The effects of *Curcuma zedoaria* and *Zingiber zerumbet* on non-specific immune responses of grouper *Epinephelus coioides*

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Abstract

This study was conducted to investigate the effects of *Curcuma zedoaria* and *Zingiber zerumbet* on non-specific immune responses of grouper (*Epinephelus coioides*). Fish were fed an experimental diet containing 0, 0.5, 1.0, 2.5 g/kg of *C. zedoaria* and *Z. zerumbet* mixed diets twice daily for two weeks. Non-specific immune parameters such as respiratory burst activity, reactive oxygen species, phagocytic activities, superoxidase dismutase activity and lysozyme activity were sampled at 0, 1, 2, 4, 7 and 14 days, respectively. Results indicated that in fish fed with *C. zedoaria* at 0.5 g/kg diet and *Z. zerumbet* at 1 g/kg and 2.5 g/kg diets the non-specific immune response was affected, especially in cellular defense which had significant effects in the short term. Thus, this study indicated that *C. zedoaria* and *Z. zerumbet* supplemented in the diets of orange-spotted grouper acted as immunostimulants and appeared to enhance the non-specific immune responses in this species.

Keywords: Herbs, Immunology, Phagocytosis, Plant extract, White blood cell

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Introduction

Generally in aquaculture, fish are reared in intensive system in order to maximize productivity. This condition often negatively affects the fish immune system and can increase susceptibility to disease (Kirubakaran *et al*., 2010). Moreover, when the diseases occur, most farmers and aquaculturists still depend on antibiotics and chemotherapeutics to treat fish diseases. This practice is actually not recommended since the use of chemical substances caused some risks of generating resistant pathogen, bioaccumulation and environment pollution (Citarasu *et al*., 2002; Sagdic and Ozcan, 2003). Furthermore, commercial vaccines are expensive for fish farmers and only specific against particular pathogens (Murray *et al*., 2003; Gopalakannan and Arul, 2006; Ardo *et al*., 2008). Therefore, one of the most promising methods of controlling disease is strengthening the defense mechanisms of fish through immunostimulants.

Immunostimulants are natural or synthetic substances that are able to activate non-specific and specific immune responses (Sakai, 1999; Esteban *et al*., 2000). According to Bricknel and Dalmo (2005) the use of immunostimulant as a dietary supplement can improve the non-specific defense of animal and can provide resistance against pathogens during periods of high stress, such as: grading, transportation and vaccination. Moreover, application of herbs as an immunostimulant is believed to contribute to enhancing immune responses and increasing fish appetite. Components such as polysaccharides, lectins, proteins and peptides in plants have been shown to stimulate the immune system (Bafna and Mishra, 2005).

Several herbs have been investigated to know their effects on fish immune response. For example: *Lactuca indica* in *E. bruneus* (Harikrishnan *et al*., 2011a); *Prunella vulgaris* in *Paralichthys olivaceus* (Harikrishnan *et al*., 2011b); *Nyctanthes arbortristis* in *Oreochromis mossambicus* (Kirubakaran *et al*., 2010); *Astragalus radix* and *Ganoderma lucidum* in *Cyprinus carpio* (Yin *et al*., 2009); *Allium sativum* in *Labeo rohita* (Sahu *et al*., 2007); *Nigella sativa* in *Oncorhynchus mykiss* (Dorucu *et al*., 2009); *Lonicera japonica* and *Ganoderma lucidum* in *Oreochromis niloticus* (Yin *et al*., 2008); *Andrographis paniculata* in *C. carpio* (Taukhid *et al*., 2007); *Picrasma javanica* in *Osphronemus gouramy* (Supriyadi *et al*., 2006); *Achyranthes aspera* in *Catla catla* (Rao and Chakrabarti, 2005); and *Psidium guajava*, *Andrographis paniculata* and *Piper betle* in *Pangasius hypophthalmus* (Giyarti, 2000). However the effects of *Curcuma zedoaria* and *Z. zerumbet* on immune responses in fish are not well known.

