



The Effectiveness of *Curcuma zedoaria* Extract in Enhancing Non Specific Immune Responses of Tiger Grouper (*Epinephelus fuscoguttatus*)

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Abstract

The present study was conducted to determine the effect of using turmeric extract (*Curcuma zedoaria*) on non specific immune of grouper (*Epinephelus fuscoguttatus*). During the study, fish were fed a diet containing 0, 0.5, 1.0, 2.5 g extract/kg feed twice a day for 14 days. Observations of non-specific immune parameters such as respiratory burst activity, chemiluminescent response, phagocytic activity, superoxidase dismutase (SOD) and lysozyme activity were sampled at 0, 1, 2, 4, 7 and 14 days. The results showed that fish fed *C. zedoaria* with 0.5 g/kg feed affected non-specific immune responses, and showed significant effect in the short term. On contrary, for long-term administration causes a decrease and inhibition of non-specific cellular defense activity. Thus, this study concluded that the administration of turmeric extract added to the feed can act as an immunostimulant and increase the non-specific immune response in grouper (*E. fuscoguttatus*).

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INTRODUCTION

Recently, aquaculture is recognized as one of promising food productions sector in some countries (Samad *et al.*, 2014; Kirubakaran *et al.*, 2016). However, in commercial aquaculture, fish mostly cultivated in intensive system with high stocking density, and this condition may lead to increase susceptibility to disease and often affect the fish's immune system (Rottmann *et al.*, 1992; Kirubakaran *et al.*, 2016). Currently, the use of antibiotics and chemicals in treating fish diseases is still commonly used, however it could cause pathogen resistance, bioaccumulation and environmental

pollution (Citarasu *et al.*, 2002; Sagdiç and Özcan, 2003). In addition, vaccines are considered too expensive for fish farmers and only specific to certain pathogens (Ardó *et al.*, 2008; Murray *et al.*, 2003; Gopalakannan and Arul, 2006). Therefore, one of methods to control fish diseases is by strengthening the fish defense responses through immunostimulants.

Esteban *et al.* (2000) mentioned that immunostimulants have the ability to activate non specific and specific immune responses. Moreover, Bricknell and Dalmo (2005) noticed that the use of

immunostimulants as dietary supplements may increase the non specific immune defense and provide resistance to pathogens during stressing periods such as grading and reproduction. Furthermore, Bafna and Mishra (2005) reported some components in herbal plants such as polysaccharides, peptides and lectins are believed to contribute in enhancing the immune response against pathogens and increasing appetite in fish.

Various plants have been examined to identify the immune response in fish. There were some medicinal plants that have been exposed to have immunostimulatory ability in fish for example: *Lactuca indica* in *Epinephelus bruneus* (Harikrishnan *et al.*, 2011); *Astragalus radix* in *Cyprinus carpio* (Taukhid *et al.*, 2007; Yin *et al.*, 2009); *Vitex negundo* in *Labeo rohita* (Sahu *et al.*, 2007; Nargis *et al.*, 2011); and *Lonicera japonica* on *Oreochromis niloticus* (Yin *et al.*, 2008).

Curcuma zedoaria is a among food source plant which usually cultivates in Indonesia. This medicinal plant is also known as white turmeric (Manfield *et al.*, 2005). The rhizome of *C. zedoaria* contains curcumin and refined oil which are considered as anti-cancer, anti-bacterial, anti-inflammatory and immunity (Hou and Jin, 2005). In accordance with the compounds in it, this plant is considered to have a positive influence to be applied in aquaculture. Thus, this study was carried out to examine the effectiveness of *C. zedoaria* extract in enhancing the non specific immune responses of *E. fuscoguttatus*.

METHODOLOGY

Place and Time

The present study was carried out from April to June 2021 in the Laboratory of Department of Aquaculture University of Samudra.

Research Materials

Materials used during experiment were tiger grouper weighed 60-80 g, *C.*

zedoaria extraction, 100 L Aquaria equipped with aeration system, dissecting set, experimental diets compounds such as: fish meal, fish oil, α -starch, vitamin and mineral mix, and cellulose. Moreover for detection of immune response, some solvents such as Percoll, Hanks solution/HBSS and NaCl. This study also used microscope, micrometer, centrifuge, pelleting machine and freezer.

Research Design

Prior to the experiment, *E. fuscoguttatus* (60-80 g) was reared and acclimatized in the hatchery of the Department of Aquaculture Universitas Samudra for 1 weeks. Water quality were retained at temperature of $28,0 \pm 1$ °C; pH 8.0 ± 1 , salinity $33,0 \pm 0.5$ ‰, and dissolved oxygen was maintained at 80-90% saturation.

