

Full Length Research Paper

# Assessment of synergistic efficacy of carvacrol and cymene against *Edwardsiella tarda* *in vitro* and in Tilapia (*Oreochromis niloticus*)

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Carvacrol, a major compound naturally present in oregano and thyme and its precursor, cymene, were tested *in vitro* for their antimicrobial activity against *Edwardsiella tarda*, a fish pathogenic bacteria causing edwardsiellosis. When used alone, carvacrol, but not cymene, inhibited the bacterium with the minimal inhibitory concentration (MIC) of 20 ppm. However, cymene was shown to be able to enhance the inhibitory ability of carvacrol indicated by the reduction of MIC to 5 ppm when used with 2.5 ppm of cymene. Based on mortalities in 2 weeks after intraperitoneal *E. tarda* injection, the median lethal dose (LD<sub>50</sub>) of *E. tarda* for tilapia (*Oreochromis niloticus*) was  $5.0 \times 10^2$  CFU/g of fish. Fish diets supplemented with carvacrol and cymene were also tested for their protective effect against *E. tarda* infection in tilapia. The results showed that carvacrol (200 ppm) but not cymene (200 ppm) when fed prophylactically decreased the mortality in *E. tarda*-challenged tilapia. However, carvacrol at the same concentration could cause no mortality of *E. tarda* infected fish when used in conjunction with 200 ppm of cymene.

**Key words:** Carvacrol, cymene, *Edwardsiella tarda*, tilapia.

## INTRODUCTION

*Edwardsiella tarda* is a bacterium of the family Enterobacteriaceae described by Ewing et al. (1965). *E. tarda* is Gram-negative, motile, non-capsulated, facultative anaerobic bacillus and has been recognized as an important pathogen causing edwardsiellosis in various commercial fish species such as chinook salmon (*Oncorhynchus tshawytscha*) (Amandi et al., 1982), eel (*Anguilla japonica*) (Minagawa et al., 1983), flounder (*Paralichthys olivaceus*) (Nagatogawa, 1983), channel catfish (*Ictalurus punctatus*) (Meyer and Bullock, 1973), carp (*Cyprinus carpio*) (Sae Oui et al., 1984), turbot (*Scophthalmus maximus*) (Nougayrede et al., 1994; Castro et al., 2008), and tilapia (*Oreochromis niloticus*) (Clavijo et al., 2002). *E. tarda* infection causes massive mortalities and large economic losses in natural environment and fish farming worldwide (Plumb, 1993). The major characteristics of edwardsiellosis include

systemic inflammatory response, generalized septicemia and eventual death. Environmental stresses such as overcrowding, malnutrition, sudden change of water temperature, pH and fluctuations in dissolved oxygen primarily contribute to the onset and severity of *E. tarda* infections in fish (Plumb, 1993). The virulence of *E. tarda* is demonstrated by its ability to protect itself to not be destroyed by the immune system of the host, to invade host cells and tissues and to produce dermatoxins and haemolysin causing disseminated septicemia conditions (Srinivasa et al., 2003).

Chemotherapy is the effective method commonly used to protect fish from edwardsiellosis. Presently, although a number of antibiotics such as oxytetracycline, norfloxacin, ciprofloxacin, gentamicin, chloramphenicol, cefazolin and aztreonam have been proven to be successful in controlling the infection, these have their own disadvantages such as the development of disease-resistant strains, high cost and dose problems as well as indiscriminate use by aquafarmers (Mohanty and Sahoo, 2007). Hence, there is a need to develop alternative approaches to control the infection.

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Recently, plant extracts and their compounds have been widely employed to protect fish from various infections. They have been shown to have antimicrobial activity against a variety of fish pathogenic bacteria. Moreover, several of them have been used to control diseases in fish such as streptococcosis (Abutbul et al., 2004; Rattanachaikunsopon and Phumkhachorn, 2007; Pachanawan et al., 2008; Rattanachaikunsopon and Phumkhachorn, 2009) and motile aeromonas septicemia (Pachanawan et al., 2008). Carvacrol is a major component of oregano and thyme essential oils. It has been shown to inhibit many strains of food-borne pathogenic bacteria (Burt, 2004). Synergism between carvacrol and its biological precursor, cymene has been observed against *Bacillus cereus* (Ultee et al., 2000) and *Escherichia coli* O157:H7 (Kisko and Roller, 2005) in rice and apple juice respectively. However, evidence on antimicrobial activity carvacrol and cymene against fish pathogenic bacteria has never been reported.

