomial infection rate in Indonesia reached 15.74%, much higher than in developed countries, ranging from 4.8 to 15.5% (Mabrurah and Hermawati 2023).

*Pseudomonas aeruginosa* is armed with a range of virulence factors that enable it to cause infection by destroying tissue and spreading within the host. One of the most formidable factors is its ability to form biofilms (Turkina and Vikström 2019). Biofilms, aggregates of microorganisms covered by an extracellular polymer matrix, provide a protective shield for bacteria, rendering them resistant to desiccation, antimicrobials, and other cleaning agents. Moreover, biofilm-forming bacteria can evade the body's immune responses, including phagocytosis and different innate and adaptive immune system mechanisms (Roy et al. 2018). Biofilm-producing bacteria are predicted to have 10,000 times higher antibiotic resistance than bacteria that do not form biofilms. This is because biofilms can limit the penetration of antibiotics to microbes (Sharma et al. 2019). The presence of a barrier to antibiotic penetration makes it challenging to treat biofilm-producing bacterial infections. Therefore, further development of anti-biofilm agents is needed to overcome this problem.

A lot of research has been done out recently focused on finding solutions to inhibit the growth of biofilms in order to reduce the infection rates caused by biofilm-forming bacteria. Among the myriad of microorganisms studied for

their potential antibiofilm properties, *Aspergillus niger* has emerged as a particularly intriguing candidate. *Aspergillus niger* is a filamentous fungus widely known for its ability to produce an array of organic acids, extracellular enzymes, secondary metabolites, and bioactive compounds (Cairns et al. 2021). Cell-free supernatant (CFS) is a promising agent for combating biofilms. Another antibiofilm mechanism of CFS involves reducing biofilm production by lowering cell density, reducing aggregation, and degrading the extracellular polysaccharide matrix (EPS). Several compounds have been investigated for their antibiofilm activity, such as the use of  $\alpha$ -amylase and  $\beta$ -glucosidase by *A. niger* (Aliyah et al. 2017). Additionally, enzymes including cellulases, xylanase, mannanase, pectinase, amylases, acylase, oxidoreductase, and alginate lyase have been studied (Kaur et al. 2020), with isolates originating from soil and designated as *A. niger* APS also showing biofilm destruction capabilities. Other research (Nisa' et al. 2013) indicates that cellulase and  $\alpha$ -amylase enzymes can be produced by *A. niger* FNCC 6018. The aim of this research was to test the effectiveness of cell-free supernatant (CFS) of *A. niger* against *P. aeruginosa* biofilms.

## MATERIALS AND METHODS

### Materials

The materials used in this research were *Aspergillus niger* FNCC 6018, (obtained from the Laboratory of the Center for Food and Nutrition Studies, Food and Nutrition Culture Collection (FNCC), Gadjah Mada University, Yogyakarta, Indonesia), *Pseudomonas aeruginosa* bacteria forming biofilm ATCC 27853 (purchased from the Laboratory of the Agitama Sinergi Inovasi (AGAVI), agar, distilled water, alcohol, aluminum foil, 30% acetic acid, blue disposal tip, ice cubes/ice pack, falcon, glucose, cotton, gauze, filter paper, crystal violet, Lugol, potato dextrose broth (PDB) media, trypticase soy broth (TSB) media, plastic wrap.

### Research design

This research was conducted in a true experimental design. The microtiter plate biofilm assay method was used to evaluate antibiofilm activity by measuring the optical density (OD) value with a microplate reader. The most important study population was *P. aeruginosa*, a biofilm-forming organism of significant scientific interest. The number of repetitions was calculated based on Federer's formula, as below, to ensure a robust and reliable study:

$$(t-1)(n-1) \geq 15$$

Where:

t: treatment group

n: number of repetitions for each treatment group

There were 5 treatment groups, namely 1 negative control group and 4 test groups. The control group used in this study was media with *P. aeruginosa* suspension without adding cell-free supernatant (CFS). The test group

used was CFS *A. niger* of 100  $\mu$ L with concentrations of 100, 50, 25, and 12.5%. The research was conducted at the Biology Laboratory, Faculty of Mathematics and Natural Sciences, Sebelas Maret University, Surakarta, Indonesia.

### Procedures

#### Preparation of cell-free supernatant *A. niger*

The culture of *A. niger* which was rejuvenated on Potato Dextrose Broth (PDB), was taken by scratching a needle across the surface of media 8 mm long. Then, the culture was inoculated in 100 mL of sterile PDB, and 1% glucose was added. For one week, the culture was placed in a shaker incubator at a temperature of 28°C at a speed of 120  $\times$  g. After one week, 5 mL of culture was taken and inoculated into 45 mL sterile PDB. Then, the culture was placed in an incubator shaker for 4 days to produce cell-free supernatant (CFS).

After incubation for 4 days, the fungal culture was filtered using sterile filter paper number 42 and supernatant was collected in a 15 mL tube. The tube was then stored at 0°C to maintain the fungal enzyme activity. The filtrate tube was placed in the freezer for approximately 15 minutes. Then, the filtrate was centrifuged for 15 minutes at a speed of 10,000  $\times$  g. The supernatant formed was filtered again using sterile 0.22  $\mu$ m membrane filter and collected in a sterile beaker surrounded by ice cubes at 0°C.

Serial dilutions of CFS *A. niger* were prepared using a multilevel dilution technique with a ratio of 1:2. A 15 mL TSB was made and mixed with 1% glucose. 3 mL of media was added to the test tube of each concentration, namely 100, 50, 25, and 12.5% (Prateeksha et al. 2020).

#### Preparation of growth media and bacterial suspension for *Pseudomonas aeruginosa*

The growth media was prepared using Trypticase Soy Broth (TSB) supplemented with 5% glucose. A total of 10 mL of TSB was sterilized by autoclaving for 15 minutes at 121°C. Additionally, 0.5 mL of TSB with 1% glucose was also sterilized, as the inclusion of 1% glucose strive to enhance the formation of *P. aeruginosa* biofilm. One cycle of *P. aeruginosa* culture was inoculated into the TSB containing 1% glucose. The culture was then homogenized and incubated for 24 hours at 37°C.

#### Biofilm formation test of *P. aeruginosa* isolates

The detection test for *P. aeruginosa* biofilm formation was carried out using the microtiter-plate biofilm assay method. The control group was a mixture of TSB media and 1% glucose. Next, 200  $\mu$ L of *P. aeruginosa* bacterial suspension was transferred into microplate test well. The microplate was closed and incubated at 37°C for 48 hours without stirring. After incubation, the contents of microplate were removed and washed with sterile phosphate buffer saline (PBS) to remove planktonic cells that were not attached to the microplate and dried them. Next, 200  $\mu$ L of 0.1% crystal violet dye was added to each well and then incubated at room temperature for 20 minutes. After incubation, the contents of the microplate were discarded, and then the microplate was washed using sterile PBS three times to remove cells that were stained

but not attached to the microplate. Then, the microplates were allowed to dry at room temperature. After drying, 200  $\mu$ L of 96% ethanol was put into each well and incubated for 20 minutes at room temperature. The microplates were measured using a microplate reader with a wavelength of 595 nm to obtain the optical density (OD) value of the test and control plates.

To determine the strength of test bacterial biofilm, the ODisolate value was compared with the Optical Density cut-off (ODcut) value. The OD value was obtained from the average ODisolate value minus the ODCut value (OD = average ODisolate value – OD cut value). ODCut was three times the standard deviation above the mean OD of the control (culture medium) (Ruchi et al. 2015). The formula for calculating ODCut was as follows:

$$\text{ODcut} = \text{ODC} + (3 \times \text{SD ODC})$$

OD value obtained on average ODisolate - ODCut  
(OD = average value ODisolate - ODCut)

Where:

ODcut: Optical density cut-off

ODC: Optical density control

SD ODC: Standard deviation optical density control

The criteria of the strength of bacterial biofilm test is presented in Table 1.

#### *Pseudomonas aeruginosa* biofilm growth inhibition test

*Pseudomonas aeruginosa* biofilm growth inhibition test was carried out using CFS *A. niger* with percentages of 100, 50, 25, and 12.5%. As a test group, 100  $\mu$ L of CFS with varying percentages and 100  $\mu$ L of *P. aeruginosa* bacterial suspension were placed in the microplate wells. 100  $\mu$ L of a mixture of TSB media with 1% glucose and 100  $\mu$ L of *P. aeruginosa* bacterial suspension were placed in the microplate wells as negative control. Then, the microplates were incubated for 72 hours at 37°C in an incubator (Tobi et al. 2022).

After incubation for 72 hours, the solution of microplate was removed and washed thrice a times with sterile PBS, then dried. After drying, 200  $\mu$ L of 0.1% crystal violet dye was added in each well and incubated for 20 minutes at room temperature. After 20 minutes, the dye was discarded and washed with sterile PBS, and then microplates were allowed to dry at room temperature. 200  $\mu$ L of 96% ethanol was added to the wells and incubated for 20 minutes at room temperature. Then, the microplates were observed using a microplate reader with a wavelength of 595 nm.

Inhibition of bacterial biofilm growth was calculated using the following formula (Nikolic et al. 2014):

$$\text{Inhibition of biofilm growth (\%)} = (\text{ODkn} - \text{ODuji}) / \text{ODkn} \times 100\%$$

Where:

ODkn: Optical Density negative control (K-)

ODuji: Optical Density test group

#### *Pseudomonas aeruginosa* biofilm destruction test

This test was carried out according to the method of Tobi et al. (2022) with slight modifications. *Pseudomonas*

*aeruginosa* biofilm destruction test used CFS of *A. niger* with 100, 50, 25, and 12.5% concentrations. As much as 200  $\mu$ L of *P. aeruginosa* bacterial suspension was added to the test well, and the negative control was placed on the microplate. The microplate was closed and incubated at 37°C for 72 hours in an incubator. After incubation for 72 hours, the contents of the microplate were removed, washed with sterile PBS thrice a times, and dried. Next, 200  $\mu$ L of *A. niger* CFS with various percentages was added to the test well, and 200  $\mu$ L of a mixture of TSB media and 1% glucose in the negative control well. The microplate was closed again and incubated at 37°C for 60 minutes. After incubation, the contents of the microplate were removed, washed using sterile PBS three times, and dried. After the microplate was dry, 200  $\mu$ L of 0.1% crystal violet dye was added into all wells and then incubated at room temperature for 20 minutes. Next, the contents of the microplate were washed with sterile PBS. Then, the microplate was allowed to dry at room temperature. Add 200  $\mu$ L of 96% ethanol into all wells and incubate at room temperature for 20 minutes. Then, the 96-well microplate was observed using a microplate reader at a wavelength of 595 nm. Biofilm destruction was calculated using the following formula:

$$\% \text{ Biofilm destruction} = (\text{ODkn} - \text{ODuji}) / \text{ODkn} \times 100\%$$

Where:

ODkn: Optical Density negative control (K-)

ODuji: Optical Density test group

#### Data analysis

Quantitative data in the form of OD values for each treatment was used to determine the effectiveness of antibiofilm against *P. aeruginosa* biofilms after being treated with the addition of cell-free Supernatant *A. niger*. Quantitative data, including test OD, control OD, cut OD, and SD, were analyzed using Statistical Product and Service Solutions (SPSS). A One-way Analysis of Variance (ANOVA) test with a confidence level of 95% was used to determine the significance of the effect of CFS on *P. aeruginosa* biofilms, followed by a post-hoc test. The Post-Hoc Test was a further use to determine significant differences between each data group. The final analysis used the Pearson correlation test to determine the relationship or correlation between CFS concentration and the OD test value.

**Table 1.** Interpretation of the strength of biofilm formation (Nikolic et al. 2014)

Average OD value	Biofilm formation strength
ODisolate $\leq$ ODCut	Not a biofilm former
ODcut < ODisolate $\leq$ 2xODcut	Weak biofilm forming
2xODcut < ODisolate $\leq$ 4xODcut	Moderate biofilm former
4xODcut < ODisolate	Strong biofilm former

Note: ODCut: Optical Density cut, ODisolate: Optical Density isolate

## RESULTS AND DISCUSSION

### Cell-free supernatant of *A. niger*

Cell-free supernatant (CFS) of *A. niger* used as antibiofilm test material was obtained from *A. niger* inoculation screening. Filtering was carried out twice and carried out at cold temperatures. The freezing temperature filtration method was selected to eliminate all cells, thereby obtaining a free-cell supernatant while preserving the enzyme content of *A. niger*. The filtration size was selected to ensure only enzymes remained in the medium. As a result of filtering, clear yellow CFS was obtained. Mani-López et al. (2022) stated that CFS refers to a transparent liquid medium produced after the growth of certain microorganisms through a centrifugation and membrane filtration process with a pore size capable of holding all bacteria, generally 0.22 or 0.45  $\mu\text{m}$ .

### Detection of biofilm growth

The OD measurement evaluated the strength of biofilm growth by *P. aeruginosa* bacteria (Table 2). Mira et al. (2022) explains that OD is a method used to measure microbial growth based on its optical density. The OD value indicates the number of microbes in the suspension, which is positively correlated with the turbidity of the medium. An increase in the OD value indicates an increase in microbial cells growing in the medium. In measuring biofilm growth, an increase in the OD value indicates an increase in the amount of biofilm formed on the surface.

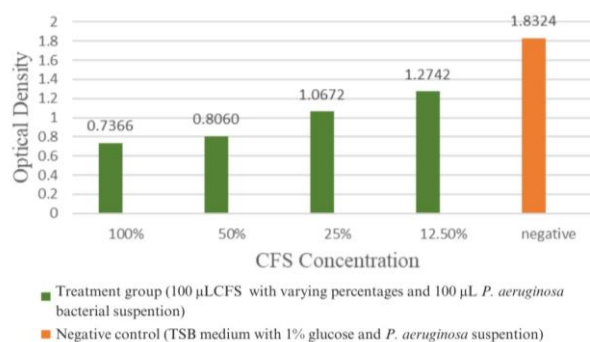
Table 2 showed the biofilm growth of *P. aeruginosa* can be assessed by comparing the optical density (OD) of the isolate with the threshold value (OD<sub>cut</sub>). The results indicate that bacteria formed strong biofilms if the condition  $4 \times \text{OD}_{\text{cut}} < \text{OD}_{\text{isolate}}$  was met, specifically  $2.972 < 3.033$ . The result suggests that *P. aeruginosa* belongs to the strong biofilm producer category. This finding is supported by research conducted by Nikolic et al. (2014) and Rehman et al. (2018), who reported that *P. aeruginosa* ATCC 27853 is indeed a strong biofilm producer. Strong biofilm-producing bacteria can develop a biofilm matrix more rapidly and thickly compared to those that produce weak biofilms. Consequently, bacteria that generate robust biofilms are often more resistant to antibiotics.

### Inhibition of *P. aeruginosa* biofilm growth

The biofilm growth inhibition test was conducted to detect the ability of *A. niger* CFS concentration to inhibit the growth of *P. aeruginosa* bacterial biofilm. The results showed (Figure 1) that the highest optical density (OD) value of 1.8324 was observed in the negative control group. The OD values for all test group concentrations were lower than that of the negative control. The highest OD value in the test group was 1.2742, recorded at a

concentration of 12.5% for the cell-free supernatant (CFS). This may be due to the minimal variation in CFS concentration, which had a limited effect on inhibiting biofilm formation, resulting in a cloudy liquid and a high OD value. Conversely, the lowest OD value of 0.7366 was observed for the test group at a concentration of 100% CFS. This lower value is likely because the highest concentration of CFS was more effective in inhibiting biofilm formation, leading to less turbidity in the liquid and, consequently, the lowest OD value among the test groups. The decrease in OD value relative to the negative control indicates the thickness of biofilm matrix was reduced when treated with *A. niger* CFS. Therefore, it is evident that CFS from *A. niger* possesses the ability to inhibit bacterial biofilm formation. The obtained OD values are then used to calculate the percentage of biofilm inhibition through the formula for inhibition of biofilm growth (%).

