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Effects of various concentrations of garlic powder and garlic extract in the diets on growth, serum chemistry and immune response of juvenile olive flounder *Paralichthys olivaceus*

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Effects of various concentrations of garlic powder and garlic extract in the diets on growth, serum chemistry and immune response of olive flounder were determined. Thirty-five juvenile fish averaging 5.1 g were randomly distributed into 21 of 180 L flow-through tanks. Seven experimental diets with various concentrations of garlic powder (GP) and garlic extract (GE) were prepared in triplicate: GP-0 without garlic supplementation, GP-0.5, GP-1, GP-2, GP-3 and GP-5 diets containing garlic powder at the concentrations of 0.5, 1, 2, 3 and 5%, respectively at the expense of wheat flour and finally, GE-0.4 diet containing 0.4% garlic extract were prepared. At the end of the 8-week feeding trial, serum chemistry of fish was measured. In addition, twenty fish from each tank were artificially infected with *E. tarda* for the following 96 h to monitor cumulative mortality. Weight gain of fish fed GP-0 diet was higher than that of fish fed GP-1, GP-2, GP-3 and GP-5 diets. No difference in serum criteria (total protein, glucose, glutamate oxaloacetate transaminase, cholesterol and triglyceride levels) of olive flounder was found among the experimental diets except for glutamate pyruvate transaminase. Lysozyme activity of fish fed GP-0, GP-1, GP-3 and GE-0.4 diets was higher than that of fish fed GP-5 diet. The highest cumulative mortality was 93.3% in fish fed GP-0 diet at 96 h after *E. tarda* infection, followed by GP-3, GP-1, GP-5, GP-2, GP-0.5 and GE-0.4 diets. In considering these results, dietary inclusion of garlic powder and garlic extract has no distinctive positive effect on improvement in growth, serum chemistry and immune response of olive flounder in this experimental conditions, therefore, its application should be carefully considered.

Key words : Olive flounder *Paralichthys olivaceus*, Garlic powder, Garlic extract, Lysozyme activity, *E. tarda*

Since olive flounder *Paralichthys olivaceus* has been one of the most important marine finfish for aquaculture in Korea, many feeding trials to determine dietary nutrients requirement (Lee *et al*., 2000; Alam *et al*., 2002; Lee *et al*., 2002), optimum feeding ratio/frequency (Lee *et al*., 1999; Cho *et al*., 2006b), alternative protein sources for fishmeal in the diets (Sato and Kikuchi, 1997; Kikuchi, 1999) for fish have been reported.

In addition, dietary additives, such as herbal medicine (Kim *et al*., 1998, 2000), culture broth of lactic acid bacteria in herb (*Acanthopanax koreanum*) extract (Jhon *et al*., 2009), green tea (Cho *et al*., 2006a, 2007), chitosan (Cha *et al*., 2008), extract of mushroom mycelium

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(*Phellinus linteus* and *Coriolus militaris*) (Kim *et al*., 2006a), microalgae, *Chlorella ellipsoidea* (Kim *et al*., 2002) and macroalga, *Hizikiafusi formis* (Pham *et al*., 2006), wood vinegar (Lee *et al*., 2008) and glucan (Kim *et al*., 2006b) were effective to improve performance and/or immune response of olive flounder. However, mortality of fish resulting from outbreak of disease frequently occurs during year-round and it eventually lowers fish production. Therefore, development of new additive for aquafeed is still highly needed.

The use of natural resources as dietary additives has several advantages, such as food safety for human consumption and minimizing the risk of side-effects. Garlic *(Allium sativum*) containing allicin, allyl cysteine, ajoene, allin and related components (Afzal *et al*., 2000; Chung, 2006) has been known to have antibacterial, antimicrobial, anti-inflammatory, antioxidant and/or antitumourigenic effects (Afzal *et al*., 2000; Shin and Kim, 2004; Chung, 2006; Wilson and Demmig-Adams, 2007). It was also known to lower cholesterol of men (Steiner *et al*., 1996) and hyperlipidemia in rats (Kang *et al*., 2008). Therefore, garlic seems to have high potential for aquafeed as an immunostimulant as well.

