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Larvicidal Activity of *Caulerpa serrulata* Against *Aedes aegypti* in Aquatic Environment

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ABSTRACT

Dengue fever causes significant numbers of victims in Indonesia every year. As of the 17th week of 2024, dengue fever has claimed the lives of 621 people out of a total of 88,593 cases across the country. This is due to environmental conditions that are suitable for the life cycle of *Aedes aegypti*. Efforts to eradicate *Aedes aegypti* larvae have been made, one of which involves using a larvicide called temephos or abate. However, abate leaves a lasting residue. This research aimed to identify natural alternatives to abate. Previous studies have explored the use of seaweed to control mosquitoes, but none have investigated *Caulerpa serrulata* (Forsskål) J.Agardh, 1837, despite its abundance in Indonesian waters and its rapid spread along the coast, much like a pest. This study analyzed the larvicidal activity of *Caulerpa serrulata* in both extract form and as titanium dioxide nanoparticles (TiO2-NPs), as well as their environmental safety. The LC50 values for the extract and TiO2-NPs against *Aedes aegypti* larvae were 0.16 ppm and 0.14ppm, respectively. The particle size of the extract ranged from 13.012 to 275.177µm, with 94.599% of the particles in the nanoparticle size range. Meanwhile, the particle size of TiO2-NPs ranged from 0.012 to 2000µm, with 96.402% in the nanoparticle size range. Further observations on the environmental safety of TiO2-NPs revealed that they are not only safe for fish but also enhance their resistance to fungi. However, the TiO2-NPs tend to settle at the bottom of the water. Therefore, further research is needed to develop methods for producing uniformly sized TiO2-NPs from *Caulerpa serrulata* and to assess their impact on non-vector organisms that live at the bottom of the water and in the soil substrate.

INTRODUCTION

Dengue fever is consistently recorded in Indonesia each year. As of the 17th week of 2024, dengue fever has caused 621 deaths out of a total of 88,593 cases throughout Indonesia. This is due to the natural conditions in Indonesia, which are favorable for the growth and reproduction of *Aedes aegypti*.

The Ministry of Health of the Republic of Indonesia has implemented several measures to prevent the spread of dengue fever outbreaks. One of these measures is the socialization of PSN 3M Plus. The first "M" stands for Menguras (drain). Draining involves emptying places that often serve as water reservoirs, such as bathtubs, jugs, water towers, drums, and other containers. The second "M" is Menutup (close). Closing involves tightly sealing water reservoirs, such as bathtubs and drums. This action also includes burying used items in the ground to prevent environmental contamination and to reduce potential mosquito breeding sites. The third "M" is Memanfaatkan (leverage). This refers to reusing waste with economic value (recycling). People are encouraged to reuse or recycle items that could become mosquito breeding grounds. The "Plus" signifies additional preventive measures, including keeping fish that consume mosquito larvae, using mosquito repellent, installing wire mesh on windows and vents, collaborating to clean the environment, checking water reservoirs, storing used clothes in sealed containers, repairing blocked water channels and gutters, planting mosquito-repellent plants, and applying larvicides to hard-to-drain water reservoirs.

The most commonly used larvicide is abate. Abate contains temephos, which is effective at killing mosquito larvae within an hour. However, it leaves a residual effect that lasts for up to three months with normal water usage patterns. According to the USEPA, temephos has moderate acute toxicity when exposed through dermal or oral routes, and low toxicity when inhaled by humans. Abate is typically applied in bathtubs. The water from these bathtubs is used for various sanitation purposes, such as bathing (dermal exposure) and brushing teeth (oral exposure), which increases the potential for acute moderate toxicity.