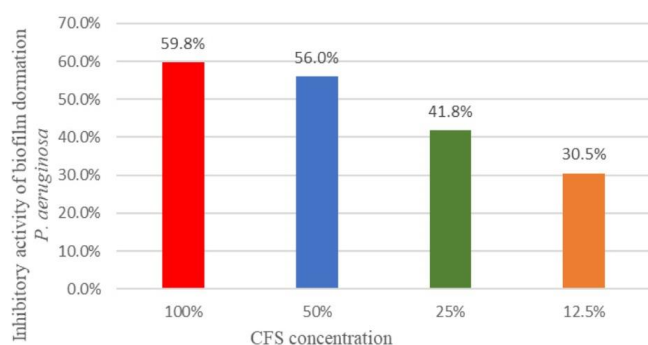
The percentage of biofilm growth inhibition activity is illustrated in Figure 2, while the microtiter plate biofilm assay is shown in Figure 3. The highest biofilm growth inhibition activity, at 60%, was observed in the cell-free supernatant (CFS) at a concentration of 100%. In contrast, the lowest inhibition, at 30%, was recorded in the CFS at a concentration of 12.5%. These results indicate that the administration of *A. niger* CFS at a concentration of 12.5% was able to inhibit *P. aeruginosa* bacteria, though this effect falls into the low category. According to Famuyide et al. (2019), anti-biofilm activity is classified as good and effective when the percentage of inhibition is 50% or greater. Conversely, an inhibition rate of 0-49% is considered low. The minimum biofilm inhibitory concentration (MBIC<sub>50</sub>) at 50% CFS concentration was found to be 56%. When CFS was administered at a concentration of 50%, the percentage inhibition met the MBIC<sub>50</sub> requirement, indicating that this concentration can inhibit biofilm growth by at least 50%, thus qualifying as excellent and effective (Sahal et al. 2020).



**Figure 1.** Inhibition of *P. aeruginosa* biofilm growth observed at various concentrations of cell-free supernatant treatment, measured by optical density (OD)

**Table 2.** Detection of *P. aeruginosa* biofilm growth by optical density (OD) parameter

Treatment group	Repetitions					Average standard deviation	OD cut
	1	2	3	4	5		
Test group	3.834	3.241	3.066	2.522	2.502	3.033±0.552	-
Control group	0.480	0.525	0.995	0.705	0.338	0.609±0.045	0.743



**Figure 2.** Percentage inhibitory activity of *P. aeruginosa* biofilm growth at different cell-free supernatant (CFS) treatment concentrations



**Figure 3.** Biofilm inhibition after 72 hours' incubation in microtiter plate biofilm assay stained with 0.1% crystal violet

**Table 3.** Post-Hoc Tukey inhibition of biofilm growth

Concentrations	Probability Value				
	K-	100%	50%	25%	12.5%
K-		0.000*	0.000*	0.002*	0.033*
100%	0.000*		0.996	0.367	0.045*
50%	0.000*	0.996		0.579	0.094
25%	0.002*	0.367	0.579		0.761
12.5%	0.033*	0.045*	0.094	0.761	

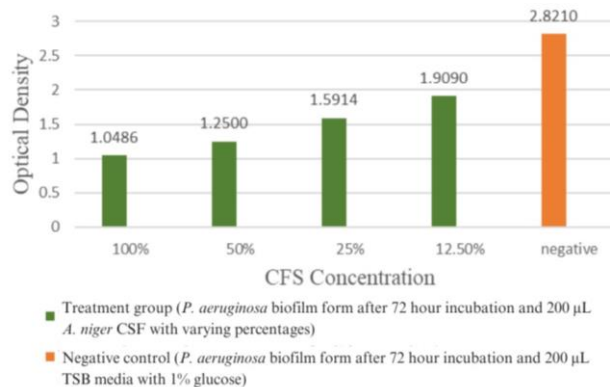
Note: K-: Negative control, \* indicates significant difference of treatment group

In One-way ANOVA test, the p-value of 0.000 was obtained ( $p < 0.05$ ), indicating a significant difference. Table 3 showed significant differences between the negative control group and all treatments with varying concentrations of CFS (cell-free supernatant). To investigate the relationship between the administration of *A. niger* CFS concentrations and the inhibition of biofilm formation in *P. aeruginosa*, Pearson correlation analysis was conducted. The Pearson correlation test yielded a value of 0.000 ( $p < 0.05$ ), demonstrating a strong correlation with a coefficient of 0.807. This positive correlation suggests a strong relationship between the concentration of CFS used and the level of biofilm growth inhibition in the tested bacteria, encouraging further research into biofilm inhibition strategies. In the biofilm formation process, bacteria irreversibly adhere to surfaces, forming micro-colonies and producing an extracellular polysaccharide (EPS) matrix. The components in *A. niger*, particularly the  $\alpha$ -amylase enzyme, can degrade the polysaccharides that constitute the biofilm structures in bacterial cells. Additionally, the  $\beta$ -glucosidase enzyme can break down glucose bonds, inhibiting bacterial movement, reducing EPS production, and disrupting cellular communication (quorum sensing) (Della Sala et al. 2019). Consequently,  $\beta$ -glucosidase can prevent bacterial attachment and inhibit growth (Gopinath et al. 2024).

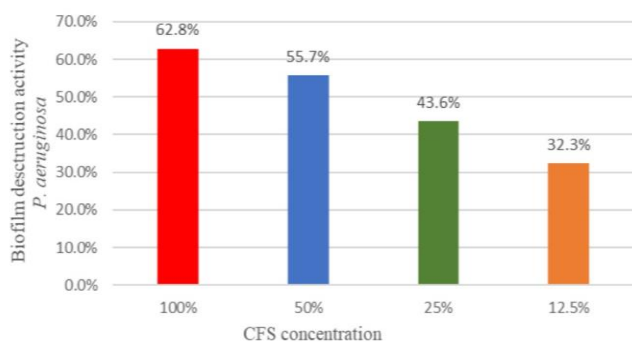
**Destruction of *P. aeruginosa* biofilms**

The results indicate that the highest optical density (OD) value recorded was 2.8212 in the negative control group. All test group concentrations showed lower OD values compared to the negative control. Among the test

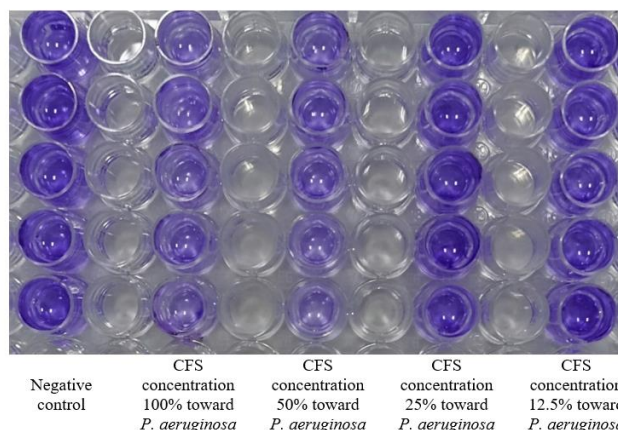
groups, the highest OD value of 1.909 was observed at a 12.5% CFS concentration (Figure 4). This can be attributed to the lower concentration of CFS, which has a minimal effect on inhibiting biofilm destruction, resulting in cloudier liquid and a higher OD reading. In contrast, the lowest OD value of 1.0486 was noted at a concentration of 100%. Here, the higher concentration of CFS significantly impacts biofilm destruction, leading to less turbidity in the liquid and, consequently, the lowest OD value among all test groups. The decrease in OD values relative to the negative control indicates that the thickness of the biofilm was reduced following treatment with CFS. This suggests that *A. niger* CFS can effectively destroy *P. aeruginosa* bacterial biofilm. Thus, higher CFS concentrations correlate with lower OD values, indicating more effective biofilm destruction.



**Figure 4.** Destruction of *P. aeruginosa* biofilm as measured by the optical density (OD) parameter



**Figure 5.** Destruction percentage of *P. aeruginosa* biofilm due to varying concentrations of cell-free supernatant (CFS) treatment



**Figure 6.** Biofilm destruction after administration of CFS and inhibition after 72 hours of incubation in a microtiter plate biofilm assay stained with 0.1% crystal violet

**Table 4.** Post-Hoc Tukey of biofilm destruction

Concentrations	Probability Value				
	K-	100%	50%	25%	12.5%
K-		0.000*	0.000*	0.000*	0.000*
100%	0.000*		0.665	0.003*	0.014*
50%	0.000*	0.665		0.058	0.230
25%	0.000*	0.003*	0.058		0.960
12.5%	0.000*	0.014*	0.203	0.960	

Note: K-: Negative control, \* indicates significant difference of treatment group

The percentage of biofilm destruction activity is illustrated in Figure 5, while the microtiter plate biofilm assay results are shown in Figure 6. The highest biofilm destruction activity, at 63%, was observed in the cell-free supernatant (CFS) at a concentration of 100%. In contrast, the lowest activity was found in CFS at concentrations of 12.5 and 32%.

The Post Hoc Tukey analysis reveals significant differences between the negative control and all treatment groups (Table 4). A Pearson correlation analysis demonstrates a relationship between the administration of CFS (Cell-Free Supernatant) concentrations of *A. niger* and the degradation of *P. aeruginosa* biofilms. The analysis yielded a p-value of 0.000 ( $p < 0.05$ ), indicating a strong correlation with a correlation coefficient of 0.846. This suggests a positive relationship, indicating that higher CFS concentrations correlate with increased destruction of the biofilm growth of the test bacteria. The effectiveness of a crude fungal supernatant (CFS) from *A. niger* in destroying biofilms relies on the ability of its active components to penetrate and break down the extracellular polymeric substance (EPS) matrix.

Research indicates that the enzyme  $\alpha$ -amylase found in *A. niger* can degrade EPS by disrupting its main structural components, which include proteins and carbohydrates. Supporting this, a study by Lahiri et al. (2021) conducted in silico revealed that the interaction between  $\alpha$ -amylase and *Pseudomonas polysaccharides* is spontaneous. This is demonstrated by a significant correlation between the energy released during the action of  $\alpha$ -amylase and the

biofilm EPS, highlighting the role of  $\alpha$ -amylase in lysing the EPS layer that surrounds the bacteria, thus weakening the biofilm. Alginate lyase is another hydrolytic enzyme that catalyzes the cleavage of  $\beta$ -elimination bonds in the alginate polymer chain, resulting in the production of short-chain oligosaccharides. This enzymatic process disrupts the three-dimensional structure of the biofilm, allowing alginate lyase to compromise the biofilm's ability to protect bacterial colonies from external disturbances (Daboor et al. 2021; Zhang et al. 2021).

In conclusion, based on the results, it can be concluded that CFS of *A. niger* had the ability to inhibit the growth and destruction of *P. aeruginosa* biofilms. It was also observed that the minimum biofilm inhibitory concentration (MBIC<sub>50</sub>) and minimum biofilm eradication concentration (MBEC<sub>50</sub>) of *A. niger* against *P. aeruginosa* were obtained at a concentration of 50% with a percentage of 56 and 55.7%, respectively. Further research is needed regarding the specific active compounds contained in *A. niger* CFS and the mechanism of action of these compounds as anti-biofilm agents.

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## Review: Phytochemical composition, biological activity, and health-promoting effects of *Avicennia* spp. (Avicenniaceae)

ARDITA RAHMANIA<sup>1</sup>, ALYA ADYSTI REVALITHA<sup>1</sup>, ABIZAN RAMADHAN TORIMBANU<sup>1</sup>,  
AMITA BELLA MUSTIKA<sup>1</sup>, GILANG DWI NUGROHO<sup>2</sup>, DARLINA MD NAIM<sup>3</sup>, AHMAD DWI SETYAWAN<sup>1,4,\*</sup>

<sup>1</sup>Department of Environmental Science, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret. Jl. Ir. Sutami 36A, Surakarta 57126, Central Java, Indonesia. Tel./fax.: +62-271-663375, \*email: volatileoils@gmail.com

<sup>2</sup>Biodiversity Study Club, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret. Jl. Ir. Sutami 36A, Surakarta 57126, Central Java, Indonesia

<sup>3</sup>School of Biological Sciences, Universiti Sains Malaysia. 11800 Pulau Pinang, Malaysia

<sup>4</sup>Biodiversity Research Group, Universitas Sebelas Maret. Jl. Ir. Sutami 36A, Surakarta 57126, Central Java, Indonesia

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**Abstract.** *Rahmania A, Revalitha AA, Mustika AB, Torimbanu AR, Nugroho GD, Md Naim D, Setyawan AD. 2025. Review: Phytochemical composition, biological activity, and health-promoting effects of Avicennia spp. (Avicenniaceae). Asian J Trop Biotechnol 22: 96-110.* Mangroves unique and valuable ecosystems, are groups of trees and shrubs that grow in coastal ecosystems exposed to tides. They play an important role in maintaining biodiversity and reducing the impact of coastal erosion. Mangroves are unique and valuable ecosystems because they provide coastal protection and carbon sequestration services. The genus *Avicennia* (Avicenniaceae) is a plant of mangrove consisting of eight species, namely *A. bicolor*, *A. germinans*, *A. marina*, *A. officinalis*, *A. schaueriana*, *A. balanophora*, *A. integra*, and *A. alba*, which are widely distributed in tropical and subtropical coastal areas. What sets *Avicennia* spp. apart is their remarkable adaptability to extreme environmental conditions, making them a key player in coastal ecosystems. Mangroves, especially *Avicennia* spp., have been known to contain many potentials and benefits. These plants are important for coastal ecosystems and have significant pharmacological potential. This study aims to identify and compile the phytochemical composition, biological activity, and health-promoting effect of *Avicennia* spp. There are 9 main phytochemical components: Alkaloids, terpenoids, steroids, phenolics, saponins, flavonoids, tannins, steroids, and glycosides. In addition, there are also several biological activity properties of *Avicennia* species, namely as antioxidants, anticancer, antimicrobial, anti-inflammatory, and antihyperglycemic. *Avicennia* spp. also offers traditional and modern treatments using various ingredients. By providing a comprehensive review of *Avicennia* spp., this study will provide a solid foundation for further research development, clinical application, and sustainable utilization and open up new opportunities in the field of natural material-based health.

**Keywords:** *Avicennia*, mangrove, medicine, pharmacology, phytochemicals

### INTRODUCTION

Mangroves are groups of trees and shrubs that thrive in tidal coastal ecosystems characterized by soft, anaerobic mud (Song et al. 2022). These ecosystems dominate tropical and subtropical coastal areas worldwide, making them essential components of coastal regions (Tomlinson 2016; Nugroho et al. 2020). Mangrove forests rank among the world's most productive ecosystems, providing habitat for diverse flora and fauna while playing a critical role in biodiversity, coastal protection, and carbon sequestration (Romañach et al. 2018; Law et al. 2020; Irwansyah et al. 2021; Wiraatmaja et al. 2022; Dewi et al. 2023; Rohman et al. 2023). These unique ecosystems are vital in reducing wave impacts, preventing beach erosion, and maintaining biodiversity. Mangroves are highly resilient plants that survive extreme environmental conditions, including high salinity, fluctuating tides, elevated temperatures, strong winds, and anaerobic soils (Thatoi et al. 2016).

Mangrove ecosystems, which consist of woody vegetation found in intertidal and brackish marine environments, are essential coastal wetlands (Hamilton and Friess 2018).

Their preservation and restoration are critical due to their ability to capture and store substantial amounts of carbon, effectively offsetting anthropogenic greenhouse gas emissions (Murdiyarso et al. 2015; Howard et al. 2017; Kauffman et al. 2020; Nur et al. 2022). Additionally, mangroves possess a remarkable diversity of bioactive compounds. For instance, *Avicennia*, a true mangrove genus from the Acanthaceae family, is distributed globally across tropical and temperate coastal regions (da Silva Pontes et al. 2020). This genus has shown significant potential in the health sector, with applications as antimicrobial, antidiabetic, anticancer, and antioxidant agents (Sumarlin et al. 2014; Ridlo et al. 2017). Overall, mangroves play an indispensable role in maintaining environmental balance by serving as habitats for diverse species, protecting coastlines, naturally filtering pollutants, and storing carbon reserves. Their multifunctional nature underscores the importance of sustainable utilization and conservation efforts (Mukherjee et al. 2014; Kurniawati et al. 2022; Sujiwo and Nurlaili 2022).

The genus *Avicennia* contains eight species, but some of those previously reported are varieties or have evolved differently with respect to their morphological adaptations

relating to leaves and roots in different geographical locations (Thatoi et al. 2016). *Avicennia* are generally 10-25 m in height, and some reach up to 30 m in height. Leaves are simple, opposite, oval to pointed, have salt-secreting glands (Chan et al. 2022), and have short, low-branched stems. Among the frontier mangrove species is *Avicennia marina* (Forssk.) Vierh. It is one of the most widespread in tropical estuaries, inhabiting the outer zones of mangrove forests (Feher and Hester 2018). The eight species are distributed in two main water areas in the tropics: the Old World (Afro-Asia and Australasia) and the New World (Eastern Pacific and Caribbean). However, only four species are common in Indonesia (Halidah 2014). In the southeastern United States, there are three common native mangrove species: *Avicennia germinans* (L.) Stearn, *Rhizophora mangle* Roxb, and *Laguncularia racemosa* (L.) C.F.Gaertn (Snyder et al. 2022). These species can be found on coastlines, lagoons, and estuaries across multiple continents, including Southeast Asia, South Asia, East Asia, East Africa, South Africa, Madagascar, Northern Australia, Eastern Australia, North America, South America, the Caribbean Islands, and the Oceania Islands (POWO 2023). Currently, *Avicennia africana* Beauverd and *Avicennia nitida* Jacq. are considered synonyms of *A. germinans*. Meanwhile, *Avicennia lanata* (from India) and *Avicennia tonduzii* (from the Americas) are no longer considered valid species (POWO 2023/). The wide distribution of *Avicennia* spp. reflects its ecological tolerance and important role in coastal ecosystems (Singh et al. 2024). Mangrove forests dominated by *Avicennia* spp. provide a habitat for a wide variety of marine and terrestrial organisms and protect coast-lines from erosion and storm surges (Wang et al. 2023).

