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The antibacterial activity of clove *Syzygium aromaticum* extract and its effects on the survival rate of hybrid grouper *Epinephelus fuscoguttatus* ♀ × *E. lanceolatus* ♂ infected with *Vibrio alginolyticus*

Aktivitas antibakteri ekstrak cengkih *Syzygium aromaticum* dan pengaruhnya terhadap kelangsungan hidup ikan kerapu cantang *Epinephelus fuscoguttatus* ♀ × *E. lanceolatus* ♂ yang diinfeksi *Vibrio alginolyticus*

Inem Ode[1,2], Sukenda[1*], Widanarni[1], Dinamella Wahjuningrum[1], Munti Yuhana[1], Mia Setiawati[1]

[1]Department of Aquaculture, Faculty of Fisheries and Marine Science, IPB University, Bogor, West Java 16680, Indonesia
[2]Water Resource Management Study program, Faculty of Fisheries and Marine Sciences, Darussalam Ambon University, Ambon 97219, Indonesia
*Corresponding author: sukenda@apps.ipb.ac.id

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ABSTRACT

Vibrio alginolyticus which becomes the main cause of vibriosis disease in grouper culture and causes great economic loss in Asian aquaculture industries. This study investigated the antibacterial activity of clove extract and the effect of adding clove powder to the diet on survival "cantang" hybrid grouper infected with *V. alginolyticus*. The clove extraction used a maceration method. Two dose levels of clove powder were used, namely 10 and 15 g/kg. The control treatments without clove powder supplementation contained positive control (CP) and negative control treatment (CN). The results found that the clove extract contained five major compounds. The highest compound was phenol, 2-methoxy-4-(2-propenyl)-eugenol (64.07%). Phytochemical analysis of clove extract contained phenolics, flavonoids, and tannins at (28.53 ± 0.00) mg/g, (0.38 ± 0.00) mg/g, and (0.15 ± 0.00) %, respectively. The diameter of the clove extract inhibition zone was significantly different in all treatments. The scanning electron microscopy (SEM) result presents that the clove extract can alter the *V. alginolyticus* cell morphology. The dietary supplementation of clove powder improves the survival rate significantly higher ($P<0.05$) post-challenge test. The conclusion of this research is that clove extract has antibacterial activity that can inhibit growth and cause cell morphological damage to *V. alginolyticus*. The application of clove powder at a dose of 15 g/kg was able to improve the survival value post-challenge test.

Keywords: antibacterial activity, clove, grouper, phytochemical, *Vibrio alginolyticus*

ABSTRAK

Vibrio alginolyticus merupakan penyebab utama penyakit vibriosis pada budidaya ikan kerapu dan menyebabkan kerugian ekonomi yang besar pada industri akuakultur Asia. Penelitian ini mengkaji aktivitas antibakteri ekstrak cengkih dan pengaruh penambahan bubuk cengkih pada pakan terhadap kelangsungan hidup kerapu hibrida cantang yang terinfeksi *V. alginolyticus*. Ekstraksi cengkih menggunakan metode maserasi. Dua dosis bubuk cengkih yang digunakan yaitu 10 dan 15 g/kg. Perlakuan kontrol tanpa suplementasi bubuk cengkih terdiri dari kontrol positif (CP) dan kontrol negatif (CN). Hasil penelitian menemukan bahwa ekstrak cengkih mengandung lima senyawa utama. Senyawa tertinggi adalah fenol, 2-metoksi-4-(2-propenil)-eugenol (64,07%). Analisis fitokimia ekstrak cengkih mengandung fenol, flavonoid, dan tanin masing-masing sebesar (28,53 ± 0,00) mg/g, (0,38 ± 0,00) mg/g, dan (0,15 ± 0,00) %. Diameter zona hambat ekstrak cengkih berbeda nyata pada semua perlakuan. Hasil *scanning electron microscopy* (SEM) menunjukkan bahwa ekstrak cengkih dapat menyebabkan perubahan morfologi sel *V. alginolyticus*. Suplementasi bubuk cengkih dalam pakan meningkatkan kelangsungan hidup ikan kerapu cantang secara signifikan lebih tinggi setelah uji tantangan. Kesimpulan dari penelitian ini adalah ekstrak cengkih memiliki aktivitas antibakteri yang dapat menghambat pertumbuhan dan menyebabkan kerusakan morfologi sel *V. alginolyticus*. Aplikasi bubuk cengkih dengan dosis 15g/kg mampu meningkatkan nilai kelangsungan hidup setelah uji tantang.