C. zedoaria or Zedoary is a perennial herb that is commonly grown in Indonesia and India. This plant is also known as white turmeric in other parts of Asia (Manfield *et al*., 2005). The rhizome of *C. zedoaria* contains curcumin substances and ethereal oils that are considered for their anti-cancer, anti-bacterial, anti-inflammatory and immunity activities (Hou and Jin, 2005). On the other hand, *Z. zerumbet* has significant advantages as an analgesic and stimulant. This herb is commonly used for its anti-inflammatory property to treat

diarrhea and rheumatic pain (Somchit and Shukriyah, 2003; Bhuiyan *et al*., 2009); as an anti-oxidant (Agrawal *et al*., 2000); and a anti-microbial (Nakatani, 2000). Corresponding to the compounds in those herbs, it is considered that these plants may be applied in aquaculture. Thus, this study was conducted to investigate the effects of a dietary supplementation of *C. zedoaria and Z. zerumbet* on the non-specific immune responses of grouper (*E. coioides*).

Materials and Methods

Fish and culture conditions

Groupers (*E. coioides*) weighing 80-100 g in body weight were acclimated in the hatchery of the Department of Aquaculture, National Taiwan Ocean University, for 2 weeks prior to experimentation. Fish were reared and fed *ad-libitum* twice a day on commercial diets. During the experiment, fish were hand fed their respective experimental feeds twice daily to apparent satiation at 08:00 and 17:00 hours. The feeding trial was carried out in a recirculation water system. Water quality parameters during the feeding trials were: temperature 29.0±1°C; pH 8.0±1, and salinity 34±1 ppt. These ranges are considered within optimal values for grouper.

Selection of herbs and dietary administration

Five herbal plants possessing screening and selection activities to enhance non- specific immunity mediated respiratory burst activity in superoxide production analysis were selected. Fresh herbal plants (whole part of *Phyllanthus niruri*) were collected based on their availability from the geographical location of Central Java region, bark (*Cinnamomum burmanii*) and fresh root (*C. xanthorriza*, *C. zedoaria* and *Z. zerumbet*) were purchased from the local market. The plant parts were shadow dried then crushed to obtain a powdered form. The extraction was conducted following the method described by Kirubakaran *et al*., 2010 (with minor modification), whereas 30 mg of each plant powder was dissolved and shaken with 60 ml of Hank's Balanced Salt Solution (HBSS, pH: 7.4) for 30 min. It was then filtered three times using filter paper (Advantec no. 2) and then stored at 4ºC until used. To study *in vitro*, leukocytes from five fish for each treatment were incubated without (control) or with different concentrations of herbs. To do this, aliquots of 100 µl of leukocytes (5 x 10^6 cells) were dispensed into 96-well microtiter plates (Nunc) and incubated with herbs in 0, 0.01, 0.05, 0.1, 0.25, 0.5 mg/ml. Then the superoxide anion production was analyzed.

Two from five herbs with good performances in enhancing the non-specific immune response *in vitro* were chosen for dietary administration. There were seven groups of diets consisting of a control diet and supplemented diets with *C. zedoaria* and *Z. zerumbet* separately at the concentration of 0.5, 1 and 2.5 g/kg diets. The ingredients of each diet were mixed together for 40 min to make a paste which was separately passed through a grinder in a paste extruder. The diet for the control group was treated similarly with the supplemented diets but no herb was added. The diets were dried in a forced-air drier at room temperature for 24 h. After drying, the pellets were stored in plastic bags at 4ºC

until further use. In all treatment groups, the immune parameters were determined six times sampling on 0, 1, 2, 4, 7 and 14 days after dietary administration. On each sampling day, five fish as replicates were sacrificed to analyze the non-specific immune parameters such as phagocytic activity assays, superoxidase production analysis, reactive oxygen species production, Superoxide dismutase (SOD) assay and lysozyme activity.