However, limited reviews are specifically discussed in-depth on *Avicennia* spp.. Therefore, a comprehensive review of *Avicennia* spp. is needed to identify and compile various studies related to its potential, especially in the medical field. Hopefully, the information presented in this study provides a solid foundation for further research, clinical applications, sustainable utilization, and new opportunities in natural material-based healthcare.

## BOTANICAL DESCRIPTION AND DISTRIBUTION

### Botanical description

*Avicennia* belongs to the Acanthaceae family. The genus *Avicennia* consists of eight species of mangrove plants (Lalitha et al. 2021), and it is the only genus of mangroves found worldwide. However, some previously reported species are now classified as varieties or have undergone distinct evolutionary changes in their morphological adaptations of leaves and roots due to variations in geographical locations.

The species of the genus *Avicennia* are *A. bicolor* Standl., *A. germinans*, *A. marina*, *A. alba* Blume, *A. officinalis* L., and *A. schaueriana* Stapf & Leechm. ex Moldenke., *A. balanophora* Staph & Moldenke and *A. integra* N.C.Duke. *Avicennia* represents the largest polymorphic genus of mangroves and is ecologically, systematically, morphologically,

and genetically different from other taxa (Sabdanawaty et al. 2021). *Avicennia* are generally 10-25 m tall, and some reach 30 m. The leaves are simple, opposite, oval to pointed in shape, have glands to secrete salt (Chan et al. 2022), and have short, low-branched stems. As a member of the Acanthaceae family, *Avicennia* is known as a mangrove tree widely distributed in tropical and subtropical regions worldwide. They are mainly in coastal ecosystems, providing protection against coastal erosion, habitat for various marine organisms, and effectively storing carbon in nutrient-rich mangrove soils. Genetic variation and environmental adaptations have led to significant variation in taxonomy and morphology among different *Avicennia* species.

*Avicennia bicolor* can grow up to 8-20 m with a dense, dark green crown. The flowers are identical to those of *A. germinans*, with hairy petals inside, but they are smaller in diameter (5-6 mm). The leaves are oval with tapered tips, varying from dark green to grayish brown. This species is often found in downstream tidal areas, where rivers meet the sea. Habitats include terrestrial, freshwater, and marine. This species has a high tolerance to hypersaline conditions.

*Avicennia germinans* is a mangrove species that lives on extensive coastlines (Lonard et al. 2017), also known as black mangrove (Pickens et al. 2022). This mangrove species has the northernmost distribution of all mangrove species in North America. It can grow up to 30-50 meters, and the leaf length is 3-15 cm and elliptical. Dookie et al. (2023) explained that *A. germinans* exhibit mesophyll characteristics and variation in leaf circumference, area, length, width, dry mass, wet mass, turgid mass, leaf-specific area, and relative water content across ecosystems. This species can grow in permanently flooded soil.

*Avicennia marina* is a medium-sized *Avicennia* species with a height ranging from 3-14 meters (Kandasamy et al. 2021), and it is the most widespread mangrove species in the world. This species has lance-shaped leaves with flat edges and a glossy surface. It can grow on soils with varying sand and clay proportions (Budiadi et al. 2022) at 0-50 m above sea level. *Avicennia marina* is known as white *api-api*. This species has small clusters of 3-5 yellow flowers, approximately one centimeter in diameter, and bears fruit with a single large, fleshy seed (Baishya et al. 2020). It also has a breath root, which is a branching root that grows at a regular distance vertically from a horizontal root that is immersed in the soil. The fruit is grain-shaped, like a mango. It forms shrubs or trees with a height of 12 to 20 meters.

*Avicennia alba* is a mangrove tree widely distributed in tropical and subtropical regions (Kar et al. 2015). This species, known as the white flame, is found on the coasts and estuaries of India, Southeast Asia, Australia, and Oceania. This species has 4 light yellow petals, 4 light green sepals, 4 broken anthers, and a stigma (Jeffry et al. 2024). It grows less than 20 meters tall and often develops under the trunk. The leaves are dark green and 15 cm long. *Avicennia alba* has been a pioneer species in the successional mangrove and is very effective in colonizing new intertidal areas. It shows the importance of *Avicennia* spp. in strengthening mangrove ecosystems, especially after geomorphological changes that cause mangrove

degradation and the establishment of potential new areas for mangrove colonization (Farshid et al. 2023).

*Avicennia officinalis* or ludic fire, a member of the Avicenniaceae family, is a mangrove tree commonly found in tidal forests of river estuaries along the coast of South Asia, including India, where it is one of the three prominent *Avicennia* species (Krishnamurthy et al. 2023). Native to Tropical Asia and extending to northern and eastern Australia, this species grows to a height of 12-18 meters and is characterized by spoon-shaped leaves with shiny green upper surfaces (Thatoi et al. 2016). Its flowers, the largest among the genus, are orange-yellow, round, and 1 cm in diameter, while the fruits are 2-3 cm long, oval, slightly beaked, smooth, and velvety, each containing a single seed that fills the capsule (Thatoi et al. 2016).

*Avicennia schaueriana* grows as a tree reaching 10 to 20 meters in height. The trunk is gray to brown. The fruits are pale sap green, rarely with a purple tinge, and flatter and more pointed than *A. germinans*. The roots of this species develop well in mud soils and brackish water, which help stabilize the soil and protect the coast from erosion. It grows in coastal and estuarine locations (POWO 2023).

*Avicennia balanophora* is native to Queensland and Australia and usually grows in coastal and estuarine locations. It is generally of modest length. The main root of this species can reach about 1 meter in length. These mangroves have aerial pencil roots and are often found in the intertidal zone of estuarial areas (Thatoi et al. 2016). This species is known for its ability to survive in wet environments and is often a habitat for various marine animals (Aluri 2022).

*Avicennia integra* is a mangrove tree species that grows in coastal areas and estuaries. It can grow up to 30 meters tall (Thatoi et al. 2016). The leaves of this species have distinctive characteristics, such as salt glands on their lower surface that produce white visible salt crystals (Li et al. 2016). These leaves are usually stalked, opposite, flat-edged, and with poorly defined veins (Thatoi et al. 2016).

### Distribution and habitat

Mangroves are intertidal ecosystems or groups of plants that are widely adapted and live in coastal environments

(Giri et al. 2014). Mangroves are important in natural disaster mitigation, protecting the coast from storms, waves, and soil erosion (Marlianingrum et al. 2021). Thriving mangrove forests shield coastal ecosystems from natural disasters and are better at carbon sequestration than other forest types (Sahana et al. 2022). Although mangrove forests hold significant social, ecological, and economic value in coastal ecosystems, a limited understanding of their sensitivity has contributed to their degradation (Sobhani and Danekkar 2023).

The distribution of *Avicennia* species extends from Africa to Asia, Australia, and the Americas, with certain species adapted to local environmental conditions. *Avicennia* species typically dominate the coastal edge of mangrove forests, forming dense forests characterized by their distinctive aerial roots, known as pneumatophores, which help facilitate gas exchange in waterlogged soil. In some locations, mangroves are found on oceanic (freshwater) islands (Crameri and Ellison 2022) and in arid areas where freshwater only originates from groundwater (Adame et al. 2021). Mangrove habitats are dynamic because they depend on the hydrological regime of tides, waves, and continental waters. Mangroves have good ecological resilience because they thrive under harsh conditions, including high salinity, extreme tides, strong winds, high temperatures, and muddy and anaerobic soils (Ribeiro et al. 2019; Ellison et al. 2020). Moteriy et al. (2015) reported that mangroves that grow in coastal wetlands have unique adaptations to changes in salinity, temperature, nutrients, and excessive radiation (Rozirwan et al. 2021).

*Avicennia* spp. has a varied distribution in tropical and subtropical regions of North and South America, including Colombia, Costa Rica, Mexico; Panama, Brazil, and Chile; the African coast; the Middle East; South and Southeast Asia, which includes the Coasts of India, Bangladesh, Malaysia, Vietnam, Thailand, Indonesia; and the coasts of TransAsian countries of Australia and New Zealand (Figure 1). *Avicennia marina* is the most widely distributed mangrove species in the Western Indo-Pacific Region (EIDohaji et al. 2020). This species may be the only species with highly evolved morphological, biological, ecological, and physiological adaptations to various environmental conditions (Das et al. 2016).



**Figure 1.** Distribution of *Avicennia* spp. Note: The higher intensity of the orange color indicates that, at that point, the presence of the species is higher and dense (source: gbif.org)

**Table 1.** Summary on *Avicennia* distribution, phytochemistry, bioactivity, and medical utilization

Species name	Distribution	Phytochemistry	Bioactivity	Medical utilization	
				Traditional	Modern
<i>Avicennia bicolor</i>	<b>Pacific coast of Central and South America (endemic)</b> <b>Central America:</b> Mexico (southern), Guatemala, El Salvador, Honduras, Nicaragua, Costa Rica, Panama. <b>South America:</b> Colombia, Ecuador, Peru.	<b>Flavonoids:</b> Quercetin, kaempferol (antioxidant, antimicrobial). <b>Triterpenoids/Steroids:</b> Lupeol, $\beta$ -sitosterol, taraxerol (anti-inflammatory). <b>Saponins/Tannins:</b> Defense against pathogens/herbivores. <b>Naphthoquinones:</b> Avicennin (roots/bark). <b>Trace alkaloids:</b> similar to other <i>Avicennia</i> spp.	Antimicrobial (leaf extracts against Gram-positive bacteria) Antioxidant (phenolic compounds in bark) Traditional wound healing (Central American folk medicine)	Used in Central America for wound healing (bark poultice) Leaf extracts applied to skin infections	<b>Antimicrobial creams:</b> leaf extracts for skin infections <b>Wound dressings:</b> bark tannins for healing acceleration
<i>A. germinans</i>	<b>Americas:</b> USA (Florida, Louisiana), Mexico, Belize, Guatemala, Honduras, Nicaragua, Costa Rica, Panama, Colombia, Venezuela, Guyana, Suriname, French Guiana, Brazil, Ecuador, Peru. <b>Africa:</b> Senegal, Mauritania, Gambia, Guinea-Bissau, Sierra Leone, Liberia, Nigeria, Cameroon, Gabon, Angola. <b>Caribbean:</b> Cuba, Jamaica, Dominican Republic, Puerto Rico, Bahamas.	<b>Iridoids:</b> Aucubin (leaves) (unique) <b>Triterpenes:</b> Lupeol, betulinic acid (bark/roots) <b>Flavonoids:</b> Vitexin (leaves), luteolin-7-O-glucoside (bark) <b>Tannins:</b> Proanthocyanidins (bark) (highest concentration in genus)	Anticancer (betulinic acid from bark against tumor cells) Antileishmanial (unique among <i>Avicennia</i> species) Antimalarial (leaf extracts) Salt-tolerance (phenolic acids in leaves)	Antimalarial (leaf decoctions in West Africa) Treatment of leprosy (bark extracts in Caribbean) Snakebite remedy (root preparations in South America)	<b>Anticancer drugs:</b> betulinic acid in clinical trials for melanoma <b>Antileishmanial:</b> bark extracts against parasitic diseases <b>Antimalarial research:</b> iridoids as lead compounds
<i>A. marina</i>	<b>Asia:</b> India, Bangladesh, Myanmar, Thailand, Vietnam, Malaysia, Indonesia, Philippines, China (Hainan), Taiwan, Japan (Ryukyu Is.), Pakistan, Iran, Oman, UAE, Saudi Arabia, Yemen. <b>Africa:</b> Egypt (Red Sea), Sudan, Eritrea, Somalia, Kenya, Tanzania, Mozambique, South Africa, Madagascar. <b>Australia &amp; Pacific:</b> Australia (all coastal states), New Zealand (northern), Papua New Guinea, Fiji. <b>Middle East:</b> Israel, Jordan, Qatar, Kuwait.	<b>Naphthoquinones:</b> Avicequinone-C (roots) (salt tolerance) <b>Limonoids:</b> Marinoids (leaves) (species-specific) <b>Flavonoids:</b> Quercetin-3-O-glucoside (leaves/bark)	Antimicrobial (avicequinone-C against marine pathogens) Antioxidant (highest in leaves among mangroves) UV-protection (leaf flavonoids) Diabetes management (bark extracts show $\alpha$ -glucosidase inhibition)	Diabetes management (bark tea in India/Middle East) Rheumatism treatment (leaf poultice in East Africa) Antidiarrheal (fruit preparations in Southeast Asia)	<b>Diabetes supplements:</b> bark extracts for $\alpha$ -glucosidase inhibition <b>UV-protective cosmetics:</b> leaf flavonoids in sunscreens <b>Antioxidant nutraceuticals:</b> standardized phenolic extracts

<i>A. alba</i>	<b>Asia:</b> India, Bangladesh, Myanmar, Thailand, Malaysia, Singapore, Indonesia, Vietnam, Cambodia, Philippines, China (Hainan), Sri Lanka. <b>Australia:</b> Northern Territory (rare).	<b>Flavonoids:</b> Kaempferol derivatives (fruits), albaflavonoid (leaves) (antiviral) <b>Diterpenes:</b> Alba-diterpene A (bark)	Antiviral (leaf flavonoids against HSV) Mosquito larvicidal (bark compounds) Cytotoxic (fruit extracts on cancer cells)	Not recorded	<b>Antiviral research:</b> leaf flavonoids against HSV-1/HSV-2 <b>Larvicidal products:</b> mosquito control formulations
<i>A. officinalis</i>	<b>Asia:</b> India, Sri Lanka, Bangladesh, Myanmar, Thailand, Malaysia, Indonesia, Vietnam, Philippines, China (Guangdong, Hainan), Taiwan. <b>Australia:</b> Northern coasts (rare).	<b>Naphthoquinones:</b> Lapachol (fruits) (antitumor) <b>Phenolics:</b> Gallic acid (bark) (highest antioxidant activity)	Hepatoprotective (fruit extracts) Antitumor (lapachol from fruits) Radioprotective (leaf phenolics) Anti-ulcer (bark extracts)	Hepatitis treatment (fruit extracts in India) Ulcer remedy (bark paste in Myanmar) Fever reduction (leaf infusions in Thailand)	<b>Hepatoprotective drugs:</b> fruit-derived lapachol in liver therapies <b>Radioprotective agents:</b> for chemotherapy support <b>Antitumor research:</b> naphthoquinones in preclinical studies
<i>A. schaueriana</i>	<b>South America:</b> Brazil, Venezuela, Guyana, Suriname, French Guiana, Colombia (Caribbean coast).	<b>Flavonoids:</b> Vitexin (leaves) (cardioprotective) <b>Triterpenes:</b> Taraxerol (bark) (wound healing)	Cardioprotective (vitexin from leaves) Wound healing (bark taraxerol) Anti-inflammatory (leaf extracts)	Not recorded	<b>Cardioprotective research:</b> vitexin for ischemic injury
<i>A. balanophora</i>	<b>Northern Australia (endemic):</b> Queensland, Northern Territory.	<b>Triterpenes:</b> Balanophorin (leaves) (Australia-endemic)	Antimicrobial (unique leaf triterpenes) Antioxidant (phenolic content)	Not recorded	Not recorded
<i>A. integra</i>	<b>Northern Australia (endemic):</b> Northern Territory, Western Australia	<b>Triterpenes:</b> Integrins (bark) (unique pentacyclic compounds)	UV-protection (high leaf phenolics) Antimicrobial (bark integrins) Nematocidal (root compounds)	Not recorded	Not recorded

Note: Distribution: Ellison et al. (1999), Giesen et al. (2007), Spalding et al. (2010), Giri et al. (2011), Tomlinson (2016), Duke (2017); Phytochemistry: Bandaranayake (2002), Wu et al. (2008), Sadeer and Mahomoodally (2022), Govindhan (2024); Bioactivity: Patra et al. (2011), Assaw et al. (2018), Fauzi et al. (2021), Ibrahim et al. (2022), Rajivgandhi et al. (2024), Ghosh et al. (2025); Traditional Medical Utilization: Bandaranayake (1998), Kathiresan and Bingham (2001), Abeyasinghe et al. (2009), Sura et al. (2011), Mazumder et al. (2016); Modern Medical Utilization: Esau et al. (2015), Das et al. (2016), Sivakumar et al. (2018), Ahmed et al. (2022, 2024), Rashno et al. (2022).