Kata kunci: aktivitas antibakteri, cengkih, fitokimia, kerapu, *Vibrio alginolyticus*

INTRODUCTION

The grouper is an economically important aquaculture organism in global trade. Nowadays, the hybrid grouper is one of the main grouper species cultured in Indonesia and one of the exported fish products. Suhana (2020) stated that Indonesia is the world's main exporter of grouper, more than 90% of which was exported to the Hong Kong market of 165.87 tons in January 2020. Hybrid grouper is a commodity that is in demand by the export market, especially for the China and Hong Kong markets (Kumalaningrum *et al*., 2022). The main obstacle in grouper culture is mortality due to vibriosis disease attacks. The dominant vibriosis that attacks grouper is *V. alginolyticus* (Lee *et al*., 2020). *Vibrio alginolyticus* are rod-shaped motile Gram-negative bacteria abundant in seawater and estuary areas, which becomes the main cause of vibriosis disease in seawater culture and causes great economic loss in Asian aquaculture industries for several years (Zhou *et al*., 2020). Vibriosis is the main disease in fish and shrimp seawater culture in Indonesia as ported since the 1990s (Istiqomah *et al*., 2020).

The treatment action that is often taken to overcome this vibriosis attack is the use of antibiotics which are considered more applicable and practical. The negative impact of continuous use of antibiotics with inappropriate doses can cause bacterial resistance to these antibiotics. According to Manage (2018), excessive use of antibiotics can cause bacterial resistance, food safety hazards and environmental problems. One of the preventive measures that are relatively safe for fish, the environment, and humans as well as more economical and applicable is the administration of immunostimulants from plant materials. Plants have important phytochemicals that act as chemical protectors against microbial infections and as immunomodulators by increasing immune cell proliferation, modulating cytokines, and increasing antibodies (Lillehoj *et al*., 2018).

A clove *Syzygium aromaticum* is a native Indonesian spice plant that was first discovered in the Maluku Islands (Sohilait, 2015). Cloves contain bioactive compounds, namely 10-13% tannins, terpenoids, glycosides, and 14-21% essential oils. Essential oil compounds contain eugenol, caryophyllene, furfural, vanillin, methyl salicylate, pyrocatechol, methyl ketone, & valeric aldehydes, eugenin, isoeugenitol, isoeugenitin, eugenitin, tannin, mucilage, sitosterol, estigmaterol, resins, oleanolic acid, resins, cellulose, and fixed oil. Clove has a strategic role in various industries including the pharmaceutical, cosmetic, food and beverage, cigarettes, vegetable pesticides, fisheries, active packaging, and other chemical industries (Cortés-Rojas *et al*., 2014). Wael *et al*. (2018) stated the content of clove compounds can induce specific and non-specific immunity and activate cellular components of the immune system, such as phagocytic functions without affecting both humoral and cellular immunity.

Previous studies have shown that eugenol is the main component of cloves that has antibacterial activity (Sutili *et al*., 2014; Islamuddin *et al*., 2016; Hariyadi *et al*., 2020; Pathirana *et al*., 2019), antioxidant and inhibiting lipid peroxide (Ghadermazi *et al*., 2017; Dibazar *et al*., 2015), and anti-inflammatory (Batiha *et al*., 2020), thereforein this study, propose their use to control *Vibrio alginolyticus* which causes vibriosis in cantang groupers. expected to produce natural products that can control vibriosis disease in marine fish farming. This study aimed to evaluate the antibacterial activity of clove extract and the effect of clove powder addition into the diet on survival "cantang" hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *E. lanceolatus* ♂) infected with *V. alginolyticus.*

MATERIALS AND METHODS

Clove extract preparation

Dried clove was obtained from Mardika Market, Ambon, Maluku Province. The clove was identified in the Center for Biological Research, Indonesian Institute of Sciences (LIPI) (No. 1114/IPH.1.01/If.07/XI/2020). Clove was extracted by using 96% ethanol through the maceration method (Adeshina *et al*., 2019). The clove extract was produced by measuring 50 g of clove powder then was extracted using 96% ethanol solvent at 1:5 ratio (w/v). Furthermore, the extracted sample was macerated for 24 hours, while being stirred using the magnetic stirrer at room temperature. The maceration product was filtered using the Whatman no. 41-filter paper. The unfiltered sample was re-macerated twice with the same method. The macerated production was concentrated by the vacuum evaporator at 30–45 °C and preserved at -20°C for further steps.