Abate (temephos) is a pesticide classified under organic phosphate compounds. This class of pesticide works by inhibiting the cholinesterase enzyme, leading to disruptions in nerve activity due to the accumulation of acetylcholine at nerve endings. Normally, the cholinesterase enzyme breaks down acetylcholine into choline and acetic acid. When this enzyme is inhibited, acetylcholine accumulation causes prolonged muscle contractions, which can lead to spasms or convulsions. Over time, continuous use of abate may result in resistance among mosquito larvae and other insects **(Suwasono, 1991)**. Resistance traits can be passed down through generations, making it a major challenge in controlling malaria vectors. This is due to the presence of resistance genes, which control the production of enzymes that detoxify insecticides **(Beaty & Marquardt, 1996)** Another factor contributing to resistance is the use of sub-lethal or insufficient doses **(Tarumingkeng, 1992)**. According to **Kasumbogo (2004)**, factors influencing mosquito resistance to insecticides include pesticide concentration, frequency of application, and the areas treated.

Therefore, it is crucial to find environmentally friendly alternative larvicides, one of which could be natural ingredients from seaweed, as seaweed produces bioactive compounds with various beneficial effects. The mosquito larvicidal potential of seaweed extract has been studied by numerous researchers, as summarized in Table (1).

Table 1. Larvicidal ability of seaweed extract

Seaweed extract	Mosquito	Reference
Caulerpa racemose	*Culex pipiens*	**Alarif *et al.* (2010)**
Bryopsis pennata	*Aedes aegypti, Aedes albopictus*	**Yu *et al*. (2015)**
Sargassum wightii	*Anopheles stephensi*	**Murugan *et al*. (2018)**
Sargassum wightii, Halimeda gracillis	*Anopheles stephensi, Aedes aegypti, Culex tritaeniorhynchus*	**Suganya *et al*. (2019)**
Champia parvula	*Aedes aegypti*	**Yogarajalakshmi *et al*. (2020)**
Jania rubens, Galaxaura elongata,	*Culex pipiens*	**Haleem *et al*. (2022)**
Gelidium latifolium, Ulva usus, Codium tomentosum, Dictyota dichotoma, Sargassum dentifolium,		
Padina boryana		
Sargassum wightii	*Anopheles subpictus, Culex quinquefasciatus*	**Mathivanan *et al*. (2023)**

The larvicidal ability of this seaweed extract has also been increased by researchers by forming nanoparticles as shown in Table (2).

Table 2. Larvicidal ability of seaweed nano particles

Seaweed	Nanoparticle	Mosquito	Reference
Sargassum wightii	zinc oxide nanoparticles (ZnONPs)	*Anopheles stephensi*	**Murugan *et al*. (2018)**
Sargassum wightii	silver nanoparticles (Ag-NPs)	*Aedes aegypti*, *Culex quinquefasciatus*	**Balaraman *et al*. (2020)**
Sargassum wightii	titanium dioxide nanoparticles (TiO_2-NPs)	*Aedes aegypti*, *Culex quinquefasciatus*	**Balaraman *et al*. (2022)**
Sargassum wightii	titanium dioxide nanoparticles (TiO_2-NPs)	*Anopheles subpictus*, *Culex quinquefasciatus*	**Mathivanan *et al*. (2023)**
Sargassum palmeri	silver nanoparticles (AgNPs)	*Aedes aegypti*	**Ghramh *et al*. (2022)**
Sargassum muticum	silver nanoparticles (AgNPs)	strain nyamuk India maupun Arab Saudi	**Trivedi *et al*. (2021)**
Sargassum myriocystum	silver nanoparticles (Ag-NPs)	*Aedes aegypti*, *Culex quinquefasciatus*	**Balaraman *et al*. (2020)**
Ulva lactuca	silver nanoparticles (AgNPs)	*Aedes aegypti*, *Culex pipiens*	**Aziz (2022)**

The larvicidal ability of the *Aedes aegypti* using *Caulerpa serrulata* has never been observed, even though this seaweed is often found in Indonesian waters and spreads quickly like a pest. Therefore, this research was conducted to analyze the larvicidal activities of extract and TiO_2-NPs *Caulerpa serrulata* along with their safety for the environment.

MATERIALS AND METHODS

1. Extraction and extract tested against *Aedes aegypti* larvae

Caulerpa serrulata was freshly collected from the beach near the Seaweed Tissue Culture Laboratory at the Center for Brackish Water Aquaculture Fisheries (BBPBAP) in Jepara, Central Java, Indonesia (coordinates: -6.584035, 110.643786) on October 26, 2023. The taxonomy of Caulerpa serrulata, according to **Amjad *et al.* (2024)**, is as follows:

Kingdom : Plantae
Phyllum : Chlorophyta
Class : Ulvophyceae
Order : Bryopsidales
Family : Caulerpaceae
Genus : Caulerpa
Scientific name : *Caulerpa serrulata*
Scientific name authorship : (Forsskål) J.Agardh, 1837.

The morphology of *Caulerpa serrulata* used in this study is shown in Fig. (1).

Fig. 1. Morphology of *Caulerpa serrulata*

Fresh seaweed was sorted, rinsed and air-dried without being exposed to direct sunlight. Drying was carried out until the water content remained under 20%. The seaweed was cut into pieces and was then mashed using a blender. The results were sieved using a sieve with a mesh size of one millimeter. Extraction was carried out based on **Handayani *et al*. (2020)** with slight modifications in the mesh size using range 365-555µm, type of solvent using ethyl acetate only, and evaporation temperature at 40°C.

The extract was tested on *Aedes aegypti* larvae at a dose of 0.1, 1, and 10ppm with 3 repetitions of each dose. *Aedes aegypti* larvae were obtained from the Research and Development Center for Disease Vectors and Reservoirs, Salatiga, Central Java, Indonesia. Larval mortality data were recorded and used as a basis for determining the upper and lower limits. Follow-up tests on larvae were carried out at 4 different doses based on the upper and lower limits with 3 repetitions for each dose. Larval death data were recorded and entered into the Probits application for determining LC_{50} **(Kumalasari *et al*., 2015)**.

2. TiO_2-NPs making process and TiO_2-NPs tested against *Aedes aegypti* larvae

In this study, we used titanium dioxide (TiO2) due to its excellent chemical stability, good adsorption properties in the ultraviolet region, and high transparency in visible light **(Rasheed, 2017)**. TiO2 is widely used as a photocatalyst for wastewater purification **(Wulandari *et al.*, 2018)**, and it also possesses good optical properties, photocatalytic activity, superhydrophilicity, high mechanical stability, and environmental friendliness **(Agustiana *et al.*, 2022)**. These environmentally friendly properties make TiO2 an ideal candidate for developing an alternative larvicide to replace Abate.

The titanium dioxide precursor used was pure analytical-grade titanium isopropoxide (TTIP) from Sigma. The process for preparing TiO2 nanoparticles (TiO2-NPs) followed **Ahmed *et al. (*2023)** with slight modifications in the rinsing step, using appropriate centrifugation techniques as described by **Ridhawati and Fajar (2017)**. The obtained TiO2-NPs were then ground using a mortar.

Exposure of *Aedes aegypti* larvae to the TiO2-NPs was conducted at doses of 0.1, 1, and 10ppm, with three repetitions for each dose. The TiO2-NPs were first mixed with tap water and were stirred until getting homogeneous, after which *Aedes aegypti* larvae were added. The number of dead larvae was recorded, and the doses served as the upper and lower limits for subsequent testing. The next phase involved testing four doses, with three repetitions per dose, based on the data from the upper and lower limits. The results were analyzed using a probit application to determine the LC50, following the methodology of **Kumalasari *et al.* (2015)**. Environmental safety tests were conducted using the LC50 dose of TiO2-NPs.

3. Particle size and form analysis

Some of the extracts and TiO2-NPs were analyzed for their particle size using a laser particle size analyzer (LPSA). The extract needed to be freeze-dried before particle size analysis. Particle morphology was examined using a Scanning Electron Microscope (SEM).

4. Environmental safety test

The TiO2-NPs were homogenized into water, which would serve as the medium for raising fish. Once homogeneous, the fish were placed into the water. In this study, 20 two-and-a-half-month-old tetra glowfish were used for both the treated and control tanks. The fish in both the treated (with TiO2-NPs added to the rearing water) and control (without TiO2-NPs added to the rearing water) tanks were kept for 30 days with strong aeration to ensure that the TiO2-NPs were incorporated into the fish. Swimming activity was observed and compared before and after 30 days of exposure. The effects of the exposure on the gill tissue were examined after 30 days.

RESULTS

1. Extraction and extract tested against *Aedes aegypti* larvae

The extraction of 1kg *Caulerpa serrulate* powder using ethyl acetate as solvent produce 0.023% extract. Lethal concentration (LC) 50 of the extracts was 0.16ppm. Larvae died within one minute after being exposed to *Caulerpa serrulata* (Fig. 2). At low doses, if there are live larvae after the first minute of exposure, the larvae will remain alive even if exposed until 96 hours.

Fig. 2. *Aedes aegypti* larvae died after exposed to extract of *Caulerpa serrulata*

2. TiO_2-NPs making process and TiO_2-NPs tested against *Aedes aegypti* larvae

LC_{50} of *Caulerpa serrulata* TiO_2-NPs against *Aedes aegypti* was 0.14ppm with the response of the larvae which sink to the bottom of the water (Fig. 3) after being exposed to TiO_2-NPs. Dead larvae sink, not responding at all to touch from the tip of the skewer. Larval death occurs in less than one minute after the larvae are exposed. At lower doses of exposure, larvae that remained alive after the first minute were proven to survive until the 96 hours.

Fig. 3. Dead larvae after exposure to TiO_2-NPs are marked with blue arrows, side (left) and top (right) views

3. Particle form and size analysis of extract and TiO_2-NPs

The extract of *Caulerpa serrulata* looks like sticky lump and after being converted into nanoparticles, it looks like chalk lump (Fig. 4). The particle morphology was observed using a SEM, as shown in Fig. (5).

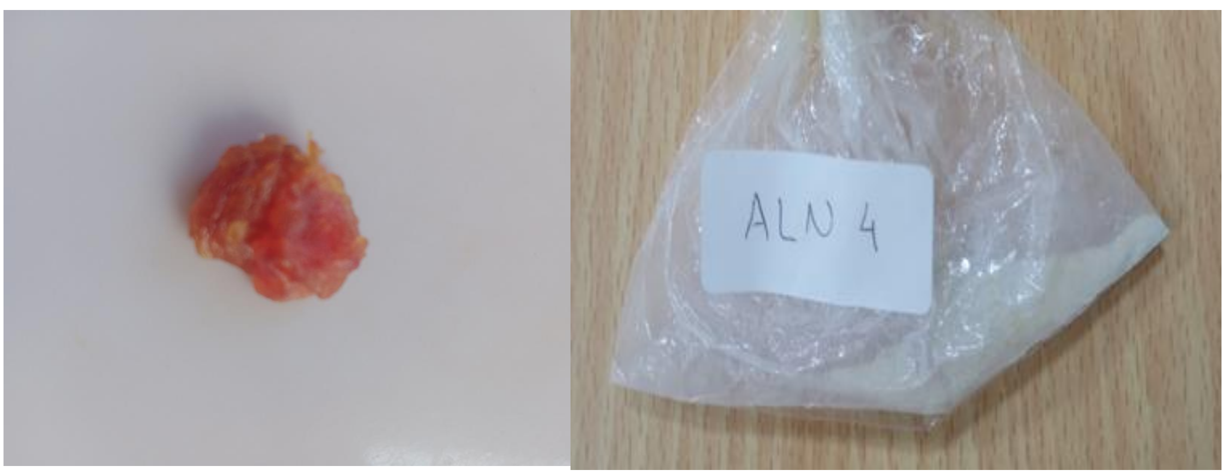

Fig. 4. Visual form of the extract (left) and TiO_2-NPs (right)

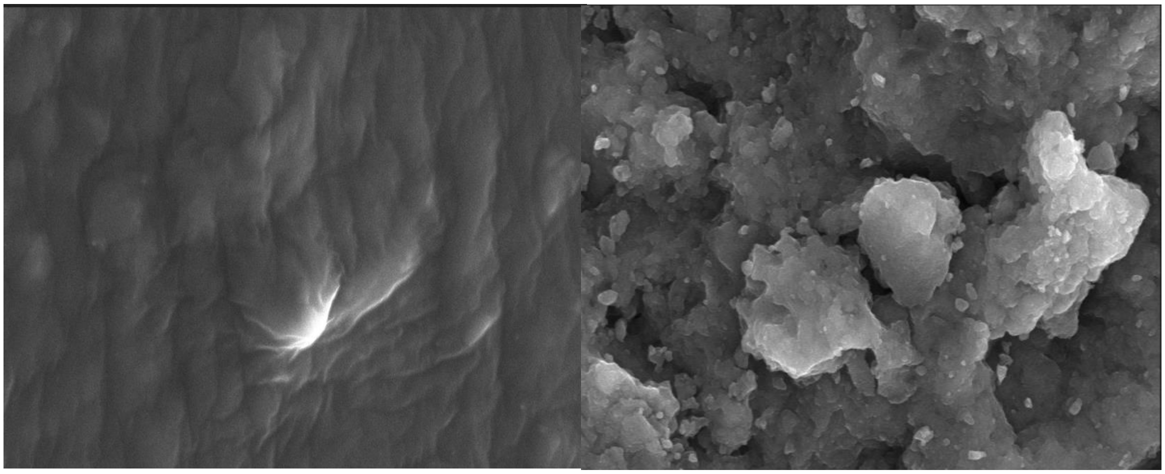

Fig. 5. SEM observation at 5000x magnification of the extract (left) and TiO_2-NPs (right) particles

Particle size of *Caulerpa serrulata* extract was in the range of 13.012-275.177μm, while particle size of the *Caulerpa serrulata* TiO_2-NPs was in the range of 0.012-2,000μm (Fig. 6). Particles are categorized into the nanoparticle group if they have a size of less than or equal to 100μm. The percentage of nano-sized particles observed using LPSA from the extract samples was 94.599%, while TiO_2-NPs samples was 96.402% (Fig. 7).

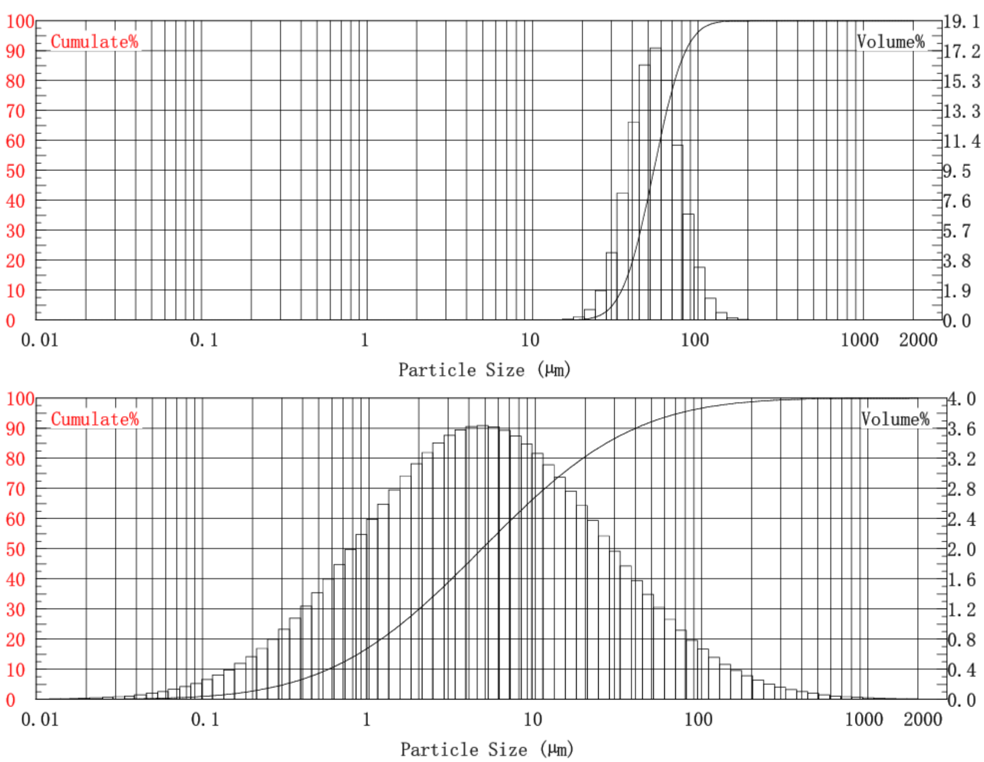

Fig. 6. LPSA observations of the extract (top) and TiO_2-NPs (bottom) particle size

Size (μm)	Volume%	Cumulate%	Size (μm)	Volume%	Cumulate%	Size (μm)	Volume%	Cumulate%
0.334	0.000	0.000	3.839	0.000	0.000	44.102	12.607	27.828
0.389	0.000	0.000	4.472	0.000	0.000	51.371	16.252	44.081
0.453	0.000	0.000	5.209	0.000	0.000	59.839	17.333	61.413
0.528	0.000	0.000	6.068	0.000	0.000	69.702	15.291	76.704
0.615	0.000	0.000	7.068	0.000	0.000	81.191	11.159	87.863
0.717	0.000	0.000	8.233	0.000	0.000	94.574	6.736	94.599
0.835	0.000	0.000	9.590	0.000	0.000	110.163	3.364	97.963
0.972	0.000	0.000	11.171	0.000	0.000	128.321	1.390	99.352
1.133	0.000	0.000	13.012	0.002	0.002	149.472	0.475	99.827
1.320	0.000	0.000	15.157	0.011	0.013	174.110	0.134	99.961
1.537	0.000	0.000	17.656	0.051	0.064	202.809	0.031	99.993
1.790	0.000	0.000	20.566	0.206	0.269	236.238	0.006	99.999
2.085	0.000	0.000	23.956	0.685	0.954	275.177	0.001	100.000
2.429	0.000	0.000	27.904	1.885	2.839	320.535	0.000	100.000
2.830	0.000	0.000	32.504	4.294	7.133	373.369	0.000	100.000
3.296	0.000	0.000	37.861	8.089	15.222	434.912	0.000	100.000

Size (μm)	Volume%	Cumulate%	Size (μm)	Volume%	Cumulate%	Size (μm)	Volume%	Cumulate%
0.012	0.005	0.005	0.717	1.806	12.601	44.102	1.593	90.965
0.014	0.006	0.011	0.835	2.006	14.607	51.371	1.409	92.374
0.016	0.009	0.020	0.972	2.209	16.816	59.839	1.235	93.610
0.018	0.011	0.031	1.133	2.414	19.230	69.702	1.074	94.684
0.021	0.015	0.045	1.320	2.615	21.845	81.191	0.926	95.610
0.025	0.021	0.067	1.537	2.808	24.654	94.574	0.792	96.402
0.029	0.028	0.094	1.790	2.991	27.644	110.163	0.671	97.073
0.034	0.038	0.132	2.085	3.158	30.803	128.321	0.564	97.637
0.039	0.048	0.180	2.429	3.307	34.110	149.472	0.470	98.108
0.046	0.064	0.244	2.830	3.434	37.544	174.110	0.389	98.496
0.054	0.084	0.328	3.296	3.536	41.080	202.809	0.319	98.815
0.062	0.105	0.433	3.839	3.610	44.690	236.238	0.259	99.074
0.073	0.136	0.569	4.472	3.654	48.344	275.177	0.209	99.282
0.085	0.171	0.741	5.209	3.668	52.013	320.535	0.167	99.449
0.099	0.214	0.955	6.068	3.651	55.664	373.369	0.132	99.581
0.115	0.265	1.219	7.068	3.604	59.268	434.912	0.104	99.685
0.134	0.325	1.545	8.233	3.527	62.795	506.599	0.081	99.766
0.156	0.396	1.941	9.590	3.423	66.218	590.102	0.062	99.828
0.182	0.480	2.422	11.171	3.294	69.512	687.369	0.048	99.876
0.211	0.572	2.994	13.012	3.143	72.655	800.669	0.036	99.912
0.246	0.681	3.675	15.157	2.974	75.629	932.643	0.027	99.939
0.287	0.804	4.480	17.656	2.790	78.419	1086.372	0.020	99.960
0.334	0.939	5.419	20.566	2.596	81.015	1265.439	0.015	99.975
0.389	1.088	6.507	23.956	2.394	83.409	1474.023	0.011	99.986
0.453	1.251	7.758	27.904	2.190	85.599	1716.987	0.008	99.994
0.528	1.426	9.184	32.504	1.986	87.586	2000.000	0.006	100.000
0.615	1.611	10.794	37.861	1.787	89.372			

Fig. 7. Percentage of particle size between the extract (top) and TiO_2-NPs (bottom) particle size

4. Environmental safety test

The results of environmental safety tests for non-vector animals show that there is no interference with swimming activity in the test animals (fish). Even when control fish were exposed to the fungus and impact to disruption of their movement and appetite, the treated fish were still healthy, active swimmers, and had a high appetite. Some of the treated fish began to be exposed to the fungus on the 21st day. Images of fish exposed to fungus can be seen in Fig. (8). Dead fish were taken from the aquarium every day. After 30 days of observation, not a single control fish was still alive. On the other hand, there were still 50% of treated fish that lived until the end of the observation period.

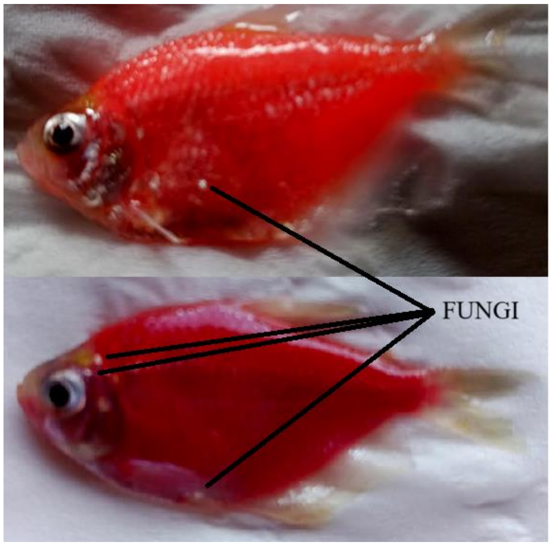

Fig. 8. Fish exposed to fungus

Observation of tissue in the gills of control fish showed damage to this organ. This result is very different from the results of observing the tissue on the gills of the treated fish which looked neater and without damage (Fig. 9). In fact, no particles of *Caulerpa serrulata* TiO_2-NPs were found in the gills of the treated fishes.

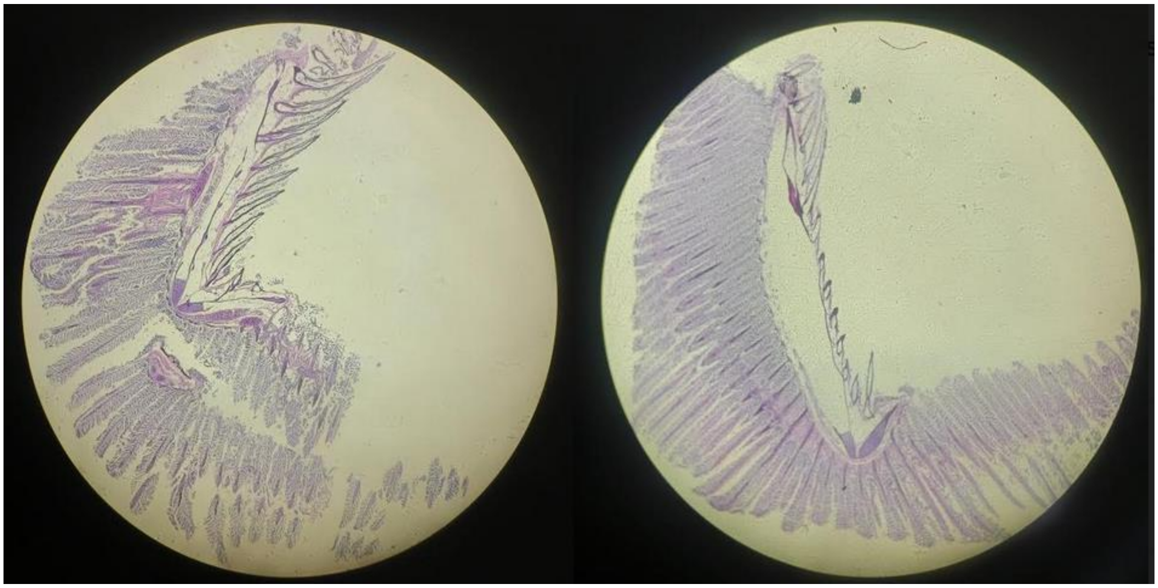

Fig. 9. Microscopic observation at 100x magnification of control (left) and treated (right) fish gills stained with Hematoxylin and Eosin (H&E)

DISCUSSION

1. Extraction and extract tested against *Aedes aegypti* larvae

The extract of *Caulerpa serrulata* exhibits larvicidal activity against *Aedes aegypti* larvae. This activity is indicated by the death of larvae after being placed in water exposed to the extract. Based on the results, the exposure effect of *Caulerpa serrulata* extract is acute, as it only requires a short contact time to kill the larvae. This demonstrates that *Caulerpa serrulata* extract is a promising alternative to abate, with larvicidal activity that falls into the strong larvicidal category.

2. TiO_2-NPs making process and TiO_2-NPs tested against *Aedes aegypti* larvae

In addition to the secondary metabolites in *Caulerpa serrulata*, TiO2-NPs also have the ability to affect the nervous system **(Hu *et al.*, 2017)**. Based on this ability, it is possible that TiO2-NPs enter the larvae's body through the siphon hole, followed by entering into the bloodstream. Once in the blood, TiO2-NPs may impair the larvae's nervous system, causing the larvae to become stiff, immobile, and fall to the bottom of the water.

The effect of TiO2-NP exposure on *Aedes aegypti* larvae is also acute. The acute nature of exposure may be influenced by the tendency of TiO2-NPs to settle at the bottom of the water, while *Aedes aegypti* larvae tend to move in the water column or remain near the water's surface. Consequently, TiO2-NPs from *Caulerpa serrulata* can only come into contact with the larvae when both are in the water column. This short but effective contact time required to kill *Aedes aegypti* larvae further supports *Caulerpa serrulata* as a strong candidate for use as a natural alternative larvicide to replace abate.

3. Particle size and form analysis

The larvicidal activity of the extract is proven to increase by reducing the particle size. Converting the extract into nanoparticle form allows for smaller particle sizes, as the natural form of *Caulerpa serrulata* extract is difficult to grind. Once converted into nanoparticles, the particles become easier to grind.

Based on observations using LPSA, 3.598% of the TiO2-NPs were still too large to be classified as nanoparticles. These larger particles tend to settle at the bottom of the water faster than nano-sized particles, making them ineffective and wasted. Therefore, further research is needed to explore alternative methods for producing TiO2-NPs from *Caulerpa serrulata* with uniformly sized particles, in order to enhance the effectiveness of the larvicidal activity of the resulting TiO2-NPs.

4. Environmental safety test

Although the presence of *Caulerpa serrulata* TiO_2-NPs in the water column has proven to be dangerous for the life of *Aedes aegypti* larvae, the results of environmental safety tests shows that TiO_2-NPs from *Caulerpa serrulata* which are added to the rearing media water, can help maintain the quality of the rearing media water so that the fish become stronger against fungal attacks.

It was noticed that TiO_2-NPs were not found in the gills of the treated fishes; this may occur due to the tendency of TiO_2-NPs *Caulerpa serrulata* to fall to the bottom of the water so that the use of strong aeration is still not able to stir the particles thus they move into the water column above. Therefore, it is necessary to carry out further research by observing the effect of using TiO_2-NPs *Caulerpa serrulata* on non-vector animals that live at the bottom of waters such as snails, as well as non-vector animals that live on the bottom substrate of waters like worms.

CONCLUSION

Caulerpa serrulata has strong larvicidal activity, both in the form of extract and TiO_2-NPs. The TiO2-NPs derived from *Caulerpa serrulata* have also been proven to be environmentally friendly. Therefore, *Caulerpa serrulata* can be an alternative candidate to replace abate.

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