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The Effectiveness of Citric Acid as an Anti-Ectoparasite of Marine Leech (*Zeylanicobdella arugamensis*) through Soaking

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Abstract. *Zeylanicobdella arugamensis* infection causes health problems for cultured grouper. This study aims to determine the effectiveness of citric acid soaking as an anti *Z. arugamensis* ectoparasite. A total of ±120 *Z. arugamensis* were isolated from sick hybrid grouper (*Epinephelus fuscoguttatus* x *E. lanceolatus*) placed in Petri dishes (6 Petri dishes). Citric acid solution with 0, 50, 100, 150, 250, and 400 ppm was added to the petri plates. Immersion of *Z. arugamensis* was carried out for 30, 60, 90 and 120 minutes. Each 30 *Z. arugamensis*/treatment/30 minutes was taken and placed in 3 Petri dishes (10 individuals/petri dish) filled with seawater. Observations were made on the response of *Z. arugamensis*. Comments were made on *cocoon* development into larvae. The citric acid safety test was conducted on cantang hybrid grouper infected with *Z. arugamensis*. A total of 3 test fish were soaked with citric acid solution in seawater at concentrations of 0, 150, 250, and 300 ppm. Observations of fish condition were made after 30 and 60 minutes of immersion. The results showed that citric acid concentrations of 250 and 400 ppm effectively killed *Z. arugamensis* (76.67±15.28-100%), and their *cocoon* development reached (0%). A citric acid concentration of 300 ppm is still safe to use for immersion of cantang hybrid grouper infected with *Z. arugamensis* for 60 minutes. Citric acid concentrations of 250-300 ppm can also release *Z. arugamensis* from the fish body. Citric acid has antiparasitic properties and lowers the pH of seawater.

Keywords: citric acid; *cocoon*; hybrid grouper; soaking; *Zeylanicobdella arugamensis*

I. INTRODUCTION

Sea leeches (Piscolidae, Hirudinea: *Zeylanicobdella arugamensis*) are one of the ectoparasites that become an obstacle in the cultivation of grouper (*Epinephelus* sp.) in hatcheries and floating net cages (KJA). Sea leech infections occur almost year-round with prevalence reaching 10-100%. The highest prevalence of sea leeches occurs in March-June [1, 17, 23].

Z. arugamensis attacks and attaches to soft parts of the fish body such as pectoral fins, pelvic fins, caudal fins, and lower head, and if in large numbers the fish looks hairy. This type of leech has a striated body, strong body walls, and two suckers that function to eat, move, and take nutrients in the host it is riding [9]. *Z. arugamensis* attaches to the skin using 2 suckers then injects histamine into the blood vessels and sucks nutrients using probocysts. Blood clotting at the attachment site can be inhibited by injecting saliva so that the host's blood clotting enzyme (thrombin) can be inhibited [19, 20].

Z. arugamensis infection can cause wounds that trigger secondary infections by pathogenic bacteria such as *Vibrio alginolyticus*. This bacterium is one of the species that cause vibriosis in grouper fish. Vibriosis outbreaks can occur due to environmental stress that makes fish weak [12, 13, 28].

Infection control of *Z. arugamensis* has been done by chemical immersion, such as formalin at 15 ppm for 30 minutes and $CuSO_4$ at 150 ppm with strong aeration [16], [18, 26, 27]. In the case of formalin use, leeches showed strong swimming ability for 60 seconds and decreased sluggishly, after 3 minutes until they finally did not move [27]. The use of formalin in high concentrations can cause disruption of fish respiration because formalin reacts with oxygen dissolved in water [20, 26].

Another chemical alternative for controlling *Z. arugamensis* infection is with chemicals that have lower toxicity effects. Citric acid which is a weak organic acid with $C_6H_8O_7$ and is a preservative and acidity regulator. Citric acid can also be used as an environmentally

friendly cleaning agent and antioxidant [24]. The use of citric acid is used to reduce metal levels in fish [29]. The function of citric acid needs to be tested as an alternative material to overcome *Z. arugamensis* infection. Therefore, a trial of *Z. arugamensis* treatment using citric acid in the laboratory was conducted. The purpose of this study was to determine the effectiveness in killing *Z. arugamensis in vitro* and *in vivo*.

II. METHOD

Research Setting

This research was conducted in November-December 2022 at the Pathology Laboratory, Fisheries Research Center, Maritime Earth Research Organization, National Research and Innovation Agency (BRIN), CWS Gondol, Gerokgak District, Buleleng Regency, Bali.

Source of *Zeylanicobdella arugamensis*

Z. arugamensis sea leeches were derived from 10 to 30 sick hybrid grouper (*E. fuscoguttatus* X *E. lanceolatus*) of 25-30 cm and 12-15cm size reared in 2 round fiber tanks of 500 liter volume with running water system. The fish have cohabited with sea leeches for 2-3 weeks until they were infected with many sea leeches on their bodies.

Collection of *Zeylanicobdella arugamensis*

Z. arugamensis was collected by sequencing the body of grouper fish that had been infected with many sea leeches. The *Z. arugamensis* was put into 6 petri dishes with a diameter of 8 cm that had been filled with seawater. Each petri dish was filled with ±120 *Z. arugamensis*. Furthermore, all Petri dishes were rinsed with new water 3 times to remove fish slime that came and attached to *Z. arugamensis*. Petri dishes are allowed to stand for 1 hour to relieve stress from *Z. arugamensis* until it sticks firmly to the inner surface of the petri dish.

Citric Acid Solution

The citric acid used was pro-analyzed citric acid (Emsure®, Germany). The citric acid powder was weighed in an analytic balance (Shimadzu AW220) as much as 0.01, 0.02, 0.03, 0.05, and 0.08 grams. Furthermore, each citric acid was dissolved in 200 mL of seawater in a 300 mL Erlenmeyer volume so that the final concentrations were 50, 100, 150, 250, and 400 ppm. One Erlenmeyer was filled with seawater for control (0%).

In vivo Treatment of *Zeylanicobdella arugamensis*

A total of 72 pieces were prepared and filled with seawater. The Petri dishes were used to hold *Z. arugamensis* after treatment. Each petri dish (6 pieces) containing *Z. arugamensis* had its rearing seawater removed and replaced with new seawater (control) and different concentrations of citric acid solution with a time lag of 10 minutes per petri dish. Next, *Z. arugamensis* was taken with tweezers slowly after 30, 60, 90, and 120 minutes of immersion in each treatment. For each treatment and control, as many as 30 fish and 10 fish each were placed in 1 petri dish (a total of 3 petri dishes/treatment). Observations were made for 1 hour on *Z. arugamensis* in each petri dish/treatment. The treatment of *Z. arugamensis* was carried out with as many as 3 replicates at different times.

In Vitro Treatment for *Z.arugamensis* Cocoons

Z. arugamensis was collected from a diseased hybrid grouper. 40-50 *Z. arugamensis* each were placed in 24 petri dishes. The Petri dishes were then incubated for 2 hours at room temperature (30°-32°C) to recover after isolation/collection and the rearing water was changed. Sea leeches and their *cocoon*s were soaked with citric acid solution at different concentrations (0, 50, 100, 150, 250, and 400 ppm with 4 petri dishes/concentration each). Every 30 minutes, 1 petri dish/treatment was replaced with seawater. Then all petri dishes were incubated at room temperature for 14 days. Maintenance water was added if evaporation occurred.

Observation of Cocoon Development

Observations of *cocoon* development were made macroscopically (visible to the eye) and microscopically using a Leica M60 microscope connected to an LG monitor to take pictures. Observations were made on the number of *cocoon*s that developed and hatched into larvae.

In Vivo Treatment

The *in vivo* test was conducted in 5 plastic tanks with a volume of 10 liters. Each was filled with 2 liters of water and citric acid solution was added until the final concentrations were 50, 200, 250, and 300 ppm. Each concentration used 1 tub and 1 other tub for control without the addition of citric acid. Then, 3 hybrid grouper fish (average total length 14.27±0.95 cm) infected with *Z. arugamensis were* placed in each tub (30 fish in total). Observations were made on the response of the test fish.

Water Quality

Measurement of water quality in each citric acid concentration treatment is carried out every day. The parameters to be measured are temperature, pH, and salinity.

Data Analisys

Data from *in vitro* tests were subjected to *One Way Anova* (*Analisys of Variant*), testing for *cocoon* development and *in vivo* tests were carried out by descriptively.

III. RESULTS AND DISCUSSION

Effectiveness of Citric Acid Soaking against *Z. arugamensis In Vitro*

The test results of citric acid immersion of *Z. arugamensis* at concentrations of 50, 100, and 150, ppm *in vitro* showed that the response of *Z. arugamensis* was still in a normal state with active body movements and suckers that were firmly attached to the bottom of the petri dish. However, immersion of *Z. arugamensis* with citric acid at concentrations of 250 and 400 ppm caused the condition of *Z. arugamensis* to be weak, the body shrank and many floated on the surface of the water in a petri dish. *Z. arugamensis* die both inactive suckers and will fall floating in the waters [22, 26].

Citric acid immersion at concentrations of 250-400 ppm caused the death of *Z. arugamensis*. The higher concentration of citric acid caused higher mortality ($P<0.05$) (Table 1). Similarly, the longer the immersion time of *Z. arugamensis* caused higher mortality, except at a citric acid concentration of 400 ppm which caused 100% mortality from the first 30 minutes. Citric acid affects the pH value of the *in vitro* test maintenance water, the higher the concentration given will reduce the pH so that there is a release of H^+ ion values, this is influenced by the mechanism of action of organic acids and different bioactive compounds [8], [14], [27].

TABLE 1.
MORTALITY OF *Z. arugamensis* (%) AFTER SOAKING WITH CITRIC ACID IN 3 REPLICATES OF TIME

Soaking times (minutes)	Citric Acid Concentration (ppm)					
	0	50	100	150	250	400
Replicate 1						
30	0 a	30,0±10,0 b	30,0±10,0 b	53,33±11,55 b	80,0±10,0 c	100 d
60	0 a	33,33±5,77 b	43,33±15,28 b	63,33±5,77 b	86,67±5,77 c	100 d
90	0 a	36,67±11,55 b	43,33±5,77 b	66,67±15,28 c	93,33±5,77 d	100 e
120	0 a	43,33±15,28 b	46,67±11,55 b	73,33±5,77 c	93,33±5,77 d	100 d
Replicate 2						
30	0 a	33,33±5,77 b	50,0±10,0 c	56,67±15,28 c	76,67±15,28 c	100 d
60	0 a	43,33±5,77 b	50,0±0 c	56,67±11,55 c	90,0±0 d	100 e
90	0 a	46,67±15,28 b	56,67±20,82 b	70,0±10,0 b	96,67±5,77 c	100 c
120	0 a	50,0±10,0 b	56,67±5,77 b	70,0±10,0 b	96,67±5,77 c	100 c
Replicate 3						
30	0 a	33,33±15,28 b	33,33±5,77 b	46,67±5,77 c	80,0±10,0 d	100 e
60	0 a	46,67±5,77 b	56,67±5,77 b	50,0±0 b	93,33±5,77 c	100 d
90	0 a	50,0±0 b	56,67±5,77 b	56,67±20,82 b	96,67±5,77 c	100 c
120	0 a	56,67±5,77 b	70,0±10,0 c	73,33±5,77 c	96,67±5,77 c	100 d

Notes: Different values in the same row indicate significantly different ($P<0.05$)

Table 1. shows that citric acid immersion at concentrations of 50-150 ppm caused mortality of *Z. arugamensis* up to 63,33 ± 5,77% in the first 60 minutes in 3 replicate times. These concentrations are less effective in killing *Z. arugamensis*, because farmers expect chemicals or drugs that are able to kill *Z. arugamensis* completely in a short time (<60 minutes) and are not toxic to fish.

Citric acid immersion at a concentration of 250 ppm in the first 30 minutes was able to kill *Z. arugamensis* as much as 76,67 ± 15,28-80,0 ± 10,0%, and increased in the next 30 minutes to 100% at the 120 minute. Citric acid immersion with a high concentration (400 ppm) was able to kill *Z. arugamensis* in a short time, less than 30 minutes. The high concentration of citric acid caused *Z. arugamensis* to shrivel when freshly soaked, followed by hyperactive movements and detachment from the bottom of the petri dish. Mortality reached 100% within 30 minutes in all three replicates conducted in the study.

The results of the Randomized Group Design (RAK) test using SPSS showed that different concentrations of citric acid had a significant effect on the mortality of *Z. arugamensis* sea leeches with a value of $F = 250.44$, $P<0.05$. Treatment using different observation times had a significant effect on mortality with a value of $F = 23.836$, $P<0.05$. The results of the interaction test between different concentrations of citric acid and the treatment time showed the mortality of *Z. arugamensis*

with a value of F = 1.261, P<0.05. The test results showed that the higher the concentration of citric acid and the longer the soaking time influenced the mortality of *Z. arugamensis*.

Effectiveness of Citric Acid Soaking on *Cocoon*s of *Z. arugamensis*

*Cocoon*s of *Z. arugamensis* stuck to the bottom of the petri dish. *Cocoon*s were produced 2 hours after *Z. arugamensis* was placed in the petri dish. These results are in line with previously reported results by [15] that 30% of *Z. arugamensis* produced eggs after 2 hours of isolation and placed in a petri dish, and as many as 60-90% of *Z. arugamensis* produced *cocoon*s one hour later. The other 10-40% did not produce *cocoon*s allegedly because *Z. arugamensis* is still immature as seen from its size which is less than 10 mm (6-8.5 mm).

Z.arugamensis maintained in room temperature conditions of 29-32ºC experienced gradual death on each day. The decline in the condition of *Z. arugamensis* began on day 5 which looked weak and swam at the surface, did not attach the sucker to the bottom of the Petri dish, and on days 6-8, the leech began to die, with a peak on days 10 and 11 where *Z. arugamensis* was already in a state of death all in the treatment of immersion with citric acid 50-150 ppm and control. [25] mentioned that the survival period of adult and juvenile *Z. arugamensis* ranged from 11 to 16 days at 25°C, which was relatively longer than the 5-13 day period at 27-30°C and 35-40°C. The osmotic structuring of the sea leech *Z. arugamensis* does not rely on the life process of its host alone but also on the conditions of its rearing water when it is not attached to and nourished by fish [15]. However, immersion with a citric acid concentration of 400 ppm showed that all *Z. arugamensis* died after immersion.

*Cocoon*s produced in each petri dish varied (19-91 *cocoon*s) from 40-50 *Z. arugamensis* with an average diameter of 0.51±0.03 mm (n: 50). *Cocoon*s appeared to be still developing in the treatment of immersion with citric acid concentrations of 50-150 ppm and the control. This can be seen from the percentage of *cocoon* hatching in these treatments, although the percentage of hatching varies (Table 2). According to [1], [10] that eggs produced by leeches take 10-11 days to hatch larvae at 27ºC. *Z. arugamensis* lays multiple eggs, and the number of eggs produced will be different.

TABLE 2.
COCOON OF *Z. Arugamensis* (%) HATCHED AFTER SOAKING WITH CITRIC ACID FOR 14 DAYS OF OBSERVATION

Soaking time (minutes)	Citric Acid Concentration (ppm)					
	0	50	100	150	250	400
30	81,81	65,62	68,75	64,7	0	0
60	68,42	60	87,5	57,14	0	0
90	51,42	78,94	52,17	46,67	0	0
120	87	48	60	66,67	0	0
Average	72,16±15,89	63,14±12,84	67,11±15,19	58,80±9,07	0	0

Table 2. shows that the average percentage of *cocoon* hatching in the control is slightly higher (72.16 ± 15.89%) than the percentage of *cocoon* hatching with citric acid soaking concentrations of 50-150 ppm (58.80 ± 9.07-67.11 ± 15.19%). However, soaking *cocoon*s with citric acid concentrations of 250 and 400 ppm did not result in any developed *cocoon*s, as indicated by the absence of hatched *cocoon*s (0%). Observations under the microscope showed that *cocoon*s that developed after immersion with concentrations of 50-150 ppm and the control hatched after 12 to 14 days.

*Cocoon*s develop from the morula, blastula, gastrula, and embryo phases. The *cocoon* development phase is in accordance with the *cocoon* development phase. The morula to gastrula phase occurs for 3-5 days followed by the embryo phase until hatching on days 10-12 at a rearing temperature of 28-31°C and salinity of 34-35 ppt, but cannot hatch at 40°C [13, 17, 19, 25, 30]. *Cocoon*s in citric acid immersion at concentrations of 250 ppm and 400 ppm had no nucleus and did not develop until the end of the study. This is due to the strong influence of high citric acid, which does not allow *cocoon*s to develop to the embryonic stage.

*Cocoon*s in this study were maintained at room temperature ranging from 29-32°C with an average of 28.67±1.36°C. This value is in accordance with the life temperature of *Z. arugamensis*, because the prevalence of parasites can decrease with increasing temperature and the temperature range for grouper rearing is 25°C-27°C [13, 30].

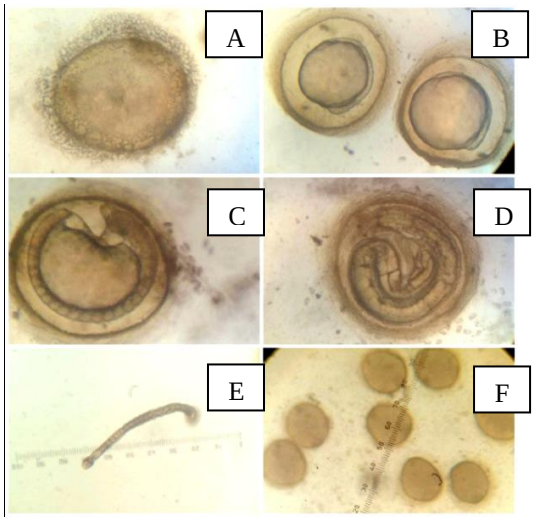

Fig. 1. A). *Cocoon* at early blastula stage in the control treatment reared on day 1 B). *Cocoon* at morula stage in the control treatment reared on day 2. C). *Cocoon* at the late gastrula stage in citric acid immersion concentration of 50 ppm on day 4. D). *Cocoon* at late embryonic stage in the control treatment on day 5 (2.5X). E). *Z. arugamensis* larvae that hatched in the control treatment on day 11. F). Unfertilized *cocoon*s in citric acid soaking at 400 ppm concentration. *400X magnification for *cocoon* observation.

Temperature observations of citric acid solution for immersion of *Z. arugamensis* and its *cocoon*s at concentrations of 100 to 400 ppm increased slightly (Table 3). The temperature of seawater used as a medium for citric acid mixture increased by 0.3-0.4°C (30.3-30.4°C) compared to the concentration of 50 ppm and the control (30°C). However, the citric acid solution did not affect the salinity of the seawater used (34 ppt) (Table 3).

TABLE 3.
SEAWATER QUALITY AFTER ADDING CITRIC ACID

Citric Acid Concentration (ppm)	Temperature (°C)	Salinity (ppt)	pH
0	30	34	8,35
50	30	34	6,73
100	30,3	34	6,05
150	30,3	34	5,23
250	30,3	34	4,09
400	30,4	34	3,79

Citric acid affects the pH of seawater, where the pH change is caused by the presence of different bioactive compounds with acidic properties. Heavy metal content in aquatic media can be reduced by citric acid because it affects chelation and changes the formation of citric acid in solution [11, 26].

High temperature affects the quality of rearing water, it is influenced by the volume of water in the petri dish used for rearing media. *Z. arugamensis* can live in various environments with extreme changes in temperature and oxygen [7, 15]. A high-salinity rearing environment causes *Z. arugamensis* to require additional energy and a lot of oxygen content. When oxygen levels are reduced, the sea leech will adapt by aerating the surface of its body by creating air bubbles on its dorsoventral body [2], [6].

Safety of Citric Acid Immersion for Hybrid Grouper Infected with *Z. arugamensis* (*In Vivo* Test)

The results of citric acid immersion at concentrations of 0, 150, 200, 250, and 300 ppm showed the condition of test fish infected with *Z. arugamensis*, which differed from normal levels to fish that looked weak but were still alive until the end of observation (Table 4). When the grouper was first put into a container containing citric acid concentrations, it showed rotating and struggling movements. The fish in all citric acid treatments were still alive with active tail and fin movements.

TABLE 4.
CONDITION OF CANTANG HIBRIDA CATFISH INFECTED WITH *Z. arugamensis* AFTER SOAKING WITH SITRAT ACID

Soaking Times (Minutes)	Citric Acid Concentration (ppm)				
	0	150	200	250	300
30	Normal	Normal	Normal	Normal	Normal
60	Normal	Normal	Normal	Normal	Weak

Table 4. shows citric acid immersion at concentrations of 150-200 ppm, the state of *Z. arugamensis* at 30 minutes of observation shows the condition will be released but it only hangs on the tip of the grouper fin. While at a concentration of 250 ppm, the leeches were released from the grouper's body but stuck to the bottom of the container. This indicates that the condition of *Z. arugamensis* is still alive. The piscicolid leech *Z. arugamensis* has been reported to have the ability to transmit hemogregarine and trypanosomes simultaneously among fish [4]. *Z. arugamensis* at concentrations of 150-200 ppm still showed protruding proboscysts on the anterior sucker. Under normal conditions, the sucker will protrude when the leech is about to suck nutrients from its host [23].

Fish body parts that are still infested with *Z. arugamensis* will experience bleeding and swelling of the skin, while the fins will become pebbles. Infestation of ectoparasitic worms in the gills results in hyperplasia and fusion of the gill lamellae. Affected host fish will be thickened/swollen and pale red in color [15, 19-21].

Z. arugamensis in citric acid immersion at a concentration of 300 ppm attached to the body of the grouper appears to be released to the bottom of the water in a shrunken and dead condition. The number of *Z. arugamensis* that were released and shrunken was 26 fish. The condition of fish in citric acid immersion at a concentration of 300 ppm after 30 minutes is still moving normally and is near aeration. However, after soaking for 60 minutes the grouper showed weak movement. According to [3], the higher the concentration of the solution used, the higher the oxygen consumption rate. Based on this, it shows that fish adapt to environmental conditions so fish move a lot and require more oxygen.

The oxygen consumption rate increased at the time of immersion at 60 minutes and 120 minutes. This is thought to be because the fish are stressed and require a lot of energy to adjust [5]. Seawater after adding citric acid (Table 5) showed the same quality in the *in vitro* test. These results indicate that citric acid solution made in large volume (2 liters) has similar water quality to citric acid solution made in small volume (200 mL).

TABLE 5.
WATER QUALITY TESTING *IN VIVO* TEST

Citric Acid Concentration (ppm)	Temperature (°C)	Salinity (ppt)	pH
0	29	35	8,15
150	29,2	35	5,74
200	29,2	35	4,70
250	29,3	35	4,17
300	29,5	35	3,94

Fish that experience stress due to citric acid immersion treatment will require high oxygen consumption, because of the respiration process that has an impact on increasing water temperature. The pH of seawater added with citric acid was seen to decrease sharply to 5.74 at a concentration of 150 ppm, because the higher the concentration of citric acid can make the lower the pH range [3], [8]. Citric acid in seawater at a concentration of 250-300 ppm was able to release *Z. arugamensis* from the body of the hybrid grouper, although it did not kill *Z. arugamensis* completely.

IV. CONCLUSION

From the results of the study it can be concluded that citric acid immersion at concentrations of 250-400 ppm *in vitro is* able to kill *Z. arugamensis* and *its cocoon*. Citric acid immersion up to a concentration of 300 ppm for 30 minutes is still safe for cantang hybrid grouper infected with *Z. arugamensis*. *Z. arugamensis* and *its cocoon* will die at pH ≤ 4.

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