Clove extract analysis

Clove extract analysis with the gas

chromatography-mass spectrometry (GC-MS). The analysis used GC-MS 5973 Pyrolysis, Agilent Technology equipment. The clove extract was dissolved in ethanol at 1:1 ratio (w/v). The sample injection volume was 3 µL with the HP-5MS column (30 m in length, 0.5 mm diameter, and 0.25 µm width). The helium gas (99.999‰) was used as a carrying gas at a total flowing of 104 µL/ minutes with 30 minutes at an oven temperature of 50 °C, injector temperature of 290 °C, and aux temperature of 290 °C. The total compounds obtained were presented from the number of peaks in the chromatogram. The compound names found were interpreted based on the mass spectrum data from each peak that were matched with the GC-MS Pyrolysis database. The total phenolic content of clove extract was measured using the spectrophotometric method (Munaeni *et al*., 2019). The flavonoid content analysis was performed using the spectrophotometric method and the tannin content analysis was performed using the titrimetric method (Mayur *et al*., 2010).

Bacterial preparation

V. alginolyticus bacteria were obtained from Brackish Water Aquaculture Development Center, Jepara, Indonesia. *V. alginolyticus* were isolated from "cantang" grouper which had clinical symptoms. *V. alginolyticus* was identified physiologically and biochemically using standard bacteriological methods. The polymerase chain reaction (PCR) method was used for the verification of *V. alginolyticus* using the primers F-gyrB 5'-ATT GAG AAC CCG ACA GAA GCG AAG-3' and R-gyrB 5'-CCT AAT GCG GTG ATC AGT GTT ACT-3 (Zhou *et al*., 2007).

Antibacterial activity

The antibacterial activity test of clove extract was performed by using the Kirby-Bauer agar diffusion method (Lay, 1994). The clove extract was diluted with the phosphate buffer saline (PBS) solution at 0.5, 1, 5, 10, 15, 25, 50, 100 mg/mL (w/v). The 0.05 mL *V. alginolyticus* at 10^8 CFU/mL concentration was spread on seawater complete (SWC) agar media. The paper disk at 6 mm diameter with a 15 µL absorption level was soaked for five minutes on each concentration of clove extraction, before being attached to the SWC agar medium. The negative control used the 5% dimethyl sulfoxide solution (DMSO), while the positive control used 30 µg/mL oxytetracycline, each of which was repeated three times. The media were incubated for 24 hours at 37 °C for 24 hours. The antibacterial activity of clove extract was measured based on the inhibitory zone formed around the paper disk. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were determined using the dilution method (Tortora *et al*., 2016).

***Vibrio alginolyticus* cell damage**

The *V. alginolyticus* membrane cell disruption was observed with scanning electron microscopy (SEM). The bacteria's morphological and structural damage patterns were observed, namely general appearance, cell size, cell-wall damage, and cytoplasmic damage. The method used to prepare the samples for SEM observation was based on (Bozzola & Russel, 1999) method. The 24-hour clove extract exposed bacterial pure cell suspension was centrifuged at 3500 rpm for 15 minutes. The specimen was washed with phosphate buffer solution and separated from the liquid after centrifugation, which was repeated twice. The filtrate was removed and added with 2.5% glutaraldehyde (pH of 7.3), then stood for two hours. The filtrate was fixated with 1% osmium tetroxide in 0.05% cacodylate buffer, pH of 7.2 for two hours, then washed with dd-H_2O three times, each of which was for two minutes. The specimen was washed with phosphate buffer solution twice, and the liquid was removed and added with serial ethanol concentrations (25%, 50%, 75%, and 100%), each of which was repeated three times for 10 minutes. Furthermore, samples were attached to the aluminum stubs through the vacuum process (6-7 Pa) for 20 minutes. The samples were observed using the SEM JEOL 5310.

***V. alginolyticus* challenge test**

This study was performed on July 2021 and located in the laboratory of Fisheries and Marine Observation Station (IFMOS), IPB University Bogor in Ancol, Jakarta. Cantang groupers were obtained from Brackish Water Aquaculture Development Center, Situbondo, East Java, Indonesia. The fish were maintained in glass aquarium sized of 60×40×40 cm (70 L volume) distributed in 16 aquariums. Cantang hybrid grouper used for treatments had an average weight size of 21.6 ± 1.93 g and the average length size of 10.82 ± 1.36 cm with the stocking density of 11 fish per aquarium. The present study fulfilled ethical requirements considering the welfare of the test animals and received prior ethical approval from the IPB University animal ethics

commission (ethical approval number 198–2021 IPB).