Dietary inclusion of fermented garlic powder was effective against *Vibrio anguillarum* and *Streptococcus ininae* infection, but not for *Edwardsiella tarda* (Kim *et al*., 2010). In above study, however, fish were grown and challenge test were performed in low temperature (16-18°C). Since those disease occurs frequently in olive flounder at high temperature (over 20°C), more studies in high temperature are needed. In addition, Lee *et al.* (2010) reported that injection of garlic extract effectively enhanced nonspecific immunity and resistance against *E. tarda* and *S. ininae* when fish were grown at 20-22°C.

In this study, therefore, effects of various concentrations of garlic powder and garlic extract in the diets on growth, serum chemistry and immune response of juvenile olive flounder were determined.

Materials and Methods

Experimental conditions

Juvenile olive flounder were purchased from a private hatchery (Taean, Chungcheongnam Do, Korea), transferred to the laboratory and acclimated for 2 weeks before an initiation of the feeding trial. During the acclimation period, fish were fed with commercial extruded pellet containing 54% crude protein and 11% crude lipid twice a day. Thirty-five juvenile fish averaging 5.1 g were randomly distributed into 21 of 180 L flow-through tanks (water volume: 150 L) and water flow rate of tank was 6.8 L/min. The water source was the sand-filtered natural seawater and aeration supplied to each tank. Water temperature ranged from 18.4 to 24.1°C (mean±SD: 21.5±1.16°C) and photoperiod followed natural condition.

Preparation of garlic powder and extract for the experimental diets Garlic powder (crude protein: 19.0%, crude lipid: 0.8% and ash: 3.1%) and garlic extract (Edentownfnb, Incheon, Korea) were used as the additives. Seven experimental diets with various concentrations of garlic powder (GP) and garlic extract (GE) were prepared in triplicate: GP-0 without garlic supplementation, which was used as control diet, GP-0.5, GP-1, GP-2, GP-3 and GP-5 diets containing garlic powder at the concentrations of 0.5, 1, 2, 3 and 5%,

respectively at the expense of wheat flour and finally, GE-0.4 diet containing 0.4% garlic extract were prepared (Table 1). Fishmeal, dehulled soybean meal and corn gluten were used as the protein source for the experimental diets. And wheat flour and fish and soybean oils were used as the carbohydrate and lipid sources, respectively. All experimental diets were prepared to satisfy dietary nutrient requirements for olive flounder (Lee *et al*., 2000, 2002). The ingredients of the experimental diets were well mixed with water at a ratio of 3:1 and pelletized with a pellet-extruder. The experiment diets were dried at room temperature overnight and stored to -20°C until use. All fish were hand-fed to apparent satiation twice a day (07:00, 17:00), seven days a week, for 8 weeks.

Table 1 Ingredients and chemical composition (%, DM basis) of the experimental diets

	Experimental diets						
	GP-0	GP-0.5	GP-1	GP-2	GP-3	GP-5	GE-0.4
Ingredients (%)							
Fishmeal	50	50	50	50	50	50	50
Soybean meal	12.5	12.5	12.5	12.5	12.5	12.5	12.5
Corn gluten meal	5	5	5	5	5	5	5
Wheat flour	25	24.5	24	23	22	20	25
Garlic powder[1]		0.5	1	2	3	5	
Garlic extract[2]							+
Fish oil	2	2	2	2	2	2	2
Soybean oil	3	3	3	3	3	3	3
Vitamin premix[3]	1	1	1	1	1	1	1
Mineral premix[4]	1	1	1	1	1	1	1
Choline	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Nutrient (*%, DM*)							
Dry matter	82.7	82.1	80.6	77.6	78.0	78.2	83.9
Crude protein	51.5	51.9	51.6	52.0	53.8	51.8	52.1
Crude lipid	8.9	9.8	9.9	10.8	10.6	10.9	10.0
Ash	8.4	9.3	9.2	9.2	9.2	8.4	9.2

[1]Garlic powder and [2]Garlic extract were supplied from Edentownfnb (Incheon, Korea).
[3]Vitamin and [4]Mineral premix were same as Cho *et al*. (2007)'s study.
+ indicated that 0.4% garlic extract was included instead of same amount of water.

Chemical analysis of the experimental diets and fish

Five fish from each tank at the termination of the feeding trial were sacrificed for proximate analysis. Crude protein was determined by the Kjeldahl method (Kjeltec 2100 Distillation Unit, Foss Tecator, Hoganas, Sweden), crude lipid was determined using an ether-extraction method (Soxtec TM 2043 Fat Extraction System, Foss Tecator, Hoganas, Sweden), moisture was determined by oven drying at 105°C for 24 h, fiber was determined using an automatic analyzer (Fibertec, Tecator, Hoganas, Sweden) and ash was determined using a muffle furnace at 550°C for 4 h, all methods were according to standard AOAC (1990).

Chemical analysis of blood

Blood samples were obtained from the caudal vein of randomly chosen three fish from each tank by syringes after they were starved for 24 h at the end of the feeding trial. Serum was collected after centrifugation (6,000 rpm for 5 min), stored freezer at -70°C as separate aliquots for analysis of total protein, glucose, glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), cholesterol and triglyceride (TG), and analyzed by using automatic chemistry system (Vitros DT60 II, Vitros DTE II, DTSC II Chemistry System, Johnson and Johnson Clinical Diagnostics Inc., New York, USA).

Lysozyme activity assay

The turbidimetric assay for lysozyme was carried out according to Parry *et al*. (1965). Briefly, test serum (100 μL) was added to 2 mL of a suspension of *Micrococcus lysodeikticus* (0.2 mg/mL, Sigma, MO, USA) in a 0.05 M sodium phosphate buffer, pH 6.2. The reaction was carried out at 25℃ and absorbance at 530 nm was measured after 0.5 and 4.5 min on a spectrophotometer. A lysozyme activity unit was defined as the amount of enzyme producing a decrease in absorbance of 0.001/min.

Challenge test

Twenty five fish from each tank were randomly chosen at the end of the 8-week feeding trial and shown to be free from bacterial infection. The bacteria used for the challenge were obtained as a reference pathogenic strain of *E. tarda* that was previously isolated from olive flounder (National Fisheries Research and Development Institute, Korea). A culture suspension of *E. tarda* was prepared after being cultured in agar for 24 h, collected, washed and suspended in a sterile 0.85% saline solution and counted. Fish were artificially infected by intraperitoneal injection with 0.1 mL of culture suspension of pathogenic *E. tarda* containing 4×10^8 bacteria/mL. Fish were monitored for the following 96 h after *E. tarda* infection and dead fish were removed every 3 h for the first 24 h, 6 h for the second 24 h and 12 h for the last 48 h.

Statistical analysis

One-way ANOVA and Duncan's multiple range test (Duncan, 1955) were used to analyze the significance of the difference among the means of treatments through SAS version 9.1 (SAS Institute, Cary, NC, USA).

Results

Survival was not significantly (P>0.05) different among the experimental diets (Table 2). However, weight gain of olive flounder fed GP-0 diet was significantly (P<0.05) higher than that of fish fed GP-1, GP-2, GP-3 and GP-5 diets, but not significantly (P>0.05) different from that of fish fed GP-0.5 and GE-0.4 diets.

Feed consumption of olive flounder fed GP-0 diet was significantly (P<0.05) higher than that of fish fed GP-1, GP-2, GP-3 and GP-5 diets, but not significantly (P>0.05) different from that of fish fed GP-0.5 and GE-0.4 diets (Table 3). Feed efficiency ratio (FER) of olive flounder fed GP-0 and GP-1 diets was significantly (P<0.05) higher than that of fish fed GP-3 and GP-5 diets. Protein efficiency ratio (PER) of olive flounder fed GP-0 diet was significantly (P<0.05) higher than that of fish fed GP-2, GP-3, GP-5 and GE-0.4 diets. Condition factor (CF) of olive flounder was not significantly (P>0.05) different among the experimental diets. However, hepatosomatic index (HSI) of olive flounder fed GP-0 diet was significantly (P<0.05) higher than that of fish fed GP-1, GP-2, GP-3 and GP-5 diets.