In this study, carvacrol and cymene were examined for antimicrobial activity against *E. tarda* both individually and in combination. This work also evaluated the effect of carvacrol and/or cymene in fish diets when administered prophylactically as well as post-challenge on tilapia intraperitoneally infected with *E. tarda*.

## MATERIALS AND METHODS

### Bacterial strain and culture conditions

The *E. tarda* used in this study was *E. tarda* PP00124 isolated from naturally infected tilapia in a fish farm in Ubon Ratchathani Province, Thailand. The identity of the bacterial strains was confirmed by polymerase chain reaction using 2 species-specific primers, Eta2-351 (5' TAGGGAGGAAGGTGTGAA 3'), and EtaEdwsp-780r (5' CTCTAGCTTGCCAGTCTT 3') according to the method described by Baird et al. (2003). Bacterial cultures were made on Trypticase soy agar (TSA) and in Trypticase soy broth (TSB) (Difco, Detroit, MI, USA) and incubated at 25°C. The bacterial stock culture was stored as a frozen culture at -80°C in TSB containing 20% glycerol (v/v).

### Chemicals

Carvacrol and cymene were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Solutions of these compounds were prepared in 95% ethanol.

### Determination of minimal inhibitory concentration (MIC)

Antimicrobial activity of different concentrations of carvacrol and cymene was studied *in vitro* against *E. tarda*. Stock solution (10.24 mg/ml) of carvacrol and cymene were subjected to a twofold serial dilution in a 96-well microtiter plate (Becton Dickinson, NJ, USA) containing TSB broth at the volume of 198 l per well. The concentrations of each compound obtained from the serial dilution ranged from 5120 to 1.25 g/ml. Two l of *E. tarda* suspension with the concentration of  $10^6$  CFU/ml (determined by  $OD_{600nm}$  which was equivalent to 0.2) was added to the wells containing the diluted compounds. Therefore, each well of the microtiter plate contained

$10^4$  CFU/ml of the *E. tarda* cells.

Positive and negative controls were included for each plate. Positive control was the bacterial culture at the concentration of  $10^4$  CFU/ml without test compounds while negative control was TSB broth without the test bacterium and compounds. After incubation at 25°C, bacterial growth was determined at 24 h by measuring absorbance at 600 nm using the EL x 800 universal microplate reader (Biotek Instrument Inc., Highland Park, Vermont, USA). The MIC of each compound was defined as the lowest concentration causing the decrease of the absorbance when compared with the positive control. Three replicates were performed for each concentration of the compounds.

### *In vitro* examination of synergistic effect of carvacrol and cymene

A series of combinations of carvacrol and cymene used are presented in Table 1. The concentrations of carvacrol used here were 0, 2.5, 5, 10 and 20 ppm while those of cymene used here were 0, 2.5, 5 and 10 ppm. Each combination (100 µl) was added to 4.9 ml of *E. tarda* culture ( $10^3$  CFU/ml). The optical density at 600 nm was determined after incubation at 25°C for 24 h. Each combination of carvacrol and cymene was tested three times.

### Fish preparation

Tilapia (*Oreochromis niloticus*) was obtained from "Nong Khon" Farm, Ubon Ratchatnai Province, Thailand. To acclimate to laboratory conditions, fish were maintained in 150 l plastic tanks for two weeks prior to experiments at 25°C with 12-h light/12-h dark photoperiod. They were fed 5% body weight twice a day with a commercial fish diet "C. P. CLASSIC" (S. W. T. Co., Ltd., Bangkok, Thailand). Fish weighing  $10 \pm 1$  g were transferred to 50 l aquaria (10 fish per aquarium) 24 h prior to the experiments. Water quality parameters were maintained within the following ranges; dissolved oxygen concentration  $6.0 \pm 0.6$  mg/l, temperature  $25.0 \pm 1.5$ °C, pH  $7.0 \pm 0.5$ , total hardness  $34.5 \pm 1.8$  mg/l as CaCO<sub>3</sub>, alkalinity  $29.5 \pm 2.0$  mg/l as CaCO<sub>3</sub>, ammonia less than 0.02 mg/l and nitrite less than 0.5 mg/l.