This study used a completely randomized design containing four treatments and four replications. The treatments used contained two levels, namely clove powder doses at 10 g and 15 g/kg, and control without clove powder supplementation containing the positive control (CP) and negative control (CN). The dose of clove powder used was converted from minimum bactericidal concentration (MBC) resulting in the antibacterial activity test. Feed production used a re-pelleting method. Commercial feed is made into powder, mixed with clove powder according to the treatment dose, then 400 ml/kg feed is added with water. Then the pellets were printed and dried in an oven for three hours at 50°C. Pellet feed is packaged in plastic containers and labeled according to treatment. Feeding was performed twice a day for 25 days with satiation at 08.00 and 16.00 GMT+7. On the 15th day, Cantang grouper was injected with 10^7 CFU/mL (LD_{50}) *V. alginolyticus* at 0.1 mL dose, intramuscularly. In the negative control, cantang grouper was injected with a phosphate buffer saline (PBS). Fish were reared for 25 days.

Survival rate and fish mortality

The survival rate (SR) was determined by using the following formula:

$$SR = \frac{Final\ number\ of\ fish}{Initial\ number\ of\ fish} \times 100$$

Fish mortality and clinical symptoms were observed every day after the challenge test.

Histopathology of the livers

The histopathology of the livers was observed after the challenge test. The liver tissue was collected and fixed with Davidson's solution for 24–72 h. These were then sliced into 1 × 1 cm squares with a thickness of 3–5 mm. This was followed by the dehydration, clearing, embedding, blocking paraffin, sectioning, and staining phases. The stain used was hematoxylin and eosin (H&E). The results were observed under a microscope at 40× magnification.

Total bacterial count (TBC) and total Vibrio count (TVC) in liver and kidney

The fish body surface was disinfected with 95% ethanol and rinsed with sterile distilled water. Fish was surged and taken its liver and kidney organ, before mashing them until refined. The liver and kidney were taken at 0.1 g and homogenized with 0.9 mL PBS solution, before spreading to the agar media. The SWC agar media as substrates for TBC, while the thiosulfate citrate bile salt sucrose (TCBS) agar media as substrates were used for TVC. Media containing the organ sample solutions were incubated at 28ºC for 24 hours. After 24 hours, the total bacterial colonies were formed and counted on the media. The total bacteria were calculated using the spread plate count method with the following formula:

$$\sum \text{bacteria} = \frac{N}{\sum \text{speared}} \times \frac{1}{f}$$

Σ bacteria = Number of bacterial cells (CFU/g)
N = Number of bacterial colonies
f = Diluent factor

Data analysis

The study data were analyzed qualitatively and quantitatively. The statistical analysis uses a one-way analysis of variance (one-way ANOVA) with the statistical program software system (SPSS) ver 20.0. All treatments with a significant difference ($p<0.05$), were subsequently analyzed with Duncan's multiple range test.

RESULTS AND DISCUSSION

Result

Clove extract analysis

Compounds of clove extract by GC-MS pyrolysis are shown in Table 1. Clove extract contained five main compounds. The highest compounds found in the result were phenols, namely 2-methoxy-4-(2-propenyl)-Eugenol (64.07%). The quantitative analysis of clove extracts phytochemical compounds obtained the value of total phenolic compounds in clove extract was 28.53 ± 0.00 mg/g, followed by the total flavonoids of 0.38 ± 0.00 mg/g, and tannins of 0.15 ± 0.00%.

Antibacterial activity

Figure 1 and Table 2 showed the inhibition zone of different clove extract concentrations against *V. alginolyticus.* The clove extract could inhibit the growth of *V. alginolyticus*. The clove extract of 100 mg/mL obtained the greatest inhibition zone at 12.9 ± 0.91 mm. The 0.5 mg/mL concentration was bacteriostatic compared to

other concentrations, which were characterized as bactericidal. The inhibition zone diameter of clove extract was significantly different (P<0.05) from the positive and negative controls, except for the 0.5 mg/mL concentration, which was insignificantly different from the negative control. The MIC value of clove extract on the *V. alginolyticus* growth was obtained from the 0.5 mg/mL concentration based on the clear solution with the presence of *V. alginolyticus* colony. The MBC value of clove extract on the *V. alginolyticus* was obtained from the 1 mg/mL concentration marked by the *V. alginolyticus* colony growth absence.