Serum total protein, glucose, GOT, cholesterol and triglyceride levels of olive flounder was not significantly (P>0.05) different among the experimental diets (Table 4). However, GPT of olive flounder fed GP-1 diet was significantly (P<0.05) higher than that of fish fed GP-0, GP-0.5, GP-3 and GP-5 diets.

Lysozyme activity of olive flounder fed GP-0, GP-1, GP-3 and GE-0.4 diets, which was the highest, was significantly (P<0.05) higher than that of fish fed GP-5 diet, but not significantly (P>0.05) different from that of fish fed GP-0.5 and GP-2 diets (Fig. 1).

Table 2 Survival (%) and weight gain (g/fish) of olive flounder fed the experimental diets with various concentrations of garlic powder and garlic extract for 8 weeks

Diets	Initial weight (g/fish)	Final weight (g/fish)	Survival (%)	Weight gain (g/fish)
GP-0	5.1±0.00	30.7±1.21	97.1±1.65^{a}	25.6±1.21^{a}
GP-0.5	5.0±0.02	27.9±0.22	98.1±1.90^{a}	22.9±0.24^{ab}
GP-1	5.1±0.01	26.2±0.77	100±0.00^{a}	21.2±0.78^{b}
GP-2	5.1±0.02	25.2±0.42	98.1±0.95^{a}	20.2±0.42^{b}
GP-3	5.0±0.02	25.0±1.75	99.0±0.95^{a}	20.0±1.77^{b}
GP-5	5.0±0.02	25.2±0.80	95.2±0.95^{a}	20.1±0.78^{b}
GE-0.4	5.1±0.04	28.0±0.47	97.1±1.65^{a}	23.0±0.43^{ab}

Values (means of triplicate±SE) in the same column sharing the same superscript letter are not significantly different (P>0.05).

Table 3. Feed consumption (g/fish), feed efficiency ratio (FER), protein efficiency ratio (PER), condition factor (CF) and hepatosomatic index (HSI) of olive flounder fed the experimental diets with various concentrations of garlic powder and garlic extract for 8 weeks

Diets	Feed consumption (g/fish)	FER[1]	PER[2]	CF[3]	HSI[4]
GP-0	22.0±0.67^{a}	1.17±0.019^{a}	2.26±0.037^{a}	0.93±0.035^{a}	2.50±0.086^{a}
GP-0.5	21.1±0.27^{ab}	1.08±0.025^{ab}	2.09±0.048^{abc}	0.86±0.027^{a}	2.17±0.074^{ab}
GP-1	19.3±0.22^{cd}	1.09±0.029^{a}	2.12±0.055^{ab}	0.91±0.011^{a}	2.04±0.095^{b}
GP-2	18.9±0.11^{d}	1.07±0.022^{abc}	2.05±0.043^{bc}	0.89±0.018^{a}	1.84±0.034^{b}
GP-3	20.7±0.67^{b}	0.96±0.056^{c}	1.79±0.105^{d}	0.88±0.012^{a}	1.99±0.217^{b}
GP-5	20.5±0.02^{bc}	0.98±0.037^{bc}	1.90±0.072^{cd}	0.89±0.023^{a}	1.99±0.074^{b}
GE-0.4	21.5±0.23^{ab}	1.07±0.023^{abc}	2.05±0.045^{bc}	0.91±0.026^{a}	2.17±0.081^{ab}

Values (means of triplicate±SE) in the same column sharing the same superscript letter are not significantly different (P>0.05).
[1]Feed efficiency ratio (FER) = Weight gain of fish/feed consumed.
[2]Protein efficiency ratio (PER) = Weight gain of fish/protein consumed.
[3]Condition factor (CF) = Fish weight (g) × 100/total length (cm)^{3}.
[4]Hepatosomatic index (HSI) = Liver weight × 100/fish weight.