### Determination of median lethal dose (LD<sub>50</sub>) of *E. tarda*

An overnight culture of *E. tarda* was centrifuged at 5,000 rpm for 10 min. Bacterial cells were washed twice with physiological saline and then resuspended in the same solution to obtain a bacterial suspension with the concentration of  $10^8$  CFU/ml. The bacterial suspension was subjected to ten-fold serial dilutions and then used to challenge groups of 10 fish. One hundred l of each dilution was injected intraperitoneally into each fish. For control, the same volume of physiological saline was used instead of the bacterial suspension. Each dilution trial was performed in five replicates. Mortalities were recorded daily for 2 weeks. Dead fish were removed from the aquaria daily. Livers and kidneys were aseptically streaked on TSA. After incubation at 25°C for 24 h, colonies grown on the agar were confirmed to be *E. tarda* by using PCR as described earlier. The LD<sub>50</sub> value was calculated by the method described by Reed and Muench (1938).

### Preparation of fish diets

Fish diets supplemented with carvacrol, cymene and combinations of carvacrol and cymene were prepared. For the diets supplemented with carvacrol alone (Diet 1) and cymene alone (Diet 2), each compound was added to the commercial fish diet to obtain

**Table 1.** Effect of carvacrol and cymene on absorbance at 600 nm of the *E. tarda* cultures grown in the presence of different combinations of both compounds for 24 h.

Concentration of carvacrol (ppm)	Concentration of cymene (ppm)			
	0	2.5	5	10
0	0.45 ± 0.01	0.44 ± 0.02	0.45 ± 0.03	0.45 ± 0.02
2.5	0.27 ± 0.01	0.18 ± 0.03	0.18 ± 0.01	0.18 ± 0.02
5	0.14 ± 0.02	0	0	0
10	0.08 ± 0.01	0	0	0
20	0	0	0	0

Results are mean ± SD values of three replicates.

the final concentration of 200 ppm. For the diets supplemented with carvacrol together with cymene (Diet 3), both compounds (200 ppm each) were added simultaneously to the commercial fish diet. Each fish diet mixture was mixed with distilled deionized water (1 ml/g) until a homogenous mixture was obtained. The mixture was passed through a minced-meat processing machine, producing extruded strings, which were dried at 30°C for 24 h and then broken down to about 2-mm-long pellets. The control fish diets were prepared using the same process as the other fish diets except adding oxytetracycline (100 ppm) for the positive control fish diet (Diet 4) and adding no additive for the negative control fish diet (Diet 5).

#### Test for adverse effect of supplemented fish diets

Groups of 10 fish free from bacterial challenge were fed 5% body weight twice a day with all of the supplemented fish diets and the control fish diets used in this study for 2 weeks. Mortality, appearance, feeding response, and behavioral alterations of the fish were observed daily. The feeding response was assessed by determining the percentage weight gain of fish reared with the supplemented and control fish diets. Each feeding was performed in five replicates.

#### Examination of protective effect of the supplemented fish diets against *E. tarda* infection

*In vivo* studies of the protective effect of fish diets supplemented with carvacrol, cymene and combinations of carvacrol and cymene on *E. tarda* infection of tilapia were performed. Groups of 10 uninfected fish were fed 5% body weight twice a day with all of the supplemented fish diets separately for 5 days. On the sixth day, fish were challenged with *E. tarda* by intraperitoneal injection of 100 µl of bacterial suspension, at a dose causing 50% mortality (LD<sub>50</sub>).

The infected fish were reared for another 14 days at the same rate with the assigned fish diets. Mortality, appearance, feeding response, and behavioral alterations of the fish were observed daily. Dead fish were removed from the aquaria daily and their livers and kidneys were subjected to bacterial isolation on TSA. *E. tarda* isolated from the dead fish were confirmed by using PCR as described earlier. Bacterial isolation was also performed with surviving fish as described above to confirm that they were free of *E. tarda* infection. The experiments were conducted in five replicates.

#### Statistical analysis

Analysis of variance (ANOVA) was performed using the general linear models procedure of Statistical Analysis System (SAS

Institute, Cary, NC). Duncan's new multiple range tests was used to obtain pairwise comparisons among sample means. Evaluations were based on a 5% significance level ( $p < 0.05$ ).