Table 1. Compounds of clove extract by GC-MS pyrolysis.

Real time (mins)	Chemical compound	Area (%)	Compound group	Function	References
8.361	Phenol, 2-methoxy-4-(2-propenyl)-Eugenol	64.07	Phenols	Antibacterial agent. Inhibiting the migration, adhesion, and virulence expression factor of *E. coli* (O157:H7) bacteria. Inhibiting the formation of *Staphylococcus aureus* biofilm. Antioxidant, inhibiting the lipid peroxidation. Inhibiting the prostate cancer cell and apoptosis.	Pathirana *et al*., 2019 Baskaran *et al*., 2016 (Yadav *et al*., 2015) (Ghadermazi *et al*., 2017) (Batiha *et al*., 2020; Gokalp, 2016)
8.521	Alfa-Copaene	0.32	Terpenoids	Antimicrobial agent. Antiproliferative, antioxidant, antigenotoxic, cytotoxic activities.	(Martins *et al*., 2015) (Türkez *et al*., 2013)
8.945	Caryophyllene	6.60	Terpenoids	Anti-inflammatory Anticancer, antimicrobial, antioxidant	(Varga *et al*., 2018) (Dahham *et al*., 2015)
9.230	Alpha Humulene	0.71	Terpenoids	Anti-inflammatory, anticancer, antioxidant and antitumor activity	(Yin *et al.,* 2019; González *et al*., 2021)
9.758	Phenol, 2-methoxy-4-(2-propenyl), acetate	28.30	Phenols	Antimicrobial and antioxidant	(Vanin *et al*., 2014)

Table 2. The antibacterial activity of different clove extract concentrations on *V.alginolyticus.*

Concentration (mg/mL)	Inhibition zone diameter (mm)
0.5	0.00^{a}
1	0.8 ± 0.32^{b}
5	3.2 ± 0.13^{c}
10	3.6 ± 0.82^{c}
15	5.3 ± 0.55^{d}
25	6.5 ± 0.48^{e}
50	8.5 ± 0.64^{f}
100	12.9 ± 0.91^{g}
Oxytetracyclin (Oxy) (30 µg/mL)	10 ± 0.67^{h}
Dimethylsulfoxide (DMSO) 5%	0.00^{a}

Note: Data are mean ± standard deviation of three replicates. Different letters over each treatment row indicates a significant difference (P< 0.05; Duncan's test).

V. alginolyticus cell damage

The SEM result presents that the clove extract causes the morphological alteration in *V. alginolyticus* cells. As the clove extract was absent, the *V. alginolyticus* cells had a small rod shape that colonized into one group with a complete cell surface. Meanwhile, at the 0.5 mg/mL clove extract concentration, half of the cell parts were still complete and adhesive, while several cells occurred a morphological alteration. At the 5 and 15 mg/mL clove extract concentrations, the bacterial cells had irregular shapes, followed by fragmented and aggregated cell damage as part of the cells was damaged, shown by the yellow arrow in Figure 2. This result indicates that the clove extract causes the *V. alginolyticus* cell damage. The positive control treatment using the 30 µg/mL oxytetracycline caused the *V. alginolyticus* to wrinkle, adhere, and shrink.

Survival rate and fish mortality

Figure 3 showed the survival rate of cantang grouper challenged with *V. alginolyticus.* The clove powder supplemented feed obtained a better survival rate than the positive control (CP). The statistical test showed that all treatments obtained a significant influence (P≤0.05) on the survival rate value. Mortality occurred on two days post-challenge test in the positive control, while feed supplemented with clove powder on a 3- days post-

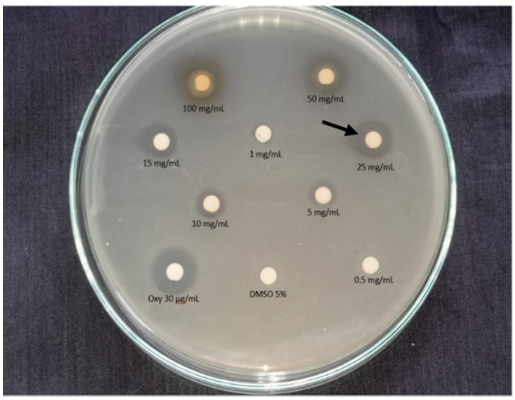

Figure 1. Inhibition zone of different clove extract concentrations against *V. alginolyticus.* The arrow showed inhibition zone (0.5–100 mg/mL: clove extract concentrations. Oxy: oxytetracyclin. DMSO: dimethylsulfoxid).