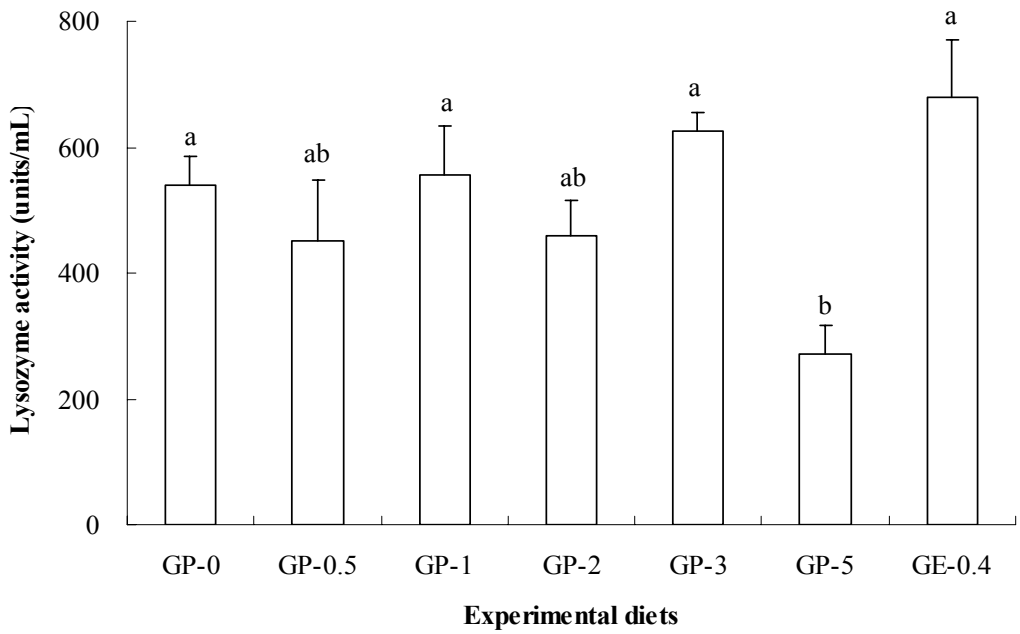

Fig. 1. Lysozyme activity (units/mL) of olive flounder fed the experimental diets containing various concentrations of garlic powder and garlic extract. Values (means of triplicate±SE) in the same letter are not significantly different (P>0.05).

Mortality was initially observed in all fish groups at 48 h after *E. tarda* infection. Mortality of fish fed GP-0 diet was relatively high compared to that of fish fed the other diets containing various concentrations of garlic powder and extract at 84 h after *E. tarda* infection, but no significant (P>0.05) difference was observed (Fig. 2). Cumulative mortality of fish fed GP-0 diet was 93.3%, which was highest, followed by 88.3, 86.7, 85.1, 85.0, 83.3 and 78.3% for GP-3, GP-1, GP-5, GP-2, GP-0.5 and GE-0.4 diets, respectively at 96 h.

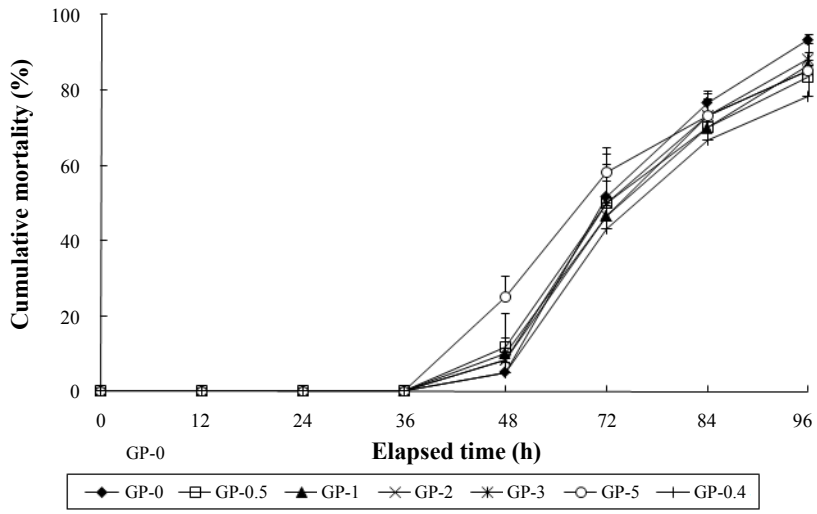

Fig. 2. Cumulative mortality (%) of olive flounder fed the experimental diets containing various concentrations of garlic powder and garlic extract after *Edwardsiella tarda* infection for the following 96 h (means of triplicate±SE).

Discussion

No adverse effect of garlic on weight gain of fish fed GP-0.5 and GE-0.4 diets, but adverse effect of fish fed GP-1, GP-2, GP-3 and GP-5 diets in this study indicated that dietary inclusion of garlic powder more than 0.5% could deteriorate growth of fish. However, administration of fermented garlic did not deteriorate growth of olive flounder when fish were administrated with 0.5, 1 and 2% fermented garlic into the diets (Kim *et al.*, 2010). This difference could be resulted from that olive flounder effectively utilized the diets containing 0.5-2% garlic powder because it was fermented with *Bacillus* spp at high temperature in the latter study.

Dietary supplementation of herb (Kim *et al*., 1998, 2000), glucan (Kim *et al*., 2006b), *C. ellipsoidea* powder (Kim *et al*., 2002) and wood vinegar (Lee *et al*., 2008) improved weight gain of olive flounder, on the contrary, Shiau and Yu (1999) reported that dietary inclusion of chitin and chitosan depressed growth of hybrid tilapia (*Oreochromis niloticus* × *O. aureus*). Therefore, effect of administrative additives on fish performance may vary

depending on fish species, dose of additives and/or fish nutritional/physiological status. Therefore, application of administrative additives to improve performance of fish should be carefully considered.

No difference in weight gain and feed consumption of fish fed GP-0.5 and GE-0.4 diets, but lower weight gain and less feed consumption of fish fed GP-1, GP-2, GP-3 and GP-5 diets compared to those of fish fed GP-0 diet in this study indicated that dietary inclusion of more than 0.5% garlic powder might depress growth of fish probably resulted from their poor palatability. Similarly, dietary inclusion of 2% fermented garlic powder lowered feed consumption of olive flounder (Kim *et al.*, 2010).

Administration of various sources of additives improved feed efficiency resulted from the increased weight gain of fish (Kim *et al*., 1998, 2000, 2002; Lee *et al*., 2008). The liver of olive flounder fed the diets containing more than 0.5% garlic powder in this study seemed to result into its shrinkage. Similarly, dietary supplementation of 3% fresh garlic powder lowered liver weight of hyperlipidemic rats compared to that fed the diet without garlic supplementation (Kang *et al*., 2008).

No difference in serum total protein, glucose, GOT, cholesterol and triglyceride levels of olive flounder among the experimental diets was probably resulted from wide variation in those values within the same treatment (Table 4). Kim *et al*. (2010) reported that hemoglobin, myeloperoxidase activity and cholesterol level of fish was not affected by concentration of fermented garlic. However, it is difficult to explain why fish fed GP-1 diet only showed high GPT level. Dietary inclusion 1% green tea lowered GPT of olive flounder (Cho *et al*., 2007). In addition, administrative *C. ellipsoidea* (Kim *et al*., 2002) and green tea (Cho *et al*., 2007) lowered serum cholesterol and low-density lipoprotein cholesterol, respectively.

Table 4. Serum chemical composition in olive flounder at the end of the 8-week feeding trial

Diets	Total protein (g/dL)	Glucose (mg/dL)	GOT (IU/L)	GPT (IU/L)	Cholesterol (mg/dL)	TG (mg/dL)
GP-0	3.0±0.21^{a}	35.3±1.91^{a}	23.3±4.77^{a}	3.3±0.54^{b}	179±15.8^{a}	129±14.1^{a}
GP-0.5	2.7±0.15^{a}	31.3±6.50^{a}	20.7±0.54^{a}	3.7±1.19^{b}	139±20.9^{a}	111±19.4^{a}
GP-1	2.8±0.12^{a}	35.3±4.01^{a}	32.7±4.72^{a}	15.7±5.86^{a}	180±5.8^{a}	173±10.9^{a}
GP-2	2.8±0.05^{a}	40.3±3.47^{a}	23.0±2.36^{a}	5.7±1.19^{ab}	165±7.1^{a}	124±15.8^{a}
GP-3	2.5±0.22^{a}	33.0±2.62^{a}	18.3±2.42^{a}	3.3±0.98^{b}	146±14.8^{a}	115±8.8^{a}
GP-5	2.3±0.07^{a}	37.0±5.50^{a}	20.3±3.31^{a}	2.7±0.27^{b}	137±8.0^{a}	108±2.8^{a}
GE-0.4	2.7±0.14^{a}	35.0±2.87^{a}	23.7±1.66^{a}	5.3±0.72^{ab}	165±10.7^{a}	139±3.6^{a}

Values (means of triplicate±SE) in the same column sharing the same superscript letter are not significantly different (P>0.05).

Higher lysozyme activity of olive flounder fed GP-0, GP-1, GP-3 and GE-0.4 diets than GP-5 diet was observed in this study (Fig. 1). Similarly, there was no positive correlation between the effect of immunostimulants and

dosage, in addition, high dosage might not enhance or inhibit the immune response (Lee *et al*., 2008). However, lysozyme activity of olive flounder tended to be increased by dietary inclusion of fermented garlic (Kim *et al*., 2010) and injection of garlic extract or immersion of garlic juice (Lee *et al*., 2010). In addition, dietary inclusion of glucan and wood vinegar improved lysozyme activity of olive flounder (Kim *et al*., 2006b; Lee *et al*., 2008).

Unlike desirable (antibacterial, antimicrobial, anti-inflammatory, antioxidant and/or antitumourigenic) effects of allicin, allyl cysteine, ajoene, allin and related components in garlic (Afzal *et al*., 2000; Shin and Kim, 2004; Chung, 2006; Wilson and Demmig-Adams, 2007) on mammals, no distinctive improvement in mortality of olive flounder fed diets containing garlic powder and garlic extract after *E. tarda* infection was found in this study (Fig. 2), probably indicating that effect of administration of garlic powder and garlic extract on immune response might vary depending on animals tested and be still controversial. Different results in administration of garlic even in olive flounder were reported. For instance, Kim *et al*. (2010) demonstrated that dietary administration of fermented garlic powder did not improve disease resistance against *E. tarda*, but did for *V. anguillarum* and *S. iniae* when fish were grown in low temperature for 5 weeks, on the other hand, Lee *et al*. (2010) reported that administration of garlic extract and garlic juice enhanced nonspecific immunity and disease resistance of olive flounder against *E. tarda* and *S. iniae* at 20-22°C. Administrative chitosan (Cha *et al*., 2008), green tea (Cho *et al*., 2006a) and wood vinegar (Lee *et al*., 2008) lowered mortality of olive flounder after artificial infection. Pham *et al*. (2006) also showed that no cumulative mortality of olive flounder fed the diet containing 6% *Hizikiafusi formis* after *S. iniae* infection and the enhanced nonspecific immune response of fish resulted from polyphenol compounds in macroalgae were observed.

Administration of dietary inclusion of garlic powder and extract did not achieve distinctive improvement in growth, serum chemistry and immune response of olive flounder in this experimental conditions, therefore, its application should be carefully considered.

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