*Avicennia germinans* species are found along the coastlines of North and South America, including the coastlines of Angola, Jamaica, Liberia, Mauritania, Mexico, Antigua and Barbuda. In addition, this species is widespread on the coastline of Africa, including Congo, Ghana, and Nigeria (Thatoi et al. 2016). *Avicennia bicolor* is widespread along the coastlines of Colombia, Costa Rica, El Salvador, Guatemala, Honduras, Mexico, Nicaragua, and Panama (Mori et al. 2015). Meanwhile, *A. officinalis* species is rarely found on the coast of South and Southeast Asia and is limited to Bangladesh, Brunei, Cambodia, India, Indonesia, Malaysia, Myanmar, Papua New Guinea, Philippines, Singapore, Sri Lanka, Thailand, and Vietnam. *Avicennia schaueriana* species can be found widely distributed in North and South American coastlines, including Anguilla, Antigua and Barbuda, Brazil, Dominica, Trinidad and Tobago, and Venezuela (Thatoi et al. 2016).

*Avicennia marina* grows along the coast of East Africa and Madagascar to India, Indo-China, South China, Taiwan, Thailand, all of Malesia, Solomon Islands, New Caledonia, Australia and northern New Zealand, in South and Southeast Asia, including Singapore, Vietnam, Middle East, African coastline including Egypt, Madagascar, Mozambique, South Africa, Tanzania (Thatoi et al. 2016). This species is the most dominant mangrove species and the primary coastal vegetation found along the shores of the Arabian Gulf in the UAE, Saudi Arabia, Bahrain, Qatar, and Iran (El-Tarabily et al. 2021). Although the plant is mainly distributed through the tropics, it is also distributed in temperate regions, including Southwest Asia, along the coast of the Arabian Gulf. The eastern and western coasts of the Red Sea. *Avicennia marina* is the most extensive in distribution due to its adaptability to a wide range of environmental conditions (Baba et al. 2016).

*Avicennia balanophora* and *A. integra* have a restricted distribution on the Australian coastline. In this region, *Avicennia* species grow on pristine flats and densely packed mudflats along the coast, in brackish coastal swamps, and on riverbanks (Thatoi et al. 2016). *Avicennia alba* is mainly found in the east coast region of India, the Pacific Ocean Islands, and Australia (Singh et al. 2024). It usually grows in tidal areas along rivers and dirt parts of the coast. *Avicennia balanophora* is widely distributed in Southeast Asia and Australia, often growing in wet and soggy coastal regions. Meanwhile, *A. integra* is also found throughout Southeast Asia and Australia, usually growing in damp and water-affected coastal areas (Singh et al. 2024).

## PHYTOCHEMICAL COMPOSITION

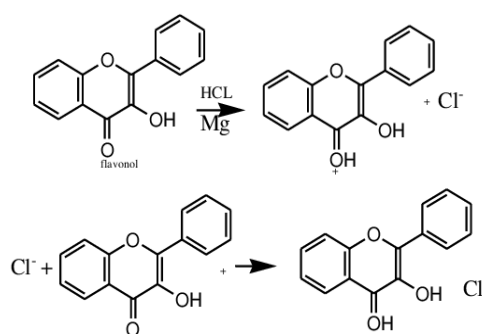
Plants have primary and secondary metabolites that can be used for medicine and other purposes. Phytochemicals, also known as phytonutrients, are chemical compounds produced by plants through primary or secondary metabolism (Roy and Datta 2019). In the search for new and natural medicines, marine plants, particularly mangroves, are an alternative source of health-essential

chemicals (Yang et al. 2014). Many reports have documented that the genus *Avicennia* spp. possess several unique metabolites of varied chemical classes, which may be responsible for their various pharmacological activities (Khan and Mubarak 2015). There are 9 major phytochemical components: alkaloids, terpenoids, steroids, phenolics, saponins, flavonoids, tannins, steroids, and glycosides. Studies on phytochemicals have revealed that most *Avicennia* spp. are rich in terpenoids, glucosides, and naphthalene derivatives (Sotheeswaran and Phomphalee 2016).

Figure 2 shows the reaction of flavonol reduction using magnesium (Mg) and hydrochloric acid (HCl). The initial flavonols are reduced by Mg in an acidic medium, producing positively charged flavonol ions (carbocations) with  $\text{Cl}^-$  as the ion partner. These positively charged ions then undergo rearrangement to form a more stable structure. Finally, the reaction produces a reduced compound with a change in the carbonyl group ( $\text{C}=\text{O}$ ) and stabilization by the  $\text{Cl}^-$  ion. Studies often use this reaction to modify flavonoids chemical or biological properties. These natural compounds are concentrated in plants leaves, stems/bark, and aerial roots.

In the research conducted by Das et al. (2018), qualitative and quantitative phytochemical analysis of Ethanol Leaf (EL) and Stem Bark (EB) extracts from *A. officinalis* identified various phytochemical compounds responsible for their bioactivity. It showed that EL and EB extracts of *A. officinalis* effectively inhibit  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes and have antioxidant, antimicrobial, and cytotoxic potential (Das et al. 2018).

The extract of *A. alba* demonstrates activity against one specific bacterium, *Erwinia herbicola*, but not against the other tested bacteria (Witoyo and Utoro 2023). This lack of effectiveness may be attributed to bacterial resistance mechanisms, such as enzymatic degradation, modification of the target site, or reduced intracellular accumulation of the drug. Additionally, the extract concentration may have been insufficient to provide an adequate amount of the active ingredient (Wulandari et al. 2022). *Avicennia alba* extract is only effective against *E. herbicola*, with a MIC value of 400  $\mu\text{g}/\text{mL}$ . The difference in sensitivity may be due to qualitative differences in the existing phytochemicals and the concentration of active ingredients in different plant extracts (Das 2020; Das and Das 2020).



**Figure 2.** Flavonoid chemical compounds in *Avicennia* spp. species

Nguyen et al. (2022) reported that *A. officinalis* leaf extracts (methanol, ethanol, ethyl acetate, acetone, dichloromethane, and chloroform) revealed an increase in yield with increasing solvent polarity, and the extract with the highest Total Phenolic Content (TPC), Total Flavonoid Content (TFC), and antioxidant activity (measured using DPPH and ABTS assays) was acetone extract. Thus, acetone is an optimal solvent for extracting polyphenols from *A. officinalis* (Nguyen et al. 2022).

*Avicennia marina* is rich in flavonoids, carbohydrates, alkaloids, glycosides, phenols, proteins, reducing sugars, and cardiac glycosides (Moghal et al. 2016). Phytochemical analysis of different species of *Avicennia* showed the presence of similar bioactive compounds, although there were variations between different species. Species such as *A. marina*, *A. alba*, and *A. officinalis* contain major phytochemical compounds such as alkaloids, flavonoids, tannins, saponins, and glycase. The difference in the concentration of these compounds can be attributed to the ecological adaptations and the specific growth environment. For example, *A. marina* showcases higher tannins and flavonoids than *A. alba* and *A. officinalis*, potentially linked to its defensive role against pathogens and herbivores. *Avicennia alba*, on the other hand, boasts a more diverse range of flavonoids, suggesting its adaptation to a broader range of environmental conditions. Then, the presence of saponins in all three species underscores their potential in biomedical applications. Saponins are renowned for their diverse biological activities, including anti-inflammatory and antimicrobial properties. Despite their specific differences, the shared phytochemical composition among *Avicennia* species underscores the need for further research to isolate and characterize these compounds. This will enable us to evaluate their pharmacological potential and health applications, a crucial step in understanding their potential benefits to human health.

The first chemical investigation of the genus *Avicennia* dates back to 1913, when Bournot isolated lapachol, a compound with antitumor activity, from *A. officinalis* growing in West Africa and India (Thatoi et al. 2016). Further research has led to the chemical profile of *A. officinalis* showing the presence of a broad class of bioactive compounds such as terpenoids, steroids, alkaloids, flavonoids, polyphenols, phenolic acids, saponins, and tannins (Mehta et al. 2021).

## BIOLOGICAL ACTIVITY

Medicinal plants, such as mangrove plants, are important in maintaining public health. Different parts of various *Avicennia* species are used in traditional medicine by indigenous communities in mangrove forests to treat several illnesses (Beniwal et al. 2024). Chemical substances in different compositions in medicinal plants provide curative properties (Kirom and Ramadhania 2017). Curative properties refer to the ability of an action, substance, or method to cure or treat a particular disease or health condition. In the medical context, curative properties are usually associated

with treatment or therapy that aims to eliminate the main cause of a disease so that it can provide a complete cure to the patient. Still, palliative care focuses more on reducing symptoms and improving the quality of life without necessarily curing the underlying disease. One of the mangrove species that has been widely studied is *Avicennia*.

Biological activities in *Avicennia* species have very complex roles and functions. *Avicennia officinalis* has many beneficial pharmacological properties, such as anticancer, antibacterial, analgesic, anti-inflammatory, antitumor, and antiviral (Islam et al. 2022). The fruit, leaves, and roots of *A. officinalis* were used to cure hepatitis, while the bark of the stem was used as an antitumor (Erwin et al. 2019).

The leaves of *A. marina* are widely used for traditional medicine due to their proven pharmacological activities, including antioxidant, antimicrobial, and anticancer properties (Huang et al. 2016; Ananthavalli and Karpagam 2017; Iranawati et al. 2018; Andriani et al. 2021; Sohaib et al. 2022; Yassien et al. 2021; Witoyo and Utoro 2023; Annas et al. 2023). Similarly, *A. officinalis* demonstrates significant pharmacological potential and is traditionally utilized for treating hepatitis through its fruits, leaves, and roots. Both *A. officinalis* and *A. marina* show immense promise in developing plant-based therapies for various health conditions, with *A. marina* being particularly notable for its potent leaf extracts. Meanwhile, *A. marina* and *A. officinalis* show the widest range of validated biological activities. *A. germinans* has unique antiparasitic properties (Bandaranayake 2002; Fauzi et al. (2021).

## Antioxidants

Antioxidants are compounds that prevent and repair the damage of cells in the body. Antioxidants are chemical compounds that can provide one or more electrons to scavenge free radicals (Binuni et al. 2020). Antioxidants help neutralize free radicals and protect against diseases like cancer, diabetes, and stroke (Lalitha et al. 2021). *Avicennia officinalis* is significant in managing complications caused by oxidative stress. Its antioxidant potential has been demonstrated through *in vitro* and *in vivo* methods. Das et al. (2018) identified antioxidants in *A. officinalis* leaf extract at a 150 µg/mL concentration. Khushi et al. (2016) reported that phenolic components contribute to the plant's high antioxidant activity.

*Avicennia marina*, a well-known mangrove species, has high polyphenol content in ethanol extract and the potential to reduce oxidative stress in diabetic rats, which plays a significant role in antioxidant potential (Al-Jaghtmi et al. 2020), as indicated by decreased lipid peroxide levels in liver tissue (Al-Jaghtmi et al. 2020). Bioactive compounds like flavonoids and steroids also exhibit antioxidant potential (Alara et al. 2019). Like other mangrove plants, *A. germinans* contain many antioxidants, such as vitamins C and E, which protect the body's cells. The antioxidant content in *A. germinans* varies based on environmental conditions and serves as a natural raw material for health products. Additionally, *A. germinans* can absorb toxins, particularly heavy metals (Halidah 2014). *Avicennia alba*

also exhibits antioxidant activity due to its phenolic compounds, which play a crucial role in maintaining the health of biological systems (Wulandari et al. 2015).

### Antimicrobial

Pathogenic bacteria and viruses are harmful microorganisms that cause infections and death (Janik et al. 2020). Antimicrobials are substances that can prevent or kill the growth of microbes and have little toxicity to humans (Cheng et al. 2016). Various studies have been conducted to explore the antimicrobial potential of mangrove plants. The leaf and bark extracts of *A. officinalis* possess a broad spectrum of antimicrobial activity (Das et al. 2018) due to diterpene excoecarin A (Sulaiman et al. 2022). In addition, other species of *Avicennia* have also shown promising antimicrobial potential.

*Avicennia marina* exhibits antimicrobial activity against seven marine pathogenic bacteria (Manilal et al. 2016). The ethanol extract of *A. marina* root had antibacterial activity against *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* and the chloroform root extract showed antibacterial activity against *E. coli*, *P. aeruginosa*, and *S. aureus*. In addition, *A. marina* leaf extract also showed antibacterial activity against *S. aureus* and *E. coli* (Okla et al. 2021; Yassien et al. 2021). *Avicennia marina* extract significantly affects *E. coli* bacteria due to antimicrobial compounds such as steroids and triterpenoids (Renaldi et al. 2018) in the bark of stems, leaves, flowers, and fruits. Triterpenoid compounds are secondary metabolite compounds that are effective as antimicrobials. Iswadi et al. (2015) found that *A. marina* leaves are effective as antibacterial agents due to their polar compounds, which can inhibit bacterial growth. These polar compounds typically include flavonoids, tannins, saponins, alkaloids, and phenols.

A study by Kar et al. (2015) suggested that methanol and chloroform extracts of *A. alba* were active against *Streptococcus mutans*, *Lactobacillus acidophilus*, *Rhizoctonia solani*, *Pseudomonas marginalis*, *Erwinia carotovora*, and *Acremonium strictum*. Research by Erwin et al. (2019) shows that *A. alba* extract can inhibit the growth of *Vibrio harveyi* and *Vibrio* sp. (MC3P5). Then, *A. germinans* inhibits the growth of pathogenic microorganisms and possess anticancer due to flavonoids, polyphenols, and triterpenoids content.

### Antihyperglycemic

The antihyperglycemic compound lowers blood glucose (Mujianti and Sukmawati 2018). *Avicennia marina* contains flavonoids, tannins, and phenols, contributing to its antidiabetic properties or antihyperglycemic effects, and can stimulate cellular activity (Aljaghthmi et al. 2017; Cerri et al. 2022). The flavonoids, tannins, and phenols in *A. marina* possess antidiabetic and antihyperglycemic effects through several interrelated mechanisms. These compounds inhibit the enzyme alpha-glucosidase in the intestines, slowing glucose absorption and reducing post-meal blood sugar spikes. They also increase the sensitivity of cells to insulin, aiding in more efficient glucose

absorption from the bloodstream. The antioxidant properties protect the beta cells of the pancreas from oxidative damage, helping to maintain adequate insulin production. Tannins can form a protective layer in the intestines, further inhibiting glucose absorption.

Flavonoids, in addition to their insulin-stimulating properties, play a significant role in reducing inflammation and oxidative stress associated with insulin resistance. This dual action provides a reassuring prospect for the potential of flavonoids in diabetes management. By combining these effects - inhibiting glucose absorption, improving insulin sensitivity, protecting insulin-producing cells, and reducing insulin resistance - the flavonoids, tannins, and phenols in *A. marina* work synergistically to lower and control blood sugar levels, providing significant antihyperglycemic effects in diabetes management.

*Avicennia alba* possessed the antihyperglycemic activity (Biswas et al. 2018). Hyperglycemia worsens insulin resistance and interferes with secretion. The antihyperglycemic activity includes free radical antidote activity, increased insulin release, insulin-mimicking activity, and changes in glucose utilization (Biswas et al. 2018), which affect diabetic patients by reducing hepatic glucose output and increasing glucose absorption by skeletal muscles. Free radical antidotes can block reactive atoms or molecules in the human body so that they can trigger various diseases (Widiawati and Asih 2015).

### Anticancer

*Avicennia marina* has the anticancer potential without any adverse effects (Behbahani et al. 2018; Tian et al. 2020). This species contains Hymecromone as an anticancer by inhibiting the synthesis of hyaluronic acid. Hymecromone is a dietary supplement that is not toxic, even in high doses. Bioactive substances with anticancer properties have been found in the ethanol and ethyl acetate extracts of *A. marina* leaves (Afshar et al. 2022). In addition to its anticancer effects, *A. marina* phytoconstituents exhibit antibacterial and antioxidant qualities. *Avicennia marina* also contains ursolic acid, oleanolic acid, and lupeol, which are pentacyclic triterpenoids. Then, *A. marina* leaf extract can lower serum urea to protect tissues such as the pancreas, liver, and kidneys. Research by Qurrohman et al. (2020) shows that *A. marina* leaf extract inhibits cancer cells due to the presence of anticancer compounds in the form of polyisoprenoids.