Measurements of non-specific immune parameter

For serum, blood samples from specimens in dietary administration were withdrawn from caudal veins of the remaining anaesthetized fish into blood collecting tubes or Eppendorf tubes without anticoagulant in the syringe. Blood samples in Eppendorf tubes (Snap Seal Graduated Microtubes, USA) were allowed to clot for 2 h at room temperature in a slanting position. The tubes were kept at 4 °C overnight and were then centrifuged at 2500 rpm for 15 min and the supernatant serum was collected. The serum was stored at −80°C until used for lysozyme activity analysis. The fish was then used for the separation of head kidney and spleen leukocytes and the liver samples for SOD activity (Samad *et al*., 2014).

The head kidneys and spleens of *E. coioides* were excised from bled fish (n=5), and passed through a 100 µm nylon mesh (Bio-Rad, Hercules, CA, USA) with Hank's Balanced Salt Solution (HBSS, pH: 7.4). The cell suspension was transferred to the tubes containing 3 mL of 30─50% Percoll (GE Healthcare, Buckinghamshire, UK). The tubes were centrifuged at 1466 rpm for 40 min at 4 °C, and the leukocytes on the interface of the 30% and 50% Percoll were collected and transferred into eppendorf tubes (SnapSeal Graduated Microtubes, USA, capacity 1.7 ml) and the volume was adjusted using HBSS solution. The leukocytes were centrifuged three times at 3000 rpm for 10 min at 4°C for complete removal of supernatant (Kuan *et al*., 2012). The white blood cells were then counted under an electric microscope (Olympus BX 41, Japan).

Phagocytic activity assays were measured using the methods described by Fujiki and Yano (1997). Briefly, 50 µL of leukocytes ($5x10^6$ cells) was placed on a glass slide, and allowed to adhere for 20 min at 25°C in a moisture incubation chamber. Then, 50 µL of latex beads (10^7beads/mL, Sigma-Aldrich) was added to the leukocytes monolayer, and incubated for 30 min at 25°C. The percentage of phagocytes ingesting beads (Phagocytic rate, PR) and the number of beads ingested per phagocyte (Phagocytic index, PI) were calculated by enumerating 100 phagocytes under a microscope. Phagocytic activity was expressed as the phagocytic index (PI) (Matsuyama *et al*., 1992). The phagocytic rate (PR) and phagocytic index (PI) were determined as followed:

PR= (Phagocytosing cell/Total cell) × 100

PI=(Total phagocytosed beads/ Phagocytosing cell) × 100

Respiratory burst activity produced by phagocytes in the head kidney was measured according to the methods described by Cheng *et al*. (2007). In brief, 100 µL of leukocytes ($5x10^6$ cells) was placed in 96-wells and incubated for 1 h at

37ºC. Then, the non-adherent cells were removed by washing the wells with Hank's Balanced Salt Solution (HBSS, pH: 7.4). Then, 100µL of zymosan solution (Sigma-Aldrich) was added to 5 wells (A-E), while 100 µl HBSS was added to other wells (F-H) and incubated for 30 min at 37ºC. Then, 100µl of nitroblue tetrazolium (NBT, Sigma-Aldrich) was added to all of the wells (A-H) and incubated at 37ºC for 30 min. Then, the HBSS was used to wash all wells (it was done gently to allow the white blood cells to still attach to the wells). Then, the reaction was stopped by adding 100 µl 100% methanol and incubated for 5 min. After washing with methanol, the formazan formed in each well was dissolved by adding 120 ml of 2 M potassium hydroxide (KOH) and 140 ml of dimethyl sulphoxide (DMSO). The NBT reduction was measured using an ELISA microplate reader at 630 nm. Cells from each fish were in triplicate wells. Respiratory burst activity was expressed as NBT-reduction.