## RESULTS

### Antimicrobial activity of carvacrol and cymene

Carvacrol was found to be able to inhibit the growth of *E. tarda* with the MIC of 20 g/ml (or 20 ppm). However, with the highest concentration of cymene used in this study which was 5,120 g/ml (5,120 ppm), the bacterium was not inactivated. At the end of the experiments, the absorbance of the bacterial cultures containing different concentrations of cymene was significantly different ( $p < 0.05$ ) from each other and also was not significantly different ( $p < 0.05$ ) from the control (the bacterial culture without cymene and carvacrol).

### Synergistic effect of carvacrol between cymene on *E. tarda*

The *in vitro* study of antimicrobial activity of different combinations of carvacrol and cymene against *E. tarda* was performed. The results are presented in Table 1. Carvacrol exhibited a dose dependent inhibitory effect on *E. tarda*. This was indicated by the decrease of absorbance at 600 nm from 0.45 to undetectable as the concentration of carvacrol increased from 0 ppm to 20 ppm. The inhibitory concentration of carvacrol was reduced by 75% (from 20 ppm to 5 ppm) with the inclusion of 2.5 ppm cymene. However, increase of cymene concentration beyond 2.5 ppm did not further promote the antimicrobial activity of carvacrol.

### Determination of median lethal dose (LD<sub>50</sub>)

The cumulative mortalities of tilapia were observed for 2 weeks after they were intraperitoneally infected with different doses of *E. tarda*. The first mortality of fish began at day 2 after infection in all the test groups.

**Table 2.** Percentage weight gain (PWG) and survival rate (SR) of uninfected tilapia fed for 2 weeks the fish diets supplemented with 200 ppm carvacol (Diet 1), 200 ppm cymene (Diet 2), 200 ppm carvacol and 200 ppm cymene (Diet 3), 100 ppm oxytetracycline (Diet 4) and no additive (Diet 5).

Fish diet	PWG (%)	SR (%)
Diet 1	57.25 ± 0.83	100.00 ± 0.00
Diet 2	56.83 ± 0.94	100.00 ± 0.00
Diet 3	57.32 ± 0.36	100.00 ± 0.00
Diet 4	56.68 ± 0.62	100.00 ± 0.00
Diet 5	57.11 ± 0.46	100.00 ± 0.00

Results are mean ± SD values of five replicates. PWG = [(final weight - initial weight)/initial weight] × 100. SR = (final number of fish/initial number of fish) × 100.

Mortality occurred continuously and the highest mortality was recorded at day 6 after infection. Based on the mortality, the calculated LD<sub>50</sub> of *E. tarda* for tilapia was  $5.0 \times 10^2$  CFU/g of fish.

#### Protective effect of the supplemented fish diets against *E. tarda* infection

Prior to examination of protective effect of fish diets supplemented with carvacol and/or cymene and with oxytetracycline on *E. tarda* infection in tilapia, the possible adverse effects of all the test diets on the normal (uninfected) fish were evaluated. Uninfected fish reared with the supplemented fish diets appeared and behaved similar to those reared with the control diet without any supplement. Furthermore, no significant difference ( $p < 0.05$ ) was observed on percentage weight gain over 2-week feeding period among the groups receiving the supplemented fish diets and between those and the control group (Table 2). No mortality in both test and control groups was detected during the 2-week observation period.

Effect on mortality of experimentally infected fish with *E. tarda* resulting from oral administration of fish diets supplemented with carvacol alone, cymene alone, carvacol plus cymene, and oxytetracycline is presented in Figure 1. Fish diets were given to fish 5 days prior to bacterial infection. After the infection, the diets were given to the infected fish for 14 more days and during this time, mortality of fish was observed. The cumulative mortality of *E. tarda* infected tilapia, fed the fish diet containing only cymene (200 ppm, Diet 2) was not significantly different ( $p < 0.05$ ) from that fed the negative control diet (without carvacol and cymene, Diet 5). However, the reduction of the cumulative mortality of the infected fish was observed in the groups fed the fish diet containing carvacol (200 ppm, Diet 1). When fish fed the diet supplemented with carvacol and cymene (Diet 3), synergistic protective effect between both compounds