Eswaraiah et al. (2020) reported that the leaf extract of *A. alba* exhibits anticancer activity in the methanol extract. In the methanol extract, phenolic compounds can inhibit the growth of lung cancer cells. Cytochrome c in methanol extracts can trigger programmed cell death (apoptosis) in cancer cells by activating cell death signaling pathways, such as cytochrome c release from mitochondria and caspase activation (Do et al. 2020). In addition, the extract may contain compounds that inhibit the cancer cell cycle, preventing uncontrolled cell division by inhibiting key enzymes in the cell cycle or growth factors. The compounds in the extract may also have powerful antioxidant properties that protect normal cells from DNA damage while

interfering with the growth of cancer cells that depend on certain levels of oxidative stress. In addition, some compounds may be able to inhibit the formation of new blood vessels that tumors need to grow and metastasize, as well as alter cell signaling pathways that are important for cancer cell growth and survival, such as the PI3K/AKT or MAPK pathways. The anticancer effects of *A. alba* include reducing chronic inflammation that is often associated with cancer progression and inhibiting cancer cell migration as well as invasion into different tissues. Some types of bioactive compounds that are commonly found include alkaloids, which are nitrogen-containing compounds with strong pharmacological activity, namely terpenoids, a large group of natural compounds consisting of isoprene units, some of which show anticancer activity, namely flavonoids. These polyphenol compounds that are known to have antioxidant and anticancer properties are steroids, organic compounds with a distinctive ring structure, some of which show antitumor activity, namely peptides, short chains of amino acids with a wide range of biological activities including anticancer, i.e., quinone, a compound that often exhibits cytotoxic activity against cancer cells, as well as phenols and polyphenols, compounds with one or more hydroxyl groups bound to an aromatic ring that frequently have antioxidant and anticancer properties *A. germinans* contained naphthoquinone which has anticancer potential (Angio et al. 2022).

### Anti-inflammatory

*Avicennia* has anti-inflammatory activity, in which inflammation could be caused by various stimuli such as pathogens or physical trauma through immunological responses (Rossi et al. 2021). Extracts from *A. officinalis* inhibit inflammatory mediators such as prostaglandins and cytokines and reduce inflammation (Borges et al. 2019). The sap of the stem in *A. marina* contains flavonoids that inhibit the activity of cyclooxygenase enzymes and lipoxygenase (Priamsarfi and Bima 2021). The flavonoid content in the leaf extract of *A. alba* plays a role in the anti-inflammatory process in two ways: by inhibiting capillary permeability and the anti-inflammatory mechanism of saponins (Wulandari et al. 2015). The anti-inflammatory activity of flavonoids limits the number of inflammatory cells that transform in the affected area so that they do not cause excessive tissue damage. *Avicennia lanata* Ridl. contains some polyisoprenoid compounds that can also be used as an anti-inflammatory (Pronin et al. 2014).

## HEALTH-PROMOTING EFFECT

### Traditional medicine

Medicinal plants have been used in traditional medicine (Dey et al. 2021) for thousands of years (Rakotoarivelo et al. 2015). The knowledge of using plants to treat diseases has been passed down from generation to generation. Mangrove plants, for instance, have long been utilized in traditional medicine for various ailments. These include asthma, arthritis, rheumatism, inflammation, and diabetes. It suggests mangrove plants have significant potential and

functions in everyday medicine (Habib et al. 2018; Alimbon and Manseguiao 2021).

The genus *Avicennia* is one type of mangrove plant that is known to have extensive and effective ethnomedical applications for various health conditions, such as use as an astringent, aphrodisiac, diuretic, and contraceptive, as well as in the treatment of asthma, cancer, antiulcer, rheumatism, malaria, bronchial ulcers, diarrhea, HIV, hepatitis, gastric ulcers, antitumor, anti-inflammatory, diabetes, smallpox, skin diseases, oxidative stress diseases, snakebite treatment, leprosy, toothache, and ulcers (Lalitha et al. 2021). *A. marina* and *A. officinalis* have the most extensive traditional applications (Bandaranayake 2002; Kathiresan and Bingham 2001; Fauzi et al. (2021).

According to Ahmed et al. (2022), *Avicennia germinans* (identified as *A. africana*) is an important ethnomedical plant that has long been used to treat various diseases, i.e., rheumatism, smallpox, asthma, ulcers, malaria, ringworm, gangrene wounds, and mouth ulcers. Many traditional societies use it as a remedy for several reasons, such as ease of access and hereditary beliefs. The wood in *A. germinans* has also been used as a supplement to the traditional oil (Genilar et al. 2021), with diverse benefits from different parts of this plant.

*Avicennia germinans* is traditionally used to treat rheumatic diseases, sore throats, and anti-inflammation (Glasesnapp et al. 2019). Leaf and twig extracts have cytotoxic effects against human cancer cell lines (Lonard et al. 2017). Its therapeutic potential of *A. germinans* could be used in both traditional and modern medicine. *A. germinans* (identified as *A. nitida*) has been traditionally used to treat various diseases, including cancer (Singh and Kathiresan 2015). Its bark or stem treats canker sores or oral infections (Thatoi et al. 2016) and accelerates wound healing in the oral cavity. This species remains a valuable resource in traditional healthcare practices.

*Avicennia marina* is known for its strong antibacterial properties, particularly in treating skin diseases caused by bacteria, such as ulcers and abscesses (EIDohaji et al. 2020). It also protects against fungal skin infections and other systems (EIDohaji et al. 2020) and antifertility drugs for family planning and reproductive health (Erwin et al. 2019). It highlights the versatility of *A. marina* as a medicinal source.

*Avicennia alba* is often traditionally used by coastal communities and has demonstrated strong antibacterial potential and antifungal activity (Purba et al. 2022). Its resin is used to treat ulcers and is a contraceptive in traditional medicine (Thatoi et al. 2016).

*Avicennia officinalis* is widely recognized in traditional medicine (Prakashamani et al. 2019) and is effective as an antibacterial and is utilized for the treatment of ulcers and abscesses, where raw seeds are applied to wounds to accelerate healing (Khushi et al. 2016; Prakashamani et al. 2019). The resin from the bark is used as a contraceptive in traditional practices (Das et al. 2018), and the decoction with added sugar and cumin is used to treat indigestion (Prakashamani et al. 2019). This species is also used to treat rheumatism, bronchial asthma, hepatitis, leprosy, gastric protection, aphrodisiac properties, antiulcer,

diuretic, and astringent (Prakashamani et al. 2019). These multifaceted benefits make *A. officinalis* a critical species in traditional medicine.

### Modern medicine

Until now, medicinal plants continue to offer great benefits, both in terms of therapeutics in traditional medicine and in developing safer and more effective modern medicines (Aljaghtmi et al. 2017). The use of medicinal plants in modern medicine has been implemented through various widely disseminated programs. This enables the modern era to better experience the benefits of traditional medicinal plants (Dwisatyadini 2017). According to Sumayyah and Salsabila (2017), various factors contribute to the growing use of herbal medicines globally, particularly in developed countries, including a longer life expectancy. Additionally, increasing access to information on herbal medicine strengthens public trust in the effectiveness and benefits of medicinal plants. Besides, modern drugs, such as cancer treatments, often face challenges like side effects and failures (Sumayyah and Salsabila 2017). Mangrove plants have become one of the traditional medicinal plants increasingly utilized in modern medicine (Saranraj and Sujitha 2015; Sasidhar 2020). The diversity of mangrove plant species offers significant opportunities to search for medicinal uses. Different types of mangroves provide the potential to develop treatments for numerous diseases, including anticancer, antibacterial, antifungal, antiviral, antidiabetic, and immune system boosters (Erwin et al. 2019). The two most widely developed species are: *A. marina* (diabetic/cosmetic) and *A. germinans* (anticancer/antiparasitic) (Bibi et al. 2019; Oliveira et al. 2020).

Recent research conducted by Ahmed et al. (2024) showed that *A. germinans* (identified as *A. africana*) leaf extract has been proven to have potential as a malaria drug. Research conducted by Dossou-Yovo et al. (2017) states that the leaves and roots of *A. germinans* are used to treat malaria. This species leaves and roots are used as an antimalaria due to the active compounds that effectively fight malaria parasites (Dossou-Yovo et al. 2017).

Previous research by Ahmed et al. (2022) showed that *A. germinans* contains various compounds, such as glycosides, terpenoids, alkaloids, phytosterols, flavonoids, tannins, and saponins. Therefore, *A. germinans* has an antimalarial activity that is effective in laboratory tests (in vitro) and living organisms (in vivo), thus supporting its utilization in the traditional treatment of malaria. The disease is spread through the bite of the *Anopheles* mosquito. In addition to mosquitoes, malaria can also be transmitted through blood transfusions and using syringes alternately (Yohannes et al. 2020). Malaria today remains a crucial disease for global health, with 3.3 billion people in 97 countries at risk, causing about 200 million cases and about 600,000 deaths each year (Cowman et al. 2016). So, malaria treatment has become a concern, and further research needs to be conducted.

*Avicennia marina* can cure skin diseases (Vinoth et al. 2019). The leaves are used for ulcers, abscesses, rheumatism, burns (Bibi et al. 2019), and digestive problems such as

constipation. The fruit has aphrodisiacs, diuretics, and hepatitis activity. The stem bark is used to treat leprosy (Erwin et al. 2019). This species leaves have strong antioxidant activity, prevent and interfere with the formation of biofilms, which can exacerbate microbial infections and cause serious problems on various surfaces (Ibrahim et al. 2022). Mitra et al. (2023) research shows that *A. marina* has significant anticancer activity. A study conducted by Huang et al. (2016) showed that *A. marina* can inhibit liver and breast cancer cell lines. Breast cancer itself is the most common type of cancer in women worldwide, accounting for 25.4% of the total new cases diagnosed in 2018 (Prahartiwi and Dari 2021). This shows that the *A. marina* is significantly needed in modern medicine.

In the recent Indian medical system, *A. alba* is used extensively for a variety of health conditions, including as an aphrodisiac, treatment of skin diseases, asthma, rheumatism, scabies, antifertility agents, paralysis, snake bites, and ulcers, demonstrating the diversity of its therapeutic applications (Das 2020; Das and Das 2020). Its crude extract is effective against polymicrobial diseases, showing the potential to fight infections caused by various pathogenic microorganisms (Kar et al. 2015). Extracts from the leaves and bark of *A. alba* stems can inhibit the growth of pathogenic bacteria such as *P. aeruginosa*, *Aeromonas salmonicida*, and *S. aureus* (Purba et al. 2022).

Furthermore, research shows that *A. alba* leaves can protect the gastric mucosa from ethanol damage, which makes it potentially an effective gastric protective agent (Amrati et al. 2021). Further research by Mitra et al. (2022) shows that *A. alba* can also treat diabetes mellitus safely. Diabetes is a chronic disease that occurs frequently and is characterized by recurrent hyperglycemia and metabolic disorders. Diabetes Mellitus (DM) is the eighth leading cause of death, affecting 4% of the world's population. Its impact on various physiological systems, especially nerves and blood flow, makes it a serious medical condition in developing and developed countries (Mitra et al. 2022). It indicates the need for modern treatment of medicinal plants such as those offered from *A. alba* extract.

The fruit, leaves, and roots of *A. officinalis* are a hepatitis remedy (Erwin et al. 2019), relieve joint pain, and accelerate wound healing (Chowdhury et al. 2021). According to Das et al. (2018), its bark is known to be effective in treating skin diseases, especially scabies, and possesses a diuretic activity. The root can be used as an aphrodisiac. The fruit of *A. officinalis* is used as a plaster for tumors, demonstrating its potential in antitumor therapy (Das et al. 2018). Globally, tumors are the second leading cause of death after heart disease. Tumors are becoming a deadly disease, especially in developing countries. Slow-growing tumors usually do not have much effect on humans. In contrast, rapidly growing tumors (malignant tumors) can cause various abnormal effects (Digdoyo et al. 2022). Overall, the different parts of *A. officinalis* do offer diverse therapeutic benefits. It supports its use and shows its potential to be developed in modern medicine.

According to Inggi et al. (2023), the negative impact of consuming herbs is relatively small compared to the

continuous use of chemical drugs (Adamczak et al. 2019). Traditional medicine is considered safer than modern medicine because it is not too harmful (Yuan et al. 2016). However, using traditional medicine must be done with caution to minimize side effects (Sumayyah and Salsabila 2017). It is crucial to ensure accuracy in choosing the proper medication for a particular condition (Lheureux et al. 2019) and pay attention to the accuracy of the dosage used. The timing of use should also be carefully observed, ensuring the drug is used at the right time for maximum effectiveness. In addition, the drug's use must follow the recommendations (Sen and Chakraborty 2017), avoiding inappropriate methods that can reduce the benefits or cause side effects (Jamshidi-Kia et al. 2017). It is also important to prevent the misuse of traditional medicine (Schrot and Hubbard 2016), ensuring that the use follows the provided instructions (Luethi and Liechti 2020). Finally, accuracy in drug selection is very important to ensure that the drugs used are appropriate and effective for treating the disease or condition. By paying attention to all these factors, traditional medicine can be safer and more effective (Sumayyah and Salsabila 2017). It shows that using medicinal plants in modern medicine is important for further study.

In conclusion, *Avicennia* is a genus in the Acanthaceae family consisting of eight species of mangroves distributed worldwide. The *Avicennia* tree grows 10-25 meters tall with simple leaves with salt secretion glands and short, low-branched stems. They play an important role in coastal ecosystems by protecting beaches from erosion, providing habitat for marine organisms, and storing carbon. Species such as *A. bicolor*, *A. germinans*, *A. marina*, *A. officinalis*, and *A. schaueriana* are widespread in tropical and subtropical regions, highlighting the importance of *Avicennia* in maintaining the biodiversity and stability of coastal ecosystems. *Avicennia* spp. is one of the types of mangroves that are used as medicinal plants. The phytochemical components in stems, leaves, and bark are terpenoids, glucosides, and naphthalene derivatives. *Avicennia* spp. has several biological activity properties: antioxidant, anticancer, antimicrobial, anti-inflammatory, and antihyperglycemic. These biological activities can be found in parts of the species, such as leaves, stems, and flowers. *Avicennia* can be used in traditional and modern medicine. In traditional medicine, *Avicennia* is used for diseases like diabetes, smallpox, malaria, ulcers, asthma, canker sores, skin diseases, and cancer. *Avicennia* provides increasing benefits and holds the potential for development into safer and more effective modern medicines. There are nine main phytochemical components that helps health, i.e.: alkaloids, terpenoids, steroids, phenolics, saponins, flavonoids, tannins, steroids, and glycosides.

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# Sustainability assessment of rural biogas systems using a rapid appraisal method in Klaten District, Indonesia

AGRA DEWI SABRINA<sup>1</sup>, AHMAD DWI SETYAWAN<sup>1,2,\*</sup>, MUHAMMAD INDRAWAN<sup>1</sup>

<sup>1</sup>Department of Environmental Science, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret. Jl. Ir. Sutami 36A Surakarta 57 126, Central Java, Indonesia. Tel./Fax. +62-271-663375, \*email: volatiloils@gmail.com, agradewi@gmail.com

<sup>2</sup>Biodiversity Research Group, Universitas Sebelas Maret. Jl. Ir. Sutami 36A, Surakarta 57126, Central Java, Indonesia

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**Abstract.** Sabrina AD, Setyawan AD, Indrawan M. 2024. Sustainability assessment of rural biogas systems using a rapid appraisal method in Klaten District, Indonesia. *Asian J Trop Biotechnol* 21: 111-122. Household-scale biogas systems are increasingly promoted as sustainable energy solutions for rural areas. However, their long-term viability depends on a range of ecological, economic, social, technological, and institutional factors that interact in complex ways. This study applied the Rap-Biogas method, a multidimensional scaling-based rapid appraisal approach, to assess the sustainability of a community-based biogas system in Mundu Village, Klaten District, Central Java, Indonesia. Data were collected through structured questionnaires, interviews, and secondary sources, covering 28 attributes across five dimensions. The overall sustainability index was 67.77%, indicating a fairly sustainable status. The ecological (81.78%), social (83.52%), and technological (77.95%) dimensions showed strong performance, driven by reductions in organic waste, high community participation, and user-friendly technology. In contrast, the economic (71.06%) and institutional (24.52%) dimensions revealed significant challenges, including limited business development, insufficient maintenance support, and the absence of long-term policy integration. Leverage analysis identified critical sensitive attributes within each dimension, providing clear targets for intervention. The study highlights the strengths and weaknesses of decentralized biogas systems. It emphasizes the need for improved institutional support, financial mechanisms, and policy alignment to enhance sustainability, with particular stress on the importance of financial mechanisms in this context. Rap-Biogas proved to be a practical and participatory tool for rapid sustainability diagnostics, and the findings offer valuable insights for replicating and scaling rural renewable energy programs in Indonesia and other developing regions.