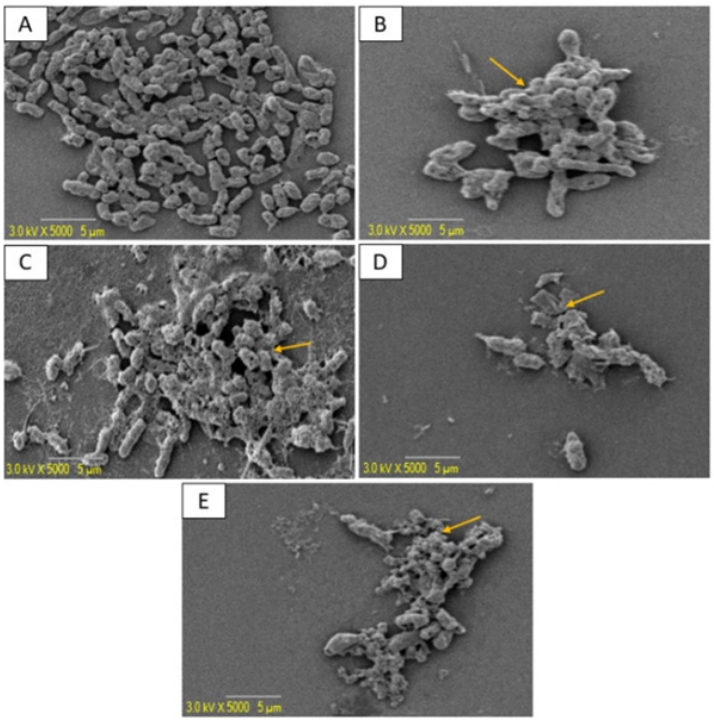

Figure 2. The *V. alginolyticus* morphology after being exposed by the clove extract. The arrows showed cell membrane damages. Negative control/PBS (a), positive control/ 30 µg/mL oxytetracycline (b), clove extract concentrations: 0.5 mg/mL (c), 5 mg/mL (d), and 15 mg/mL (e).

challenge test. Mortality pattern occurred until a 7- day post-challenge test in the positive control (CP) and 15 g treatment, while 10 g treatment still occurred until an 8 days post-challenge test (Figure 4). The highest mortality percentage occurred on a 4- to 6- days post-challenge test.

Clinical symptoms

On the first and second day after the challenge test, the fish feeding response decreased, followed by inactive movement, remaining in the container base, and loss of balance found in some fish, as these conditions occurred in all treatments except the negative control. Figure 5 shows morphological changes post the challenge test on infected fish (positive controls, 10 g, and 15 g treatments), namely lesions on the operculum to the abdomen, wounds on the abdomen and damage on the fins. Uninfected fish (negative control), no clinical symptoms occurred.

Histopathology of liver of cantang grouper infected with V. alginolyticus

The observation of histopathological change in the liver of cantang grouper on day three of post-challenge is shown in Figure 6. The histopathological change in the liver in the positive control, hydropic degeneration (hepatocyte enlargement) and necrosis. The 10 g, and 15 g of treatment was fatty degeneration. More damage

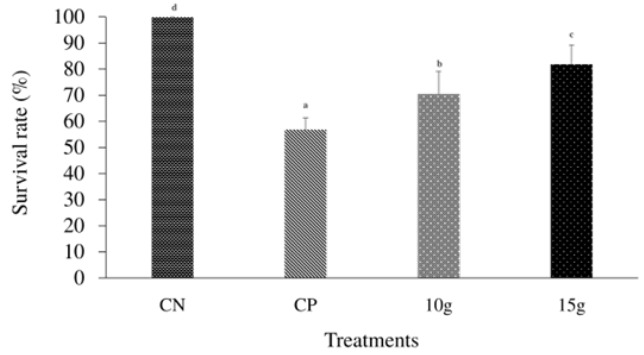

Figure 3. Survival of cantang grouper challenged with *V. alginolyticus* via intramuscular injection. Negative control (CN), positive control (CP), clove powder dose: (10 g), (15 g). Data are mean ± SD, different letters over each treatment bar indicate a significant difference (P<0.05; Duncan's test).