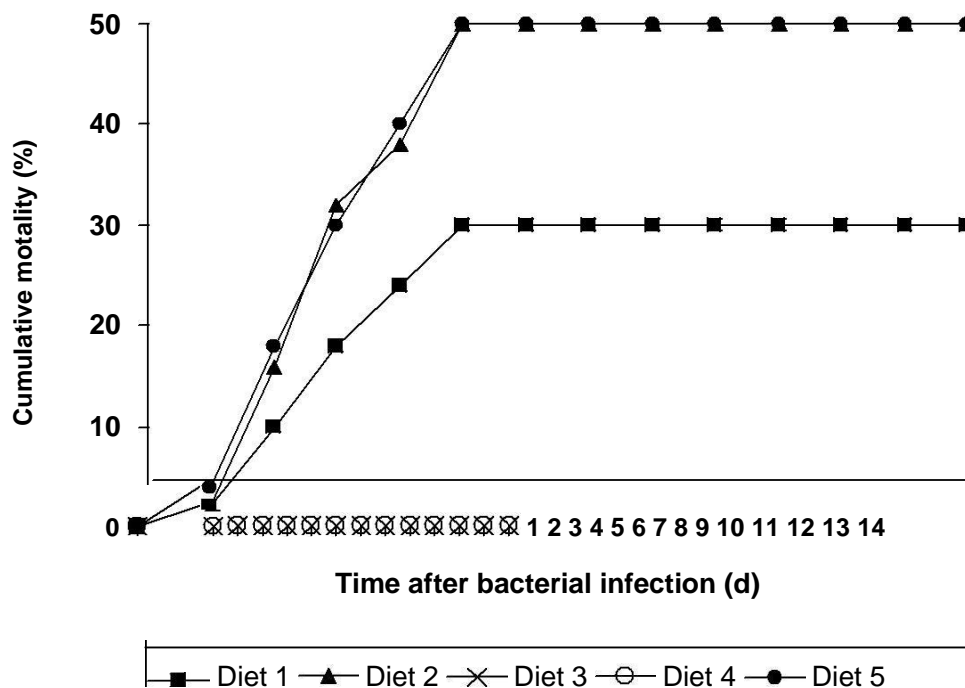
was observed. For this fish diet containing 200 ppm of cymene, carvacol at the concentration of 200 ppm could cause no mortality of *E. tarda* infected fish. Similar result was obtained from the group fed the fish diet supplemented with oxytetracycline (100 ppm, Diet 4). According to bacterial isolation from livers and kidneys and bacterial identification, *E. tarda* was found in all dead fish, but not from the survivors.

#### DISCUSSION

*E. tarda* is a pathogen causing edwardsiellosis or emphysematous putrefactive disease leading to massive mortalities and large economic losses in the natural environment and fish farming worldwide. Antibiotics and chemicals are commonly used to control the disease. However, there is much concern about their adverse effects. Their accumulation in the environment and in fish can be potentially harmful to consumers and other organisms (Wakabayashi, 1993; Aldermann and Hastings, 1998). Furthermore, prolonged use of antibiotics will favor the development of bacteria resistant to antibiotics, thereby reducing drug efficacy (Aldermann and Hastings, 1998).

Therefore, many efforts have been spent to find alternative approaches to control the disease. Pirarat et al. (2006) used a probiotic bacterium *Lactobacillus rhamnosus* to control *E. tarda* infection in tilapia. They found that cumulative mortality of experimentally *E. tarda* infected fish was significantly lower in probiotic-supplemented fish than in control fish. Taoka et al. (2006) have also reported that administration of commercial probiotics containing *Bacillus subtilis*, *Lactobacillus acidophilus*, *Clostridium butyricum* and *Saccharomyces cerevisiae* enhanced the non-specific immune system and reduced mortality due to *E. tarda* infection. Several vaccination attempts have been made to induce protection against *E. tarda* including vaccines comprised of whole cells, disrupted cells, cell extracts and attenuated strains as immunogens (Salati et al., 1983; Mekuchi et al., 1995; Kwon et al., 2006; Lan et al., 2007; Castro et al., 2008). These vaccination studies have been performed in many fish species such as Japanese flounder (*Paralichthys olivaceus*), eel (*Anguilla japonica*), tilapia (*Oreochromis mosambicus*) and turbot (*Scophthalmus maximus*). Recently, there is increasing interest in the use of plant-derived antimicrobial compounds as therapeutic and prophylactic agents in aquaculture (Abutbul et al., 2004; Rattanachaiakunsopon and Phumkhachorn, 2009). This study is the first study presenting the successful use of carvacol in combination with cymene in inhibiting a fish bacterial pathogen, *E. tarda in vitro* and in protecting against *E. tarda* infection in tilapia.