**Keywords:** Biogas sustainability, community-based renewable energy, institutional support, Rap-Biogas, rural energy

## INTRODUCTION

Energy plays a vital role in supporting human activities, ranging from lighting, cooking, and transportation to industrial operations. Over the years, global energy demand has surged due to population growth and industrial expansion (Muzayanah et al. 2022). Indonesia, as a developing country, faces significant challenges in meeting its energy needs while also ensuring sustainability. The heavy reliance on fossil fuels such as petroleum, coal, and natural gas has raised concerns about environmental degradation and energy security (Kahraman et al. 2024).

The consumption of fossil fuels is a major contributor to greenhouse gas emissions, which are accelerating global climate change. The combustion of these fuels releases carbon dioxide and methane, leading to air pollution and associated health risks (Wang and Azam, 2024). Moreover, fossil energy sources are finite; according to the Ministry of Energy and Mineral Resources (ESDM), Indonesia's oil reserves are projected to last only 9.5 years and its natural gas reserves for 19.9 years, if no new reserves are discovered (Ministry of Energy and Mineral Resources, 2021). These constraints underscore the urgent need to shift towards cleaner, renewable energy sources.

One promising alternative is biogas, which is produced through the anaerobic digestion of organic matter by

microbial communities under oxygen-free conditions. This process is a key component of the carbon cycle and is facilitated by methane-producing bacteria (Sugiarto et al. 2013). Biogas not only serves as a renewable energy source but also reduces environmental pollution by converting organic waste into usable energy. Cow dung, a common agricultural waste product, is among the most effective substrates for biogas production (Batistuta et al. 2021).

The use of cow manure in biogas systems offers dual benefits: it mitigates environmental hazards and provides a low-cost energy source for rural households. Daily manure output can reach up to 12% of a cow's body weight, posing serious environmental threats if left untreated (van den Oever et al. 2021). In many rural areas, including those in Indonesia, improper disposal of livestock waste contaminates water bodies and degrades soil quality. Biogas technology, with its innovative approach, helps alleviate these issues by transforming waste into clean energy while also producing bio-slurry. This by-product can be utilized as an organic fertilizer (Listyawati et al. 2014).

Despite its potential, the adoption and long-term sustainability of biogas systems depend on various factors beyond just technological feasibility. Sustainability assessments must incorporate ecological, economic, social, technological, and institutional dimensions to capture the

full complexity of implementation (Batistuta et al. 2021). The Rapfish method, initially developed for fisheries sustainability assessment, has been successfully adapted into Rap-Biogas for evaluating biogas systems through multidimensional scaling (Pitcher and Preikshot 2001; Fauzi and Anna 2002). This method enables rapid appraisal of sustainability status and helps identify key leverage points for improvement.

In recent years, biogas has gained popularity across Indonesia, including in remote and mountainous regions (Rianawati et al. 2021). The government has recognized the importance of renewable energy through policies, such as the National Energy Policy (PP No. 79/2014), which aims to achieve a 23% share of renewable energy in the national energy mix by 2025 and 31% by 2050. However, as of 2020, the actual share stood at only 11.31% (Setyono and Kiono 2021), indicating a significant gap between policy targets and on-the-ground implementation. This highlights the need for localized, community-driven renewable energy initiatives.

Mundu Village in Klaten District, Central Java, stands out as one of Indonesia's energy-independent villages, which has been utilizing livestock waste for biogas production since 2013 (Ningtyas et al., 2020). The village is located near the Pusur River Basin and has an economy that is largely based on agriculture and livestock. With a high cattle population, the village generates large amounts of manure that were previously disposed of improperly, leading to water contamination and health issues (Istikhomah and Riyadi 2021). The community's adoption of biogas technology represents a shift toward sustainable rural development.

Currently, Mundu Village operates 55 biogas units of varying capacities (6 m<sup>3</sup> to 20 m<sup>3</sup>), reflecting both the scalability and practical application of biogas systems in rural Indonesia. However, questions remain regarding the long-term sustainability of these systems across different dimensions. While some previous studies have evaluated

micro-scale biogas sustainability (Ristianingsih et al. 2018; Batistuta et al. 2021), there remains a lack of comprehensive, multidimensional assessments in Central Java, particularly using rapid, participatory methods.

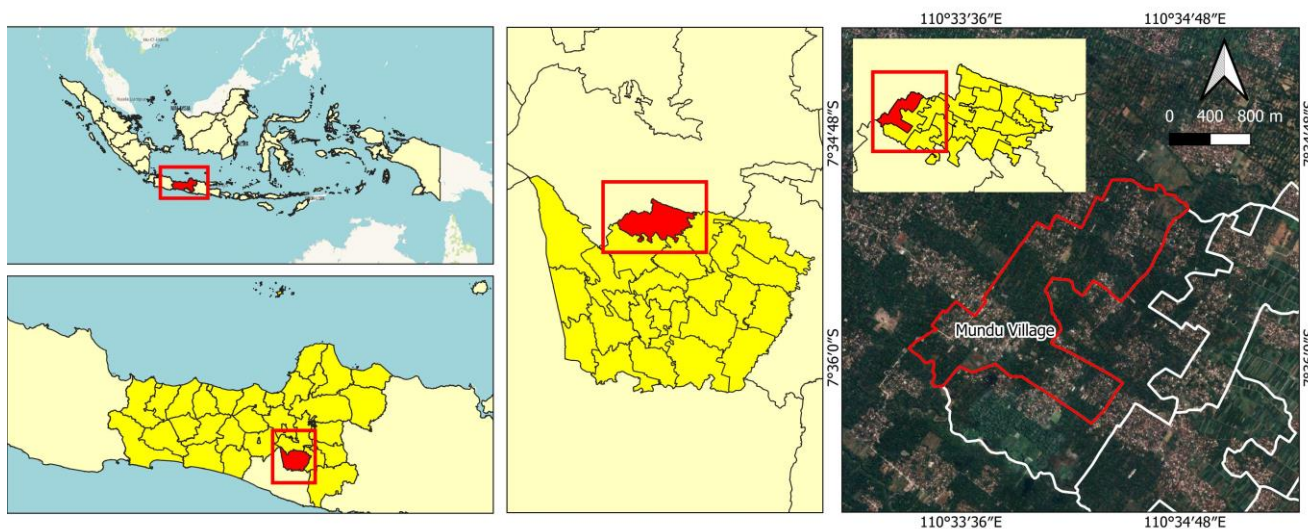
This study attempts to address that gap by evaluating the sustainability status of the biogas system in Mundu Village using a rapid appraisal method based on the modified Rapfish framework. The assessment focuses on five key dimensions—ecological, economic, social, technological, and institutional—to identify the most sensitive attributes that influence sustainability. Using the Rap-Biogas approach, this research aims to provide evidence-based recommendations for policy and community-level action to strengthen biogas sustainability in rural Indonesia.

Ultimately, this study not only contributes to the growing body of literature on sustainable rural energy but also offers a methodological framework that can be replicated in other contexts. The findings are expected to support local and national policymakers in enhancing renewable energy programs, especially in agricultural communities. Through a robust and multidimensional analysis, the study highlights the potential of microbially mediated biogas systems to advance both environmental and socio-economic goals in developing regions.

## MATERIALS AND METHODS

### Study area

The study was conducted in Mundu Village, Tulung District, Klaten District, Central Java, Indonesia (Figure 1). Geographically, the village is located approximately 15 kilometers northeast of Mount Merapi and situated within the upstream area of the Pusur River Basin. This location situates the village in a strategic yet environmentally sensitive zone, where land-use practices directly impact watershed health.



**Figure 1.** Map showing the research location in Mundu Village, Klaten District, Central Java, Indonesia

Mundu Village is characterized by a predominantly agrarian economy, with most residents engaged in farming and livestock breeding. The community maintains substantial numbers of cattle, which serve as both a primary source of livelihood and a contributor to organic waste accumulation. Traditional practices of livestock waste disposal have historically contributed to environmental degradation, particularly water pollution in nearby river systems.

The introduction of biogas technology in Mundu Village has provided a viable alternative to conventional waste management and fossil fuel dependence. Since 2013, the community has developed 55 household-scale biogas units of varying capacities, ranging from 6 m<sup>3</sup> to 20 m<sup>3</sup>. These systems convert livestock manure into biogas for daily household energy needs while producing bio-slurry, a nutrient-rich fertilizer, as a valuable by-product. The biogas program has significantly enhanced local energy resilience and exemplifies the potential of community-based renewable energy systems in rural Indonesia.

### Research design

This study employed a descriptive-quantitative research design using a multidimensional assessment approach. The design was selected for its importance in facilitating a comprehensive evaluation of various factors influencing the sustainability of rural biogas systems. The multidimensional framework allowed for an integrated analysis of ecological, economic, social, technological, and institutional factors, each of which contributes uniquely to the overall sustainability of the system.

The research was conducted over seven months, from December 2022 to July 2023, covering the entire research cycle, including planning, field data collection, stakeholder engagement, data analysis, and report writing. The fieldwork was centered in Mundu Village, where biogas users were observed, interviewed, and surveyed directly to obtain primary data.

The main objective of this research was to evaluate the sustainability of household-scale biogas systems operated by the rural community in Mundu Village. This evaluation aims to identify strengths, weaknesses, and sensitive attributes in each dimension that significantly affect the long-term viability of the biogas program. The results of this study were expected to support both local development planning and broader efforts to promote the adoption of renewable energy in Indonesia.

### Sampling technique and respondents

The respondents in this study were selected using a purposive sampling technique. This method was considered appropriate because it allowed the selection of individuals who had direct experience with the biogas system and could provide relevant information based on practical use. The sampling focused on households that actively operated biogas digesters as part of their daily domestic energy needs.

A total of 55 households were selected as respondents from Mundu Village, each of which had been using biogas units with digester capacities ranging from 6 m<sup>3</sup> to 20 m<sup>3</sup>.

The respondents were chosen based on several inclusion criteria: they had to be permanent residents of the village, actively use the biogas unit for at least six months prior to the survey, and be involved in livestock activities, particularly cattle farming. This ensured that the data collected was representative of the actual user experiences and system performance.

Local administrative records and preliminary consultations with community leaders and village officials provided support for the selection process. Prior to administering the questionnaire, all selected respondents were briefed on the purpose of the study, and their informed consent was obtained. This approach not only strengthened the ethical foundation of the research but also increased the accuracy and relevance of the data gathered from the field.

### Data collection

This study employed both primary and secondary data sources to ensure comprehensive and reliable results. Primary data were obtained through structured questionnaires and semi-structured interviews conducted directly with selected biogas users. The questionnaire was designed to explore five sustainability dimensions—ecological, economic, social, technological, and institutional—and included multiple attributes within each dimension based on validated indicators from previous studies (Ristianingsih et al. 2018; Batistuta et al. 2021).

Each questionnaire item was rated using a Likert-type scale, allowing respondents to express their perceptions of sustainability-related attributes. Prior to full deployment, the questionnaire was tested for clarity and reliability through a small pilot study involving non-sample respondents in a nearby village. Revisions were made accordingly to improve language, structure, and attribute relevance. Additionally, interviews were conducted to gain deeper insights into the contextual and behavioral aspects of biogas adoption and maintenance.

Secondary data were collected from various sources, including village administrative records, technical reports, scientific publications, and government documents. These data supported the contextual understanding of the biogas program and provided benchmarks for evaluating sustainability indicators. Information such as the number and size of installed biogas units, demographic profiles, and government support programs was obtained from village documentation and official statistics.

The combination of structured surveys and supporting documents ensured that both quantitative and qualitative aspects of biogas sustainability were captured. This multi-source data collection approach enhanced the triangulation of findings and strengthened the validity of the subsequent sustainability analysis.

### Sustainability assessment using Rap-Biogas

The sustainability assessment in this study employed the Rap-Biogas method, a modified version of the Rapid Appraisal for Fisheries (Rapfish) framework originally developed to evaluate marine resource sustainability (Pitcher and Preikshot 2001; Fauzi and Anna 2002). Rap-

Biogas applies the Multidimensional Scaling (MDS) technique to assess the sustainability of biogas systems across five core dimensions: ecological, economic, social, technological, and institutional. This method enables a rapid yet structured appraisal by converting qualitative judgments into quantitative indices.

Each dimension was composed of several attributes selected based on literature review, expert consultation, and field relevance. These attributes were then scored by field researchers and verified through respondent inputs, using a scale of 0 to 3 or 0 to 4, depending on attribute specificity. The scoring reflected the current status of each attribute in the village's biogas program. The data were then processed in Microsoft Excel to create input matrices, which were analyzed using Rapfish software to generate sustainability indices.

The index values generated for each dimension range from 0 to 100 and are interpreted according to four sustainability categories: unsustainable (0.00-25.00), less sustainable (25.01-50.00), fairly sustainable (51.01-75.00), and sustainable (75.01-100.00), as shown in Table 1. These thresholds were adopted from previous applications of Rapfish and modified appropriately for the biogas context (Batistuta et al. 2021).

In addition to calculating index values, leverage analysis was conducted to identify the most sensitive attributes influencing sustainability within each dimension. Sensitivity was determined based on the Root Mean Square (RMS) value, where attributes with RMS values above the median were considered key leverage points. These attributes represent critical areas for targeted intervention and improvement.

A Monte Carlo simulation was also performed to test the robustness and reliability of the MDS results. By running repeated simulations with randomized input variation, the analysis assessed the stability of the index values within a 95% confidence interval. The model was considered valid if the difference between MDS and Monte Carlo results was less than 5%, the stress value was below 0.25, and the coefficient of determination ( $R^2$ ) was close to 1. These parameters ensured that the findings accurately reflected the real sustainability conditions in Mundu Village.

### Statistical analysis

Several statistical procedures were applied to support the interpretation of sustainability index values. The primary tool was a leverage analysis, which aimed to identify the most sensitive attributes affecting sustainability in each dimension. The sensitivity of each attribute was calculated using the Root Mean Square (RMS) value, with attributes exceeding the median RMS value within a dimension classified as influential. These sensitive attributes indicate where management improvements or policy interventions would have the greatest impact.

Following the leverage analysis, a Monte Carlo simulation was performed to test the reliability and robustness of the Multidimensional Scaling (MDS) results. This simulation introduced controlled variability into the attribute scoring and assessed how much the final index

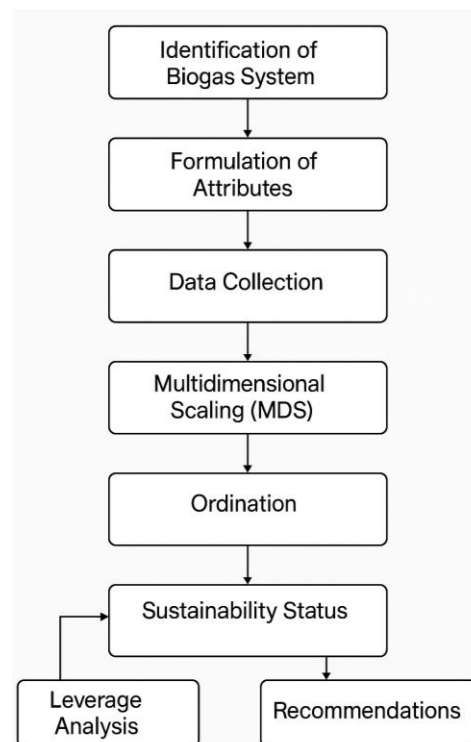
values changed under repeated resampling. The validity of the model was confirmed if the difference between the Rap-Biogas index and the Monte Carlo index was less than 5%, which is the standard threshold for acceptable error in rapid appraisal studies (Kavanagh and Pitcher 2004).

In addition to numerical consistency, the goodness-of-fit of the model was assessed using two metrics: the stress value and the coefficient of determination ( $R^2$ ). A stress value below 0.25 indicates that the model adequately represents the distance relationships between attributes in multidimensional space. Likewise, an  $R^2$  value close to 1.00 suggests that the variance in the data is well explained by the MDS configuration (Fauzi and Anna 2002). These statistical indicators ensured that the Rap-Biogas method provided a robust and valid representation of biogas sustainability in the research context.

All computations were carried out using Rapfish software and Microsoft Excel 2019. The combination of MDS ordination, leverage analysis, and Monte Carlo simulation provided a comprehensive analytical framework to diagnose the sustainability performance of the biogas system in Mundu Village.