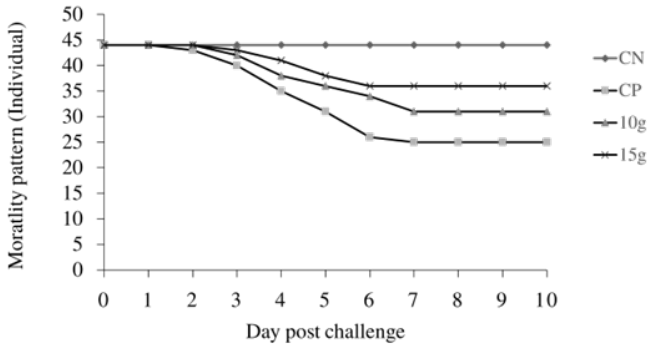

Figure 4. Mortality pattern of "cantang" hybrid grouper challenged with *V. alginolyticus*. Negative control (CN), positive control (CP), clove powder dose: (10 g), (15 g).

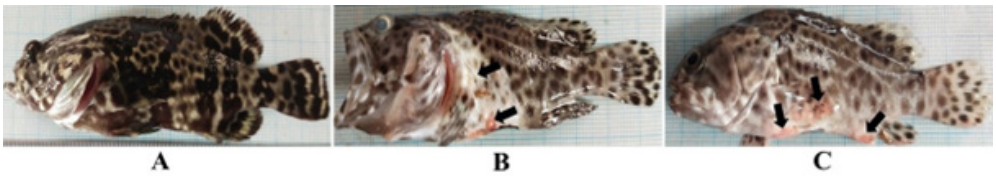

Figure 5. **"**Cantang" hybrid grouper morphological changes post challenge test. Uninfected fish (A), infected fish (B, C). Lesions on the operculum to the abdomen, wounds on the abdomen (B), damage on the fins (C).

occurred in the treatment without clove powder supplementation.

Total bacterial count (TBC) and Total Vibrio count (TVC) in the liver and kidney of cantang grouper

Table 3 shows the total bacterial count and total *Vibrio* count in the liver and kidney of cantang grouper on day 3 of the post-challenge test. The dietary supplementation of 15 g/kg clove powder resulted in significantly lower total Vibrio counts ($P<0.05$) in liver and kidney than positive control.

Discussion

The inhibitory activity of *V. alginolyticus* by clove extract was related to the secondary metabolites. Eugenol is the main metabolite bioactive in cloves demonstrating the inhibitory activity of Gram-negative and Gram-positive bacteria, as their mechanisms are associated with biofilm synthesis, virulence factor gene expression, migration, and adhesion inhibitions (Hu *et al*., 2018). This study indicates that the inhibition zone and cell damage level of *V. alginolyticus* depend on the clove extract concentration. The SEM presents cell morphological alteration due to the antibacterial activity of clove extract against *V. alginolyticus*. It indicated that without the clove extract exposure, the *V. alginolyticus* cells have a short rod shape that colonizes in a group with a complete cell surface.

The 15 mg/mL clove extract concentration caused the most serious damage compared to other concentrations. The *V. alginolyticus* cells present morphological alterations; irregular shape, fragmentation, and damaged cell

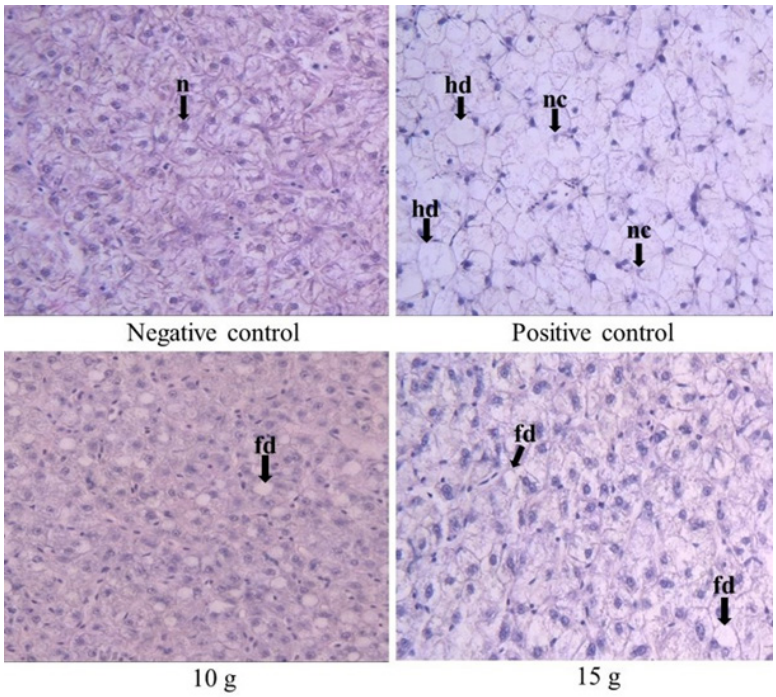

Figure 6. Histopathological changes of liver of cantang grouper on day 3 post challenge test. Negative control, positive control, treatment (10g) and and (15g). Note: (n) normal hepatocyte; (hd) hydropic degeneration; (nc) necrosis; (fd) fatty degeneration. The stain used was hematoxylin and eosin (H&E); magnification × 40 (scale bar: 50 μm).

Table 3. Total bacterial count (TBC) and Total *Vibrio* Count (TVC) in liver and kidney of "cantang" hybrid grouper on day three post-challenge test.

Parameter (Log 10 CFU/g)	Treatments			
	CN	CP	10g	15g
TBC (Liver)	6.47 ± 0.08^{d}	5.53 ± 0.06^{a}	5.66 ± 0.06^{b}	5.81 ± 0.03^{c}
TVC (Liver)	2.34 ± 0.04^{a}	2.58 ± 0.04^{bc}	2.64 ± 0.06^{c}	2.48 ± 0.12^{a}
TBC (Kidney)	5.62 ± 0.01^{a}	6.70 ± 0.02^{c}	5.80 ± 0.05^{b}	6.76 ± 0.05^{c}
TVC (Kidney)	2.40 ± 0.06^{a}	3.39 ± 0.06^{d}	3.26 ± 0.03^{c}	2.54 ± 0.03^{b}

Note: negative control (CN), positive control (CP), clove powder dose: (10 g), (15 g). Values are presented as mean ± standard deviation. Different superscript letters in the same line show a significantly different effect ($P<0.05$).

aggregation, followed by the presence of damaged cell parts. Eugenol possess a free hydroxyl group in the molecule which is mostly responsible for antimicrobial activity. Several authors reported that eugenol could change cell morphology, disrupt cell wall, alter membrane permeability, and exerting comprehensive damage to various cellular contents, eventually leading to cell death (Ashrafudoulla *et al.,* 2020).

Feed supplemented with clove powder could increase the survival rate of hybrid grouper against the *V. alginolyticus* infection. The results of previous studies showed that the addition of clove oil with a concentration of 3% (equivalent to 3 g/100 g) in tilapia feed, could increase the survival rate up to 100% after being challenged with *Lactococcus garvieae* (Rattanachaikunsopon & Phumkhachorn, 2009). Clove extract in feed was able to increase the survival rate of African catfish after being challenged with *Aeromonas sobria* (Ghaly *et al*., 2015). Clove powder supplementation treatment could reduce these organ damages. Cell damage can be avoided because of the presence of eugenol in clove powder which can increase lymphocyte proliferation and macrophage production (Wahhab & Aly, 2005). Clove powder 3% in feed can protect *Oreochromis niloticus* from pathological changes due to *Proteus mirabilis* infection (Rahman *et al*., 2018). The phenolic compounds in cloves have antioxidant effects that can protect rat liver from aflatoxins (Intan *et al*., 2020).

The use of 15 g/kg clove powder in this study reduced the *Vibrio* population in the liver and kidney on day 3 of the post-challenge test. The decrease in the number of vibrios compared to controls was due to one of the effects of adding clove powder to inhibit bacterial growth. The plant bioactive components played a role as a chemical protector against microbial infections and an immunomodulator by enhancing the immune cell proliferation, cytokine modulation, and antibody (Lillehoj *et al*., 2018). Herbs and medicinal plants are promising to be an important source of therapeutics in fish culture since these products provide a cheaper source for treatment and greater accuracy without causing toxicity (Madhuri *et al*., 2012).

The present study demonstrated new properties of clove extract as a potent antibacterial agent, it can be used in aquafarming as an alternative to vaccines, antibiotics, and chemical drugs. However, further research is needed to find the optimum dose of clove powder as a feed supplement and its effect on the growth and health status of fish.

CONCLUSION

This study revealed that the phytochemical compounds of clove extract could inhibit the growth and cause cell morphological damage of *V. alginolyticus*. The application of clove powder in feed a dose of 15g/kg was able to improve the survival value which was a higher post-challenge test with *V. alginolyticus.*

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