Reactive oxygen species was measured using the method of Secombes (1990). In brief, 100µl of leukocytes suspension was placed into 96-wells. Then, 100 µl of 1 mM luminal suspension liquid and 100µlof 1 mg/ml zymosan (Sigma-Aldrich) was added. Respiratory burst induced by phagocytosis of zymosan particles was measured in relative luminescence unit (RLU) per second. Optical density was measured using the microplate reader (PowerWave XS, BioTek Instruments, Inc., Winooski, Vermont, USA) at 650 nm.

The SOD assay was conducted using the Ransod kit (Randox Laboratories, Crumlin, UK) following the manufacturer's instruction. In brief, 850 µl of the reaction substrate containing xanthine and INT (2-(4-iodophenyl)-3-(4-nutrophenol 3-5-phenltetrazolium) was mixed with 25 µl of liver tissue solution obtained from fish fed with the test diets, or with 25µl of HBSS as a control, followed by the addition of 125 µl of xanthine oxidase (XOD). During the reaction, xanthine was reduced by XOD to produce uric acid and superoxide radicals, and further reacted with INT to produce formazan dye. The SOD in the sample solution would compete with INT for the superoxide radicals, thus the SOD activity could be determined based on its ability to inhibit formazan dye formation. The rate of formazan formation was measured by detecting the absorbance at 505 nm at 30 and 210seconds after the initiation of reaction. The rate of formazan formation inhibition was calculated by comparing the formazan formation rate of the liver tissue solution treated groups with the HBSS treated control group. The specific activity was defined as a unit of SOD that could cause a 50% reduction in the rate of formazan dye formation.

The percentage of inhabitation was calculated by the following formula:

$\Delta A_{sample/min} = (A2-A1)/3$

Inhibitation (%) = $100 - (\Delta A_{sample/min} / \Delta A_{s1/min}) \times 100$

$\Delta A_{sample/min}$ = the change of value sample absorbance per minutes.

$\Delta A_{s1/min}$ = the change of value phosphate buffer absorbance per minutes.

Lysozyme activity was measured based on turbidimetric assay according to methods described by Ellis (1990). Briefly, a standard suspension (0.2 mg/ml) of *Micrococcus lysodeikticus* (Sigma-Aldrich) was prepared in 0.05 M sodium phosphate

buffer (pH 6.2). 10 µl test plasma was added to 200 µl of the bacterial suspension in a 96-well microplate, and the decrease in absorbance at 530 nm was recorded after 1 and 6 min at 22 ºC. Standard solution containing 0, 10, 20, 30, 50 and 100µl^{-1} of hen egg white lysozyme (Sigma-Aldrich) was used to form a standard curve. The results were expressed as mg/ml equivalent of hen egg white lysozyme activity.

Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA). When the differences were significant at $p<0.05$ level, Tukey's test was used to compare the means between individual treatments. Statistical analysis was performed using the SAS software (SAS Inc. Cary, NC, USA).

Results

Superoxide production analysis of *E. coioides* leukocytes tends to enhance after being incubated with 0.05 mg/ml of *C. xanthorriza* extract, 0.1 mg/ml of *C. zedoaria* extract, and 0.1 to 0.5 mg/ml of *Z. zerumbet* extract. Significant enhancement in superoxide production was found on *P. niruri* extract (0.25 and 0.5 mg/ml). However, there was no effect on leukocytes when incubated with *C.burmanii* extract (Fig. 1A). Based on their ability to enhance immunity in low doses as the reason for the economic factor, *C. zedoaria* (0.1 mg/ml) and *Z. zerumbet* (0.1 – 0.5 mg/ml) were then used as immunostimulators in *in vivo* experiments.

In *in vivo* tests, the effect of *C. zedoaria* and *Z. zerumbet* extract on respiratory burst activities producing superoxide anion (Fig. 1B) showed that treating groups with 0.5 g/kg of *C. zedoaria* extract in diet significantly enhanced on day 4 and 7 compared with the control group. However, there was no significant difference in the group treated with 1 g/kg *C. zedoaria* extract diet during experiment. Fish receiving 1 g/kg obtained the highest value on day 2, followed by fish treated with 0.5 g/kg on day 4.

The rate of phagocytic activities of *E. coioides* fed with experimental diets is shown in Table 1. Fish receiving 0.5 g/kg of *C. zedoaria* significantly enhanced the rate of phagocytic activity on day 2 and 4, while fish fed with 2.5 g/kg *Z. zerumbet*, showed significant enhancement on day 1 and 2. Phagocytic rate (PR) of fish fed with *C. zedoaria* tends to be higher than fish fed with *Z. zerumbet* on day 2 and 4. PR activity increased directly with the increasing of *C. zedoaria* and *Z. zerumbet* dosage. The phagocytic index (PI) of *E. coioides* fed with experimental diets is shown in Table 2. The phagocytic index of fish receiving 0.5 g/kg *C. zedoaria* was significantly different from that of the control group from day 1 to 7. It showed the highest value (2.62 latex beads/cell) on day 2. However, it decreased sharply from day 7 to 14.

Inclusion of different dosages of the herbs can induce phagocyte reactive oxygen species (ROS) which was detected by the chemiluminescent reactions method (Fig. 1C). Supplementing feeds with 2.5 g/kg *C. zedoaria* showed significant enhancement on day 7, whereas fish receiving 1 g/kg significantly increased in chemiluminescent response and were able to maintain this enhancement from day 1 until the end of the experiment. Furthermore, in the group fed with 1 g/kg

of *Z. zerumbet*, ROS significantly increased from day 1 to day 4, with the highest point obtained on day 4 followed by a decrease afterward.

SOD enzyme activity was observed on day 2 and 4 when fish were treated with 0.5 g/kg *C. zedoaria*. However, no significant difference was observed in fish fed with 1 g/kg diets. In addition, the highest dose (2.5 g/kg diets) caused enhancement in SOD activity on day 7. On the other hand, fish receiving *Z. zerumbet* showed an increase in the enzyme activity on day 14 (Fig. 1D) compared with the control group.

Variance in serum lysozyme activity is seen in Fig. 1E. There was a significant difference in serum lysozyme activity on day 2 and 4, when fish were treated with 0.5 g/kg *C. zedoaria.* Moreover, inclusion of 1 g/kg diet showed the highest value among all treatment groups on day 14. While, fish fed with 0.5 g/kg *Z. zerumbet* gradually increased the serum lysozyme activity and had significant differences with that in the control from day 4 until the final day of experiment.

Table 1: Phagocytic rate of *Epinephelus coioides* fed with experimental diets.

Treatments	Dose g/kg in feed	Time (day)				
		1	2	4	7	14
Control	0	1.37±0.02^{d}	1.42±0.05^{d}	1.47±0.10^{bc}	1.52±0.04^{b}	1.43±0.06^{ab}
C. zedoaria	0.5	1.75±0.02^{c}	2.62±0.13^{a}	2.34±0.03^{a}	1.64±0.04^{a}	1.44±0.01^{ab}
	1.0	1.32±0.03^{de}	2.11±0.12^{bc}	1.51±0.01^{b}	1.56±0.03^{ab}	1.51±0.16^{a}
	2.5	1.64±0.02^{c}	2.33±0.05^{b}	1.57±0.01^{b}	1.28±0.02^{de}	1.49±0.09^{a}
Z. zerumbet	0.5	1.19±0.02^{e}	1.96±0.07^{c}	1.45±0.04^{bc}	1.41±0.06^{c}	1.15±0.01^{c}
	1.0	2.14±0.07^{b}	1.97±0.09^{c}	1.32±0.02^{c}	1.36±0.02^{cd}	1.23±0.13^{c}
	2.5	2.50±0.02^{a}	2.12±0.04^{bc}	1.45±0.08^{bc}	1.19±0.04^{e}	1.26±0.04^{bc}

Values are means of triplicate groups' ± S.D. Within a column, means with different letters are significantly different (p<0.05). Means with the same letters indicate not significantly different between the treatments.

Table 2: Phagocytic index of *Epinephelus coioides* fed with experimental diets.

Treatments	Dose g/kg in feed	Time (day) 1	2	4	7	14
Control	0	26.89±1.77^{d}	37.99±1.28^{c}	36.75±1.59^{b}	36.04±0.77^{a}	29.24±1.94^{ab}
C. zedoaria	0.5	29.14±0.59^{cd}	60.74±0.64^{a}	58.36± 0.31^{a}	34.72±2.41^{a}	28.81±1.27^{ab}
	1.0	40.02±2.35^{b}	58.38±2.15^{a}	34.61±0.55^{bc}	38.34±1.25^{a}	31.51±0.65^{a}
	2.5	65.45±1.05^{a}	41.72±1.52^{c}	33.56± 0.78^{bc}	29.97±1.62^{b}	27.02±1.19^{bc}
Z. zerumbet	0.5	31.37±0.48^{c}	40.51±2.29^{c}	33.34±3.50^{bc}	34.84±1.31^{a}	24.44±0.96^{c}
	1.0	25.30±0.20^{d}	47.17±0.67^{b}	29.51±4.16^{c}	27.54±1.38^{b}	23.26±2.46^{c}
	2.5	58.39±0.68^{a}	50.67±1.15^{b}	38.04±0.09^{b}	29.33±0.14^{b}	24.47±1.39^{bc}

Values are means of triplicate groups'±S.D. Within a column, means with different letters are significantly different (p<0.05). Means with the same letters indicate not significantly different between the treatments.

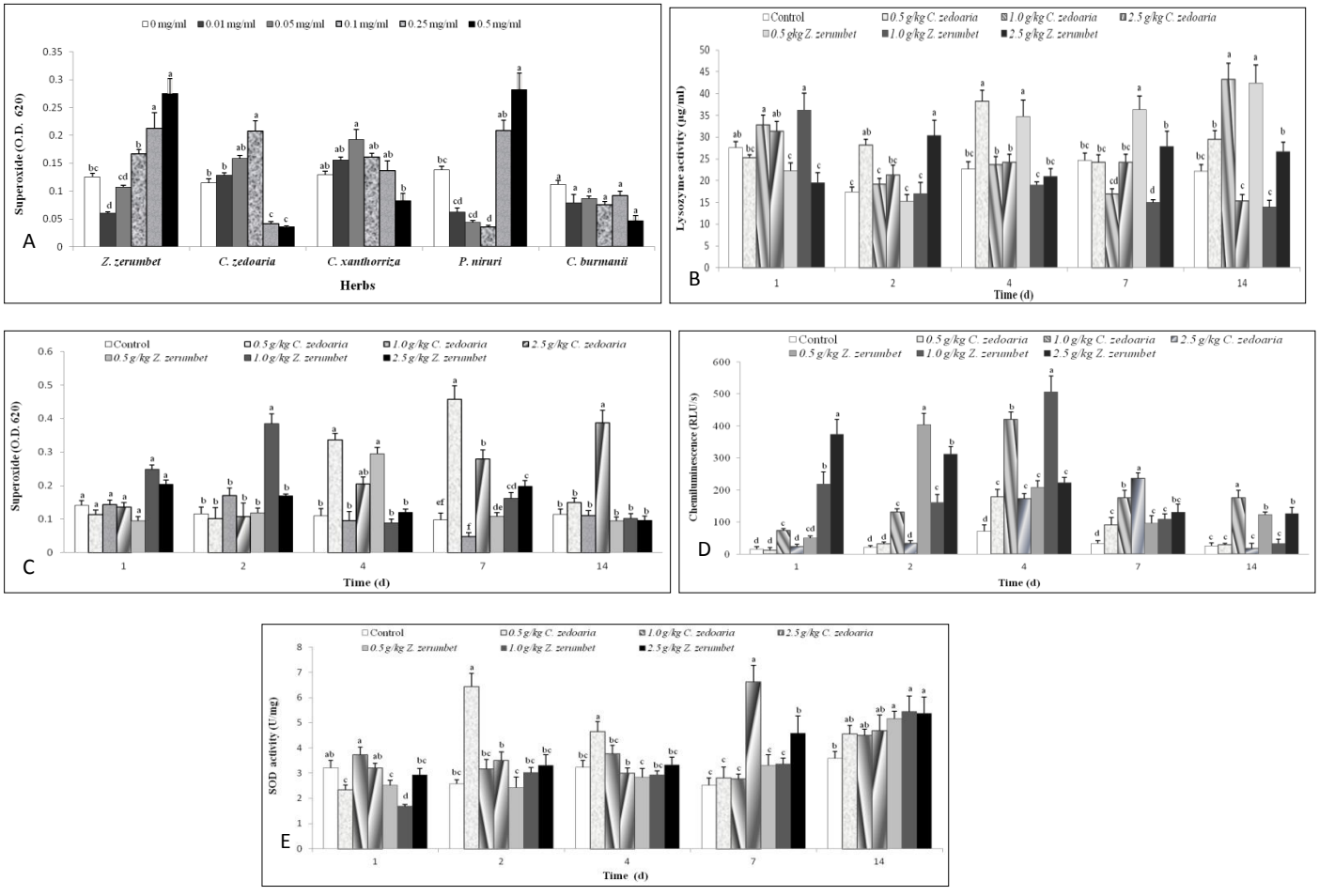

Figure 1: Superoxide production *in vitro* (A), superoxide production *in vivo* (B), chemiluminescence test (C), SOD activity (D) and lysozyme activity (E) of Table 2: Phagocytic index of *Epinephelus coioides* fed with experimental diets.

Discussion

Herbs are currently used in commercial aquaculture as growth promoting substances and anti-microbial agents (Galina *et al*., 2009). The herbal biomedicine active in aquaculture has the characteristics of growth promoting ability, tonic to improve the immune system, anti-microbial capability, appetite stimulation and anti stress characteristics due to the active compounds such as alkaloids, flavonoids, pigments, phenolics, terpenoids, steroids and essential oils (Citarasu, 2010). In the present study, two herbal plants were screened for their ability to enhance the non-specific immunity after incubation with the head kidney leukocytes of *E. coioides* mediated in respiratory burst activity in superoxide production analysis. Further study was by supplementing the herbs powder in fish diet. According to Sakai (1999) oral administration is non-stressful and allows mass administration regardless of fish size.

Enhancement of pathogen killing is the most important in macrophages of fish treated with immunostimulants (Sakai, 1999). This parameter usually shows after oral administration of immunostimulant as has been reported by Dugenci *et al*. (2003) that phagocytic activity of leukocytes increased in rainbow trout after being fed with 1% *Z. officinale*. This effect also has been observed in Nile tilapia when fed with 0.l% *Astragalus* extract (Ardo *et al*., 2008), and with sodium alginate at 20 mg/kg in *E. coioides* (Cheng *et al*., 2007). In this experiment, a significant increase of phagocytic rate was shown in all treated groups, whereas the lowest dosage of *C. zedoaria* (0.5 g/kg) showed the highest point from day 2 to day 4. Increasing dosage of *C. zedoaria* may shorten the time of induction ability in phagocyte rate of *E. coioides*. Phagocytosis and production of oxygen free radicals via the respiratory burst activity are important events in bactericidal pathways in fish, but mechanism are not well established (Sharp and Secombes, 1993). Oxygen-dependent killing mechanisms as mediated by reactive oxygen species (ROS) can be detected by the chemiluminescent and the NBT test (Sakai, 1999). In this study, we carried out two main methods to measure reactive oxygen activity; those are the superoxide production analysis by NBT test and reactive oxygen species production by chemiluminescent. Intracellular respiratory burst activities in fish leukocytes fed with *C. zedoaria* and *Z. zerumbet* were significantly enhanced. The low dosage of *C. zedoaria* (0.5 g/kg) could stimulate the superoxide production, and this activity was maintained from day 4 to 7. The fish receiving 1 g/kg *Z. zerumbet* diet had significantly enhanced superoxide production on day 2. It was also reported that administration of sodium glutamate at 20 mg/kg (Galina *et al*., 2009); 1-2 g/kg of sodium alginate in *E. coioides* (Yeh *et al*., 2008), and administration of *Astragalus* and *Lonicera* extract 0.1% in *Oreochromis niloticus* (Ardo *et al*., 2008) showed significantly enhanced respiratory burst activity.

The respiratory burst activity was also studied by a chemiluminescent method. Respiratory burst induced by phagocytosis of zymosan particles was measured in RLU per second. Enhancement of chemiluminescent also found in juvenile oil

flounder (*P. olivaceus*) after dietary intake 1% of *P. japonica* (Lee *et al*., 2002). In this study, respiratory burst activity was significantly enhanced in all treatment groups with different time of induction and different RLU/s value. Among the group treated with *C. zedoaria* extract, fish receiving 1 g/kg showed significantly stimulated chemiluminescent response. This enhancement reached the highest point on day 4 and returned to show inhibitory effects after day 7. Similar results of enhancement response were observed in fish supplemented with 1 g/kg *Z. zerumbet* diet. Significant increase was observed from day 1 to 14, whereas, the highest point was obtained on day 4. It should be noted that the NBT test and chemiluminescent responses revealed different results in their period to induce the respiratory burst activity. The fish fed 0.5 g/kg *C. zedoaria* showed a sharp increase in superoxide production on day 4 and 7. However in the same group, when tested with chemiluminescent it showed only a slight increase at the same time.

SOD is metalloenzymes that play major roles in protection of cells against oxidative damage (Metaxa *et al*., 2006). A significant difference in SOD activity was observed in juvenile of *E. fuscoguttatus* (Chiu *et al*., 2008) and *E.coioides* (Yeh *et al*., 2008) using dietary sodium alginate. The result showed enhancement of SOD activity in both species. In this study, dietary administration of *C. zedoaria* at 0.5 g/kg showed a significant enhancement in SOD after 2 and 4 days of treatment. Dietary administration of *Z. zerumbet* showed increased SOD on the final day of experiment (14 days). it can be correlated that SOD tends to be higher after which inducement of reactive oxygen species goes to resting phase.

A number of non specific humoral factors contribute to the fish's natural resistance to infections. They act in a variety of different ways to inhibit the growth and spread of pathogenic organisms. It was observed that the lysozyme activity was obtained by treatment with several Chinese herbal extracts in *P. crocea* (Jian and Wu, 2003), *C. carpio* (Jian and Wu, 2004), and *O. niloticus* (Ardo *et al*., 2008). As shown in this study, both *C. zedoaria* and *Z. zerumbet* significantly increase the lysozyme activity. There was a significant difference in serum lysozyme activity on day 2 and 4 when fish were fed with 0.5 g/kg *C. zedoaria* diet, while inclusion of 1 g/kg *C. zedoaria* in feed significantly increased lysozyme activity on day 14. On the other hand, treatment with *Z. zerumbet* at 0.5 g/kg significantly increases lysozyme activity after 4 days of feeding and maintained this level until final day of experiment.

In conclusion, we have demonstrated that supplementing *C. zedoaria* and *Z. zerumbet* in fish diets has the ability to enhance some of the non-specific immune responses of *E. coioides*. Supplementing those herbs in fish diet at low dosage has shown enhancement and positive effects in all tested non-specific immune parameters of *E. coioides*. It is recommended to use 0.5 g/kg of *C. zedoaria* extract diet or 1 – 2.5 g/kg *Z. zerumbet* extract diet. Moreover considering its low cost and immunostimulatory effects, *C. zedoaria* and *Z. zerumbet* could be suggested to be used for farmed fish to enhance their

immune system especially against pathogens.

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