**Table 1.** Categories of sustainability status based on index values

Index Value (%)	Sustainability Category
0.00-25.00	Unsustainable
25.01-50.00	Less Sustainable
51.01-75.00	Fairly Sustainable
75.01-100.00	Sustainable



**Figure 2.** Analytical flow of sustainability assessment using Rap-Biogas

## RESULTS AND DISCUSSION

### Overall sustainability status of the biogas system

The overall sustainability index of the household-scale biogas system in Mundu Village was 67.77%, placing it in the fairly sustainable category. The ecological (81.78%), social (83.52%), and technological (77.95%) dimensions were classified as sustainable, while the economic dimension (71.06%) was considered fairly sustainable. The institutional dimension (24.52%) was categorized as unsustainable. These scores, along with the low-stress values (<0.20) and high  $R^2$  (>0.90) for all dimensions, confirm the reliability of the multidimensional scaling results.

To ensure the validity of the results, a Monte Carlo simulation was conducted. The simulation yielded an overall sustainability index of 66.66%, compared to the original MDS score of 67.77%, resulting in a difference of 1.11%. This refers to the total score difference, not the average of per-dimension differences. Since this difference is below the 5% threshold, the model is considered statistically robust (Kavanagh and Pitcher 2004). Furthermore, all five sustainability dimensions demonstrated stress values below 0.25 and coefficients of determination ( $R^2$ ) close to 1.00, confirming the goodness of fit of the model.

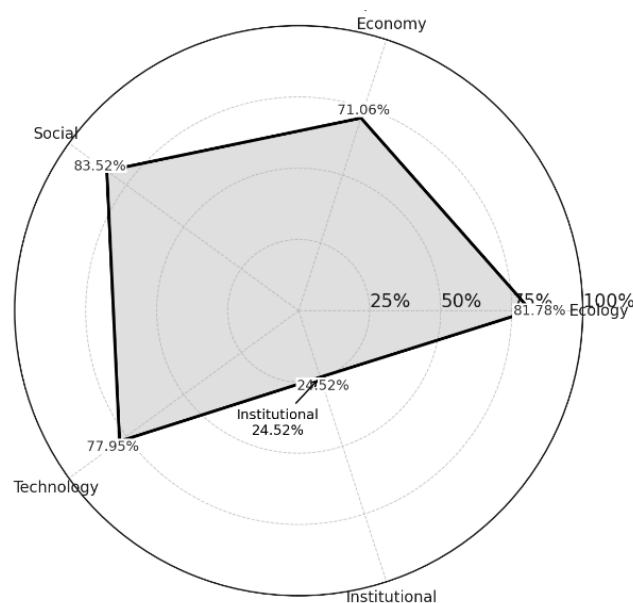
The kite diagram provides a visual representation of the sustainability index values across all five dimensions. The diagram indicates that the social, ecological, and technological dimensions are positioned furthest from the center point, suggesting a sustainable status in these areas. In contrast, the institutional dimension is positioned closest to the center, reflecting its unsustainable status and identifying it as a critical area for targeted intervention. These results provide a comprehensive baseline for understanding the multidimensional sustainability performance of biogas systems in rural Indonesia. The findings also underscore the need for enhanced institutional frameworks and effective stakeholder engagement to foster the long-term success of such initiatives.

The multidimensional sustainability performance of the household biogas system is illustrated in Figure 4, which reveals notable strengths in the ecological, social, and technological dimensions, but contrasts with a critically weak institutional component. The validity of the MDS model used in this assessment is supported by low-stress values and high  $R^2$  across all dimensions (Table 2), indicating strong goodness-of-fit. It is important to note

that the Difference (%) values in Table 2 refer to each dimension individually; the overall difference between MDS and Monte Carlo scores (1.11%) is derived from the total sustainability index (67.77% vs. 66.66%), not from averaging the per-dimension differences. Furthermore, the key leverage attributes that most influence sustainability outcomes—based on RMS values relative to each dimension's median—are summarized in Table 3. These leverage attributes are further visualized in Figure 4, which displays the comparative RMS values across all dimensions.

### Ecological dimension

The ecological dimension of the biogas system in Mundu Village achieved a sustainability index of 81.78%, which falls into the sustainable category (Table 2). This result indicates that the biogas program has had a positive environmental impact, particularly in reducing organic waste and improving household sanitation. Compared to similar studies, such as Batistuta et al. (2021), which reported an ecological index of 56.35% in Cisondari Village, West Java, the ecological performance in Mundu Village is significantly higher.



**Figure 3.** Kite diagram showing the sustainability index in five dimensions

**Table 2.** Summary of sustainability index, stress, and  $R^2$  for each dimension

Dimension	Sustainability Index (%)	Monte Carlo (%)	Difference (%)	Stress Value	$R^2$	Sustainability Category
Ecology	81.78	79.77	2.01	0.17	0.93	Sustainable
Economy	71.06	69.86	1.20	0.18	0.95	Fairly Sustainable
Social	83.52	81.44	2.08	0.16	0.95	Sustainable
Technology	77.95	75.81	2.14	0.16	0.93	Sustainable
Institutional	24.52	26.41	1.89	0.14	0.97	Unsustainable
Overall	67.77	66.66	1.11	0.16	0.95	Fairly Sustainable

Note: The overall difference of 1.11% between MDS and Monte Carlo scores is calculated from the total sustainability index (67.77% vs. 66.66%), and not from the average of the five dimension-wise differences.

**Table 3.** Sustainability index and sensitive attributes for all dimensions

Attributes	Attribute	RMS value	Sensitivity category
Ecology	Environmental comfort and cleanliness	7.79	Sensitive
	Impact on firewood or LPG usage	7.51	Sensitive
	Utilization of biogas by-products	6.96	Sensitive
	Waste odor pollution	5.82	Less sensitive
	Fertility of agricultural land	4.93	Less sensitive
	Knowledge about waste impacts	3.75	Less sensitive
	Median RMS Value	6.39	–
Economy	New business opportunities	13.66	Sensitive
	Maintenance costs of biogas systems	8.00	Sensitive
	Fossil fuel energy savings	7.76	Sensitive
	Decrease in daily expenses	5.42	Less sensitive
	Government subsidies	4.37	Less sensitive
	Fund creativity	3.81	Less sensitive
	Median RMS Value	6.59	–
Social	Active community participation	10.53	Sensitive
	Perceived benefits of biogas use	10.19	Sensitive
	Availability of technical guidance	7.65	Sensitive
	Shared responsibility for maintenance	6.05	Less sensitive
	Community support for environmental goals	4.89	Less sensitive
	Interpersonal conflicts	4.26	Less sensitive
	Median RMS Value	6.85	–
Technology	Availability of alternative energy	8.90	Sensitive
	Ease of use and maintenance	8.01	Sensitive
	Technology adaptability to user needs	6.94	Sensitive
	Functional status of biogas units	5.52	Less sensitive
	Access to repair services	4.66	Less sensitive
	Quality of installation materials	3.92	Less sensitive
	Median RMS Value	6.23	–
Institutional	Presence of follow-up programs	10.20	Sensitive
	Budget support from local authorities	8.76	Sensitive
	Technical assistance from institutions	7.88	Sensitive
	Community institutional awareness	5.98	Less sensitive
	Policy integration with renewable energy	5.21	Less sensitive
	Legal framework for energy self-reliance	4.62	Less sensitive
	Median RMS Value	6.93	–
	Community institutional awareness	5.98	Less sensitive
	Policy integration with renewable energy	5.21	Less sensitive
	Legal framework for energy self-reliance	4.62	Less sensitive
Median RMS Value	6.44	–	

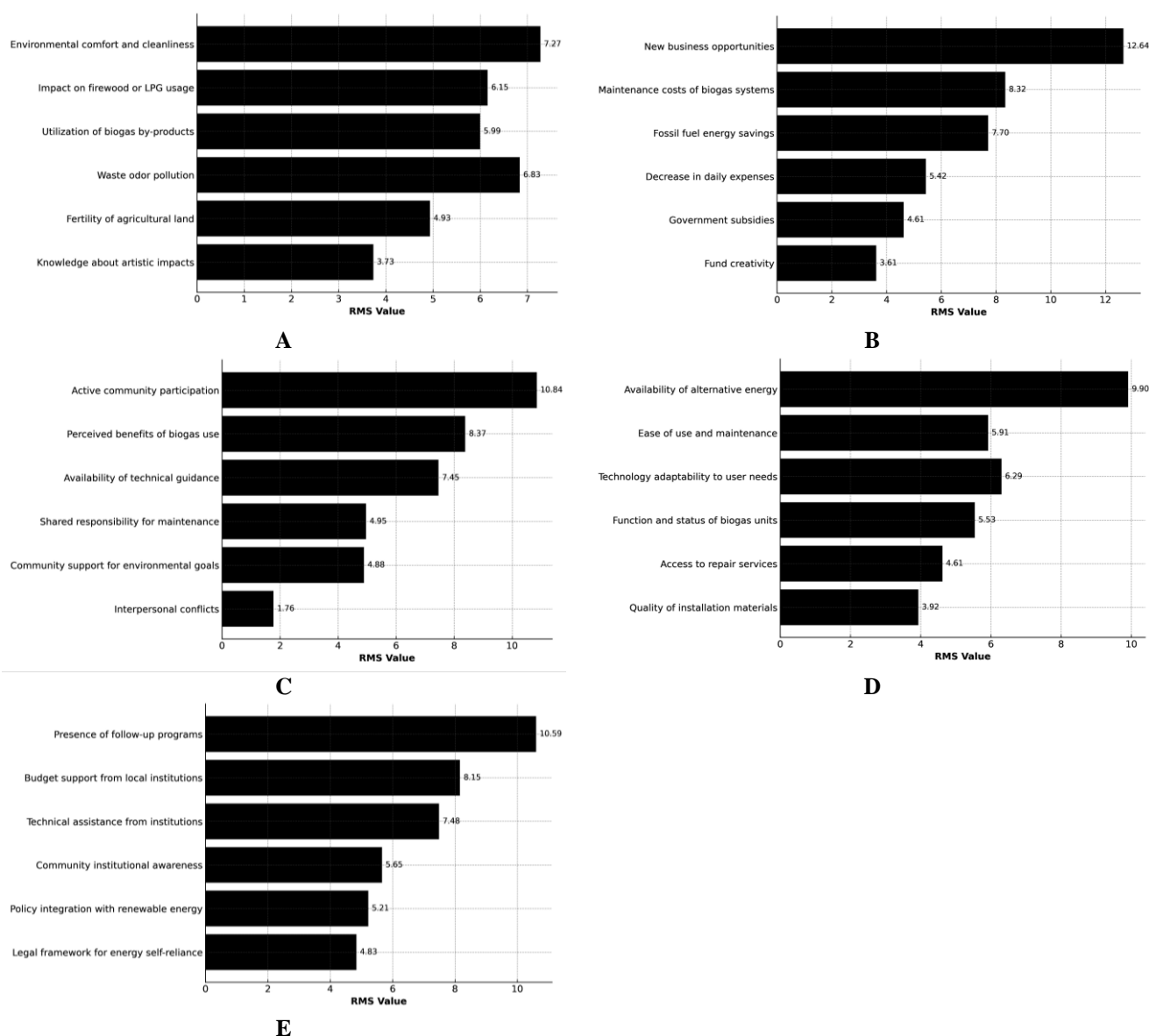
Leverage analysis was conducted to identify the most influential attributes contributing to the sustainability of the ecological dimension. The analysis revealed three sensitive attributes with Root Mean Square (RMS) values above the revised median threshold of 6.39: environmental comfort and cleanliness (7.79), impact on firewood or LPG usage (7.51), and utilization of biogas by-products (6.96). These attributes represent key leverage points that have the most substantial influence on the ecological outcomes of the biogas system (Table 3; Figure 4.A).

The results of this analysis suggest that reducing dependence on fossil fuel alternatives and improving household waste management are critical ecological benefits of the biogas program. Furthermore, the reuse of bio-slurry as organic fertilizer not only minimizes pollution but also supports agricultural productivity, providing a closed-loop resource cycle within the village. These findings reinforce the ecological value of the biogas system, especially in terms of reducing environmental

pollution and improving rural household energy behavior. Continued efforts to enhance the use of biogas by-products and raise environmental awareness are expected to sustain or even improve ecological performance in the long term, offering a pathway toward long-term ecological sustainability.

#### **Economic dimension**

The economic dimension of the biogas system in Mundu Village obtained a sustainability index of 71.06%, categorizing it as fairly sustainable (Table 2). This score suggests that while the system has generated economic benefits for users, there are still opportunities to improve its financial viability and enhance its positive impacts on livelihoods. Compared to the study by Ristianingsih et al. (2018), which reported an index of 50.37% in Banyumas, the findings from Mundu Village indicate better performance but also highlight areas requiring further development.



**Figure 4.** Leverage analysis for all dimensions. A. Ecology, B. Economy, C. Social, D. Technology, E. Institution

Leverage analysis revealed three sensitive attributes within the economic dimension, each with RMS values exceeding the updated median of 6.59. The most influential was new business opportunities (13.66), followed by maintenance costs of biogas systems (8.00) and fossil fuel energy savings (7.76). These attributes directly affect household expenses and the broader financial attractiveness of biogas systems (Table 3; Figure 4.B).

The high sensitivity of the new business opportunities attribute reflects the current underutilization of bio-slurry and other by-products as economic commodities. While the community has begun using biogas for domestic purposes, the transformation of waste into marketable organic fertilizer remains limited. Meanwhile, the financial burden associated with maintaining biogas equipment—particularly the digester and stove—remains a concern for many users, which may impact system longevity and user

satisfaction. The findings suggest that with proper institutional support and market linkage, the commercialization of bio-slurry and local entrepreneurship could significantly enhance the economic value of biogas systems. In the long term, reducing maintenance costs through improved technical training and equipment design may also contribute to greater economic sustainability.

### Social dimension

The social dimension of the biogas system in Mundu Village achieved a sustainability index of 83.52%, placing it within the sustainable category (Table 2). This result indicates that community participation, perception, and knowledge regarding the biogas program are relatively high. The high social score aligns with the village's status as an energy self-sufficient community, supported by a shared awareness of environmental and energy issues.

Leverage analysis identified three sensitive attributes with RMS values above the median of 6.85: active community participation (10.53), perceived benefits of biogas use (10.19), and availability of technical guidance (7.65). These elements are essential in maintaining public engagement and collective responsibility for biogas system operations (Table 2; Figure 4.C).

Despite the positive outlook, interviews revealed that not all community members had equal access to capacity-building programs. Some households expressed concern over the lack of routine maintenance training and the limited number of technicians available. Although these limitations have not yet significantly impacted community enthusiasm, they may pose future challenges if not addressed through inclusive outreach and educational initiatives. These findings highlight the importance of maintaining strong social cohesion and trust in the success of community-based biogas programs. Sustained engagement through education, open communication, and local leadership will be key to ensuring long-term social sustainability.

### Technological dimension

The technological dimension of the Mundu Village biogas system obtained a sustainability index of 77.95%, placing it in the sustainable category (Table 2). This result reflects the community's capacity to operate and maintain biogas units with acceptable technical performance. Most households can utilize biogas for their daily cooking needs, and the majority of units remain functional with minimal technical disruption.

Leverage analysis identified three sensitive attributes that exceeded the recalculated median RMS value of 6.23: the availability of alternative energy (8.90), ease of use and maintenance (8.01), and technology adaptability to user needs (6.94). These factors determine user-friendliness, accessibility, and long-term feasibility of the biogas systems from a technical perspective (Table 3; Figure 4.D).

Despite generally positive findings, field observations and interviews revealed recurring issues related to digester clogging, gas pressure instability, and the limited availability of spare parts. Furthermore, while most users expressed satisfaction with the ease of use, a minority reported difficulties due to inadequate training or unclear installation guidelines. These challenges suggest a need for more structured and continuous technical support from relevant institutions. These results underscore the importance of enhancing the technical reliability and service infrastructure surrounding household-scale biogas systems. Technical sustainability will be strengthened through local technician training, standardized construction guidelines, and broader integration of user feedback in future system upgrades.

### Institutional dimension

The institutional dimension received the lowest sustainability index among all five dimensions, scoring only 24.52%, which places it in the unsustainable category (Table 2). This score indicates significant deficiencies in organizational support, policy integration, and institutional

responsiveness related to biogas program implementation. While community-level initiatives have shown promise, the absence of consistent external support remains a critical barrier to sustainability.

Leverage analysis revealed that three attributes exceeded the corrected median of 6.93, making them key leverage points for the institutional dimension: presence of follow-up programs (10.20), budget support from local authorities (8.76), and technical assistance from institutions (7.88). These aspects represent significant gaps that require urgent institutional strengthening (Table 3; Figure 4.E).

Interviews with community leaders and users revealed that, following the initial program launch in 2013, structured institutional involvement has declined. There has been a lack of periodic evaluation, funding allocation, and technical oversight. Furthermore, coordination between local government agencies, NGOs, and the village community remains limited, resulting in gaps in maintenance, upgrading, and capacity-building activities. These findings emphasize that the success of community-based biogas systems depends not only on technical or social factors but also on enabling institutional frameworks. Strengthening formal-informal linkages, ensuring routine support programs, and integrating biogas into local policy agendas will be vital to lifting the institutional dimension from its current unsustainable status.

### Discussion

#### *Interpretation of overall sustainability performance*

The biogas system in Mundu Village achieved an overall sustainability index of 67.77%, which places it within the fairly sustainable category. This classification indicates that the system has performed well across several dimensions, particularly ecological, social, and technological, but still faces limitations in economic and especially institutional aspects. The result reflects a commendable level of community commitment and technological functionality, although the supporting governance structure remains weak. Such a profile is typical of community-based renewable energy projects initiated from the bottom up with minimal long-term institutional engagement.

When compared to similar studies in Indonesia, the performance of the Mundu Village biogas system is relatively strong. For example, Batistuta et al. (2021) reported an overall index of 56.75% in Cisondari Village, West Java while Ristianingsih et al. (2018) found even lower sustainability levels in Banyumas District, Central Java. These studies highlight the recurring challenges faced by rural biogas programs, particularly in sustaining economic viability and formal institutional support. The higher score observed in Mundu Village may be attributed to a combination of strong social participation and continuous household-level commitment to system maintenance and use.

From an international perspective, community-scale biogas programs in developing countries often demonstrate similar patterns: high ecological and social benefits, constrained economic returns, and limited policy integration (Amigun et al. 2008; Bond and Templeton

2011). These trends suggest that the case of Mundu Village is not isolated but rather representative of broader structural realities in decentralized renewable energy initiatives. The partial success of such programs emphasizes the need for integrative, multi-actor strategies that extend beyond infrastructure provision to include institutional capacity-building, post-installation monitoring, and value-chain development.

The implications of this finding are substantial for renewable energy development in rural contexts. While household-scale biogas has clear potential to reduce fossil fuel dependence, improve sanitation, and enhance local resource use, its long-term sustainability hinges on holistic program design. This includes reliable technical support, viable economic incentives, and consistent policy alignment at village and regional levels. Therefore, the "fairly sustainable" status achieved by Mundu Village should be viewed not only as a measure of current performance but also as a call to strengthen and replicate its successful aspects while addressing existing gaps through targeted interventions.

#### *Ecological implications*

The ecological dimension of the biogas system in Mundu Village was rated as sustainable, with an index of 81.78%, reflecting strong environmental benefits resulting from biogas adoption. One of the most immediate impacts observed was the reduction in organic waste accumulation and associated pollution. Livestock manure, which was previously discarded into waterways or left unmanaged, is now processed into biogas, significantly improving household sanitation and reducing odor pollution. This aligns with findings from Hnyine et al. (2016) and Rianawati et al. (2021), who emphasized the role of biogas in reducing environmental health risks in rural Indonesia.

A key ecological advantage of biogas systems is their ability to contribute to circular resource use. In Mundu Village, the use of bio-slurry—the semi-liquid residue of anaerobic digestion—as organic fertilizer has supported more sustainable agricultural practices. This substitution of chemical fertilizers reduces the risk of groundwater contamination and soil degradation, supporting long-term agroecological balance (Sugiarto et al. 2013). In addition, the use of biogas has led to a notable reduction in firewood consumption, helping to mitigate pressure on local forest resources and contributing to emissions reduction.

Compared to other regions, the ecological performance in Mundu Village is relatively high. In West Java, Batistuta et al. (2021) found ecological index values below 60%, primarily due to limited bio-slurry utilization and persistent issues with waste disposal. The success in Mundu appears to be supported by stronger awareness of environmental impacts and more consistent use of system by-products. This reinforces the importance of environmental education and demonstration in enhancing the ecological benefits of biogas adoption.

Ecologically, biogas systems also serve broader climate mitigation goals by capturing methane emissions that would otherwise be released from decomposing manure. Methane is a potent greenhouse gas with a global warming

potential of more than 25 times that of CO<sub>2</sub> over 100 years (IPCC 2021). Thus, the biogas program not only benefits local household environments but also contributes to Indonesia's national commitments to climate action under its Nationally Determined Contributions (NDCs). In this way, the ecological success of the system in Mundu Village illustrates how localized energy interventions can have both immediate and long-term environmental value.

#### *Economic opportunities and constraints*

The economic sustainability index of the biogas system in Mundu Village was 71.06%, placing it in the fairly sustainable category. This suggests that the system offers tangible financial benefits, particularly in reducing household energy costs. Most users reported a significant decline in expenditures on firewood and liquefied petroleum gas (LPG) after switching to biogas for cooking. These findings are consistent with those of Rachmawati et al. (2022), who documented similar savings in household fuel costs among biogas users in Sleman, Yogyakarta.

Beyond direct savings, biogas systems also present economic opportunities through the utilization and marketing of bio-slurry. In theory, the conversion of organic waste into high-quality fertilizer could stimulate rural entrepreneurship, reduce input costs for farmers, and open access to organic agriculture markets. However, in Mundu Village, this potential remains largely unrealized. As noted in the leverage analysis, the attribute new business opportunities was highly sensitive, indicating that this economic function has not yet been optimized. Limited access to processing facilities, packaging, and marketing channels hampers the commercial viability of bio-slurry.

One of the persistent economic constraints is the cost of maintenance and repairs. Although the initial installation of biogas units was supported by government and NGO programs, users are ultimately responsible for the ongoing maintenance and operation of these units. Interviews revealed that the cost of replacing stove components, repairing digesters, or unclogging gas lines could pose a financial burden, especially for low-income households. These costs, when unexpected, may reduce user satisfaction and compromise the system's long-term operability. Similar concerns were raised by Ristianingsih et al. (2018), who found that financial resilience has a strong impact on system continuity.

Another challenge is the limited availability of financial support schemes for upgrading or expanding biogas units. Unlike urban renewable energy initiatives, rural biogas programs often lack formal financing instruments such as microcredit, subsidy matching, or community revolving funds. Without institutional backing, most users rely solely on personal funds, which restricts their ability to innovate or scale up. This situation is not unique to Indonesia; Amigun et al. (2008) reported comparable financing limitations in decentralized biogas programs across sub-Saharan Africa.

To unlock the full economic potential of biogas systems, future interventions must integrate livelihood strategies into system design. This includes linking biogas users with agricultural cooperatives, building capacity in

bio-slurry value chains, and facilitating access to financial services. With such measures, biogas can transition from a subsistence technology into a catalyst for rural economic development.

#### *Social engagement and community dynamics*

The biogas system in Mundu Village demonstrated strong social sustainability, with an index of 83.52%, the highest among all five dimensions. This reflects a high degree of community awareness, acceptance, and involvement in managing biogas technology. Active participation by local residents—from system use to minor maintenance—has been critical in sustaining biogas operations since their installation in 2013. The strong performance in this dimension supports previous findings by Dhliwayo et al. (2020), who emphasized the importance of social capital in sustaining rural energy initiatives.

A key social strength is the perceived benefit of biogas adoption. Most users reported improvements in household health, convenience, and gender equity, particularly in reducing the domestic workload traditionally borne by women. The use of biogas has also enhanced household status within the community, contributing to a sense of shared progress. These findings align with studies by Bond and Templeton (2011), which highlight that social acceptance is often driven more by perceived non-monetary benefits than by financial incentives alone.

However, several challenges remain, as despite high community enthusiasm, technical knowledge and capacity are unevenly distributed. Some households lack sufficient understanding of system maintenance and troubleshooting, leading to a dependence on a small group of trained individuals. Moreover, interviews revealed that technical training sessions were conducted only during the early implementation phase and have not been followed by regular refresher programs. This situation risks creating a knowledge gap that may undermine system reliability in the future.

Another issue concerns the management of shared resources and responsibilities. Although the systems are installed on a per-household basis, community-wide coordination is still necessary—especially in managing spare parts, facilitating shared learning, and promoting collective troubleshooting. Inadequate facilitation in this area may lead to social tension or disengagement over time. Similar dynamics were observed by Ristianingsih et al. (2018), who noted that without continuous community facilitation, the sense of joint ownership and accountability tends to decline.

The case of Mundu Village illustrates how social cohesion, user trust, and community-driven motivation are foundational to the long-term sustainability of decentralized energy systems. To maintain these strengths, future programs should prioritize continuous education, local leadership development, and participatory monitoring mechanisms. Strengthening the social fabric around biogas usage can also serve as a buffer against weaknesses in other dimensions, particularly institutional support.

#### *Technological adaptability and limitations*

With a sustainability index of 77.95%, the technological dimension of the biogas system in Mundu Village is categorized as sustainable. This score reflects the generally high adaptability of the technology to local conditions and user needs. Most respondents reported that the system was relatively easy to operate, and the technology was sufficiently reliable for daily household energy requirements. These findings are consistent with Rianawati et al. (2021), who found that user-friendly design and low operational complexity are key drivers of biogas adoption in rural areas.

High performance in this dimension is closely related to the integration of the system into the daily routines of livestock-rearing households. Since cattle are a consistent source of feedstock, the input supply chain is secure, which enhances the reliability of gas production. Moreover, the technology is compatible with local cooking habits and does not require significant changes in infrastructure or behavior. This compatibility has enabled widespread and sustained use over more than a decade.

However, several technical limitations remain, particularly regarding system durability and maintenance logistics. Field observations revealed issues such as digester clogging, inconsistent gas pressure, and difficulties in obtaining replacement parts. These problems, although not frequent, can disrupt daily use and may discourage users without technical support. In some cases, users reported relying on informal solutions or temporary shutdowns of the system. Similar barriers were noted in studies by Amigun et al. (2008), especially in rural programs with limited technical backstopping.

Another constraint is the lack of standardized construction and repair protocols. Although the initial installations adhered to general biogas design principles, variations in construction quality, materials, and installer expertise resulted in inconsistent performance across households. This variability increases the burden on users to troubleshoot issues independently, often without formal guidance. In the absence of a structured maintenance system or trained local technicians, these weaknesses could gradually reduce system efficiency and user confidence.

The Mundu Village experience highlights the importance of technical training, standardization, and post-installation services in sustaining biogas technologies. To maintain and scale up the program, regular capacity-building is essential for both users and local technicians. Additionally, the development of local service centers or village-level technician cooperatives could provide timely repairs and access to spare parts, thereby enhancing the overall quality of service. By enhancing the technological resilience of biogas systems, communities can ensure greater energy security and reduce dependency on external assistance.

#### *Institutional challenges and policy gaps*

The institutional dimension recorded the lowest sustainability index in this study—24.52%, classified as unsustainable. This finding reveals fundamental weaknesses in the governance, support structures, and

policy integration of the biogas program in Mundu Village. While external partners supported the initial program implementation in 2013, there has been no consistent follow-up by formal institutions. The absence of routine monitoring, funding, and capacity-building initiatives has left the community to manage technical and managerial issues largely on its own.

Among the most sensitive attributes identified through leverage analysis were the absence of follow-up programs (RMS = 10.20), limited budgetary support from the local government (8.76), and a scarcity of technical assistance from formal institutions (7.88). These results highlight a significant institutional gap that could compromise the long-term viability of otherwise promising renewable energy projects. Without a structured support system, biogas users may struggle to address system failures, adapt to changing conditions, or scale up their operations. These findings mirror those of Ristianingsih et al. (2018), who found that institutional neglect was a key reason for stagnation in similar rural energy initiatives.

The lack of integration of biogas into local policy frameworks also contributes to weak institutional support. Interviews with local leaders revealed that biogas is not explicitly included in the village's Medium-Term Development Plan (RPJMDes) or prioritized in local environmental or energy agendas. As a result, there are no dedicated funds or institutional responsibilities assigned to sustaining the program. This administrative invisibility contributes to its marginalization and hampers opportunities for cross-sectoral collaboration.

The problem is not unique to Mundu Village. Across Indonesia, many community-scale renewable energy programs are launched with enthusiasm but falter due to insufficient policy alignment and administrative oversight (Fauzi and Anna 2002; Silaen et al. 2020). While technical feasibility and social acceptance are often high, institutional follow-through remains a weak link. The fragmentation of responsibilities between national, regional, and village-level agencies further complicates coordination and accountability.

Addressing these gaps requires a multi-tiered institutional strategy. At the village level, biogas governance must be formalized—for example, by incorporating it into annual work plans (RKPDDes) and assigning responsibilities to village institutions such as BUMDes. At the district and provincial levels, local governments should allocate budget lines and technical resources to support the biogas program. Finally, partnerships with universities, NGOs, and private sector actors can provide ongoing innovation and supervision. By strengthening the institutional ecosystem, biogas programs can transition from pilot projects into resilient, policy-backed systems.

#### *Methodological reflections and policy relevance*

The application of the Rap-Biogas method, adapted from the Rapfish framework developed by Pitcher and Preikshot (2001), proved effective in rapidly assessing the multidimensional sustainability of household-scale biogas systems. Its strength lies in its ability to integrate

qualitative perceptions and quantitative scores across ecological, economic, social, technological, and institutional dimensions (Fauzi and Anna 2002). This makes it particularly suitable for community-based renewable energy evaluations, especially in rural contexts where formal datasets are limited and participatory diagnosis is essential.

Another advantage of Rap-Biogas is its visual and communicative power. Tools such as kite diagrams, leverage analysis, and Monte Carlo simulations make the results more accessible to stakeholders at multiple levels (Kavanagh and Pitcher 2004). These visual outputs enhance engagement and facilitate participatory planning by enabling communities and decision-makers to easily interpret and respond to key sustainability findings. Furthermore, the use of Root Mean Square (RMS) in leverage diagnostics has been proven effective in identifying critical attributes in multidimensional scaling analyses (Batistuta et al. 2021).

The policy relevance of Mundu Village's findings is substantial. First, they demonstrate that community-driven biogas programs can yield sustainable outcomes even in the absence of continuous institutional oversight, as long as strong local participation and basic technical capacity are present (Bond and Templeton 2011). However, the institutional fragility exposed in this study points to the urgent need for formal policy integration and programmatic follow-up, as also emphasized in studies of biogas programs in India and sub-Saharan Africa (Amigun et al. 2008; Katuwal and Bohara 2009).

The study also highlights the importance of cross-sectoral coordination among the agriculture, energy, and rural development sectors. At the village level, integrating biogas into development planning tools such as RPJMDes and RKPDes could ensure dedicated resources and clearer accountability lines (Ristianingsih et al. 2018). Meanwhile, provincial and national stakeholders must provide supportive legal frameworks, technical training pathways, and financial mechanisms to sustain and replicate successful models, such as that of Mundu Village.

As a semi-quantitative method, it depends on the accuracy of attribute selection and consistency in scoring, which can introduce subjective bias (Pitcher and Preikshot 2001; Fauzi and Anna 2002). Furthermore, while effective at the system level, the approach may underrepresent intra-household differences or sociocultural variables influencing sustainability. Future research should consider combining Rap-Biogas with longitudinal ethnographic studies or economic modeling to generate more granular insights.

Despite these limitations, this study confirms that Rap-Biogas is a practical, participatory, and policy-relevant tool for evaluating sustainability in decentralized energy systems. The insights from Mundu Village may serve as a reference for rural communities, development agencies, and governments aiming to implement effective, resilient, and inclusive renewable energy strategies.

In conclusion, this study assessed the sustainability of a household-scale biogas system in Mundu Village, Klaten District, using the Rap-Biogas method across five

dimensions: ecological, economic, social, technological, and institutional. The system scored an overall index of 67.77%, placing it in the fairly sustainable category. Strong ecological, social, and technological performance reflected benefits such as reduced waste, active community involvement, and ease of use. However, economic potential through bio-slurry utilization was underdeveloped, and institutional aspects were critically weak, particularly due to the absence of structured follow-up programs, policy integration, and local government support. These institutional limitations pose serious risks to long-term sustainability. Interventions should include regular monitoring, technician training, and integration of biogas governance into village development plans to strengthen the system's viability. Improving market access and financial support for bio-slurry products would also improve economic outcomes. Despite these challenges, the system shows that community-based biogas can be a viable model for rural renewable energy when supported by strong social participation, appropriate technology, and environmental compatibility. Addressing institutional and economic gaps is a key factor. This case provides valuable lessons for scaling sustainable biogas programs in other rural areas of Indonesia and similar contexts in developing countries. Policymakers should consider integrating household biogas systems into village-level energy planning instruments (e.g., RPJMDes) to ensure long-term institutional support and resource allocation.

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