Carvacol is a biological compound naturally present in plants such as oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*) and savory (*Satureja hortensis*). It has



**Figure 1.** Cumulative mortality (%) of experimentally infected tilapia fed the fish diets supplemented with 200 ppm carvacol (Diet 1), 200 ppm cymene (Diet 2), 200 ppm carvacol and 200 ppm cymene (Diet 3), 100 ppm oxytetracycline (Diet 4) and no additive (Diet 5).

well-known antibacterial (Friedman et al., 2002), antifungal (Chami et al., 2005), insecticidal (Panella et al., 2005), and antiparasitic (Lindberg et al., 2000) properties as well as antitoxigenic effect (Ultee and Smid, 2001). It has been shown to have a broad spectrum of antimicrobial activity against both Gram-positive and Gram-negative bacteria (Burt, 2004). However, its antimicrobial activity against fish pathogenic bacteria and its potential use as a protective agent against bacterial infection in fish have never been reported. Carvacrol was shown in this study to inhibit *E. tarda* *in vitro* with the MIC of 20 g/ml (or 20 ppm). It was also found that the fish diet supplemented with 200 ppm of carvacrol was able to reduce the mortality of *E. tarda* infected tilapia.

On the other hand, cymene, a biological precursor of carvacrol, did not have antimicrobial activity against *E. tarda* both *in vitro* and *in vivo*. This was indicated by the results obtained from this study. In the MIC determination assay, the absorbance at 600 nm of the *E. tarda* cultures containing cymene was indistinguishable from the control (the bacterial culture containing no cymene and carvacrol). In the *in vivo* studies, the *E. tarda* infected tilapia receiving the diets supplemented with cymene alone had cumulative mortality of about 50% which was not significantly different ( $p < 0.05$ ) from the control *E. tarda* infected fish receiving the diet with no supplement. Our results are in agreement with previous reports

presenting the ineffectiveness of cymene in inhibiting the growth of bacteria (Burt, 2004).

Although cymene by itself does not have antimicrobial activity, it has been found to be able to enhance the bactericidal activity of carvacrol when used together (Ultee et al., 2000; Kisko and Roller 2005). Therefore, it is of interest to examine the synergistic antimicrobial effect of carvacrol and cymene against *E. tarda*. The results in Table 1 showed that the inhibitory concentrations of carvacrol against *E. tarda* were decreased from 20 ppm when used alone to 5 ppm when used together with cymene. Furthermore, 200 ppm of carvacrol added into the fish diets resulted in no mortality of *E. tarda* infected fish only when it was used together with cymene (200 ppm) (Figure 1). These results suggested that cymene was able to enhance the inhibitory effect of carvacrol against *E. tarda*.

The actual cause of the synergism between carvacrol and cymene is still unknown although the mechanism of action of both compounds has been extensively studied. Carvacrol can cause cell death because it is able to damage cytoplasmic membrane which leads to the collapse of the proton motive force and depletion of the ATP pool (Ultee et al., 2002). On the other hand, cymene is ineffective in killing cells because it does not affect pH gradient and the ATP pool (Ultee et al., 2002). Having a high preference for cytoplasmic membranes, cymene can

cause swelling of the membrane. Based on characteristics and mechanism of action of carvacrol and cymene, it is possible that the synergistic effect between both compounds may be due to the fact that cymene accumulating in and causing the expansion of the plasma membrane enables carvacrol to be more easily transported into the cell. However, further investigation is required for a better understanding of this unclear issue.

In conclusion, this study is the first report describing the synergistic antimicrobial effect against *E. tarda* both *in vitro* and *in vivo*. It also presented the potential of using carvacrol in conjunction with cymene in controlling *E. tarda* infection in tilapia.

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