During experimental periods, all treated fish were fed experimental diets mixed with 0, 0,5, 1,0 and 2,5 g/kg *C. zedoaria* extract diets. Data on non specific immune parameters such as: respiratory burst activity, phagocytic activity, lysozyme activity, superoxyde dismutase activity, and chemiluminescent were collected at 0, 1, 2, 4, 7 and 14 days of experiment. The experiment was carried out for 14 days. Application of feed was 3% of the biomass with the frequency of twice a day, at 07.00 and 17.00.

Work Procedure

Extract Preparation and Feed Supplementation

The fresh *Curcuma zedoaria* was cleaned and cut into small pieces and then dried at 37 °C for 3 days. After drying, the white turmeric is grounded into powder. 30 mg of this powder then dissolved and stirred in 60 ml of HBSS for 30 minutes, then filtered 3 times using filter paper and stored at 4 °C. Furthermore, experimental feed was prepared by mixing *C. zedoaria* extract into feed ingredients in 0, 0.5, 1, and 2.5 g extract/kg feed (Samad *et al.*, 2014) and then adding sufficient water. All feed ingredients are mixed and stirred

for 40 minutes, then put into a pellet press machine to make experimental diets. The respective diets were then dried in an oven for 24 hours at a 40 °C. After drying, the pellets were wrapped in plastic bags for further experimental use. In all treatment groups, immunity parameters were determined in 6 times of sampling at 0, 1, 2, 4, 7 and 14 days after feeding administration. 5 fish were taken per treatment group in each sampling time and then analyzed for non specific immune parameters such as superoxidase anion production, phagocytic rate, reactive oxygen, superoxidase dismutase and lysozyme activity.

Hematological Sampling

For hematological sampling, the blood from each specimen was taken from the tail vein using a 1 mL syringe, allowed to clot at 4 °C and serum was isolated and kept frozen at -80 °C for lysozyme activity analysis. Isolation of leukocytes was carried out by taking the head kidney, and filtered through a 100 mesh using Hank's balanced saline solution (HBSS, Sigma). The cell suspension was then placed on a Percoll solution at density gradient of 30–50% and centrifuged at 500x g rcf for 40 min at 4 °C. To analyze superoxidase dismutase activity, isolation of the liver was performed by using 1 g of the organ, then dissolved and mixed with SOD buffer (1% HBSS, 1% glycerol, 0.3% Triton X-100). Then grounded to a homogeneous liquid form followed by centrifugation at 3000 rpm for 30 minutes at 4 °C. The centrifuged supernatant was used for superoxidase dismutase activity analysis (Kuan *et al.*, 2012).

Non Specific Immunity Measurement

For respiratory burst activity, firstly put 100µl of leukocyte suspension (5×10^6 cells/ml) in 96 wells and sustained for 1 hour at 37 °C. To measure the reduction of nitro blue tetrazolium (NBT) was using an ELISA microplate reader at 630 (Cheng *et*

al., 2007). For Phagocytic measurement, put 50 µl of leukocyte cell suspension of kidney (5×10^6 cells/ml) on a glass slide, and then allowed to adhere for 20 min at 25 °C. Then, add 50µl of latex beads suspension (10^7 /ml, Sigma) to a single layer of leukocytes, then incubated for 30 min at 25 °C (Fujiki and Yano, 1997).

In this study, reactive oxygen was measured by chemiluminescent response. It was carried out by stored 100µl of leukocyte suspension into 96 wells, then added 100µl of 1 mM of luminal suspension and 100µl of 1 mg/ml zymosan HBSS. Superoxidase dismutase (SOD) activity was measured by its ability to inhibit superoxide radical-dependent reactions using the RANSOD kitt (Randox, Crumlin, UK). The activity of superoxide dismutase is measured by the degree of this reaction. Specific activity is expressed as units per mg protein. Lysozyme activity was measured based on the turbidimetric test according to the method described by Ellis (1990). A standard suspension (0.2 mg/ml) of *Micrococcus lysodeikticus* (Sigma) was prepared in 0.05 M sodium phosphate buffer (pH 6.2). One unit of lysozyme activity is defined as the amount of serum that causes a decrease in absorbance of 0.001/min.

Data Analysis

Data were analyzed using analysis of variance (ANOVA). If the overall difference is significant at the level of less than 5%, Tukey's test is used to compare the means between the individual treatments. Statistical analysis was performed using SAS software (SAS Inc. Cary, NC, USA). Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

The rate of phagocytic activity of *E. fuscoguttatus* administered different *C. Zedoaria* extract on 0, 1, 2, 4, 7 and 14 days is shown in Table 1. Fish fed 0.5 g extract/kg feed significantly increased activity rate phagocytic on days 2 and 4.

Table 1. The rate of phagocytic activity (%) in leukocytes of *E. fuscoguttatus* fed different doses of *C. zedoaria* extract.

Treatments	Dosage (g/kg)	Days					
		0	1	2	4	7	14
Control	0,0	32.39±	28.11±	39.23±	37.99±	37.28±	30.48±
		1,53	1,27 ^d	1,22 ^c	1,43 ^b	0,77 ^a	1,26 ^{ab}
<i>C. zedoaria</i>	0,5	32.39±	30.37±	61.97±	59.59±	35.95±	30.048
		1,53	0,93 ^{cd}	0,87 ^a	0,43 ^a	2,10 ^a	±1,29 ^{ab}
<i>C. zedoaria</i>	1,0	32.39±	41.25±	59.62±	35.85±	39.58±	32.751
		1,53	2,14 ^b	2,35 ^a	0,67 ^{bc}	1,32 ^a	±0,58 ^a
<i>C. zedoaria</i>	2,5	32.39±	66.68±	42.95±	34.79±	31.20±	28.258
		1,53	1,02 ^a	1,25 ^b	0,87 ^{bc}	1,51 ^b	±1,21 ^{bc}

*Data in the same column with different letters show significant differences (P<0.05) between different treatments.

The effect of *C. zedoaria* on respiratory burst activity that produces superoxide anion showed that at a dose of 0.5 g/kg extract feeding increased

significantly on day 4 and day 7. The lowest anion production was seen in the treatment of 1 g/kg feed on day 7 (Table 2).

Table 2. Production of superoxide anion (O₂⁻) in *E. fuscoguttatus* leukocytes fed different doses of *C. zedoaria* extract.

Treatments	Dosage (g/kg)	Days					
		0	1	2	4	7	14
Control	0,0	0,18±0,00	0,15±0,06 ^a	0,12±0,01 ^a	0,13±0,00 ^b	0,10±0,00 ^c	0,12±0,00 ^b
		0,18±0,00	0,12±0,02 ^a	0,11±0,01 ^a	0,35±0,02 ^a	0,46±0,00 ^a	0,16±0,02 ^b
<i>C. zedoaria</i>	1,0	0,18±0,00	0,19±0,05 ^a	0,13±0,03 ^a	0,10±0,01 ^b	0,03±0,01 ^c	0,12±0,00 ^b
		0,18±0,00	0,15±0,05 ^a	0,21±0,01 ^a	0,22±0,01 ^{ab}	0,23±0,01 ^b	0,37±0,00 ^a

*Data in the same column with different letters show significant differences (P<0.05) between different treatments.

Different doses of *C. zedoaria* extract also affected reactive oxygen species (ROS) phagocytes. It was detected by the chemiluminescent reaction method (Table

3). Fish administered a mixed diet of *C. zedoaria* showed a significant increase on the 4th and 7th day.

Table 3. Chemiluminescent response (RLU/s) in *E. fuscoguttatus* leukocytes fed different doses of *C. zedoaria* extract.

Treatments	Dosage (g/kg)	Days					
		0	1	2	4	7	14
Control	0,0	0,18±0,00	0,15±0,06 ^a	0,12±0,01 ^a	0,13±0,00 ^b	0,10±0,00 ^c	0,12±0,00 ^b
		0,18±0,00	0,12±0,02 ^a	0,11±0,01 ^a	0,35±0,02 ^a	0,46±0,00 ^a	0,16±0,02 ^b
<i>C. zedoaria</i>	1,0	0,18±0,00	0,19±0,05 ^a	0,13±0,03 ^a	0,10±0,01 ^b	0,03±0,01 ^c	0,12±0,00 ^b
		0,18±0,00	0,15±0,05 ^a	0,21±0,01 ^a	0,22±0,01 ^{ab}	0,23±0,01 ^b	0,37±0,00 ^a

*Data in the same column with different letters show significant differences (P<0.05) between different treatments.

Table 4 explained that the activity of the superoxide dismutase enzyme was seen to be highest on day 2 of the 0.5 g/kg

extract treatment. However, the activity showed decrease on day 4 and 7.

Table 4. Superoxide dismutase (SOD) (U/mg protein) activity in *E. fuscoguttatus* leukocytes fed different doses of *C. zedoaria*.

Treatments	Dosage (g/kg)	Days					
		0	1	2	4	7	14
Control	0,0	3,24±0,11	4,33±0,12 ^{ab}	2,86±0,12 ^{bc}	3,39±0,32 ^c	2,34±0,15 ^d	3,41±0,04 ^b
<i>C. zedoaria</i>	0,5	3,24±0,11	3,23±0,21 ^c	8,89±0,86 ^a	5,71±0,29 ^d	3,95±0,15 ^c	5,30±0,23 ^b
<i>C. zedoaria</i>	1,0	3,24±0,11	3,99±0,00 ^a	3,96±0,36 ^{bc}	3,24±0,17 ^a	3,11±0,17 ^b	5,18±0,25 ^c
<i>C. zedoaria</i>	2,5	3,24±0,11	3,35±0,00 ^{ab}	3,61±0,01 ^b	3,17±0,37 ^b	6,24±0,30 ^a	5,82±0,51 ^a

*Data in the same column with different letters show significant differences (P<0.05) between different treatments.

Variations activity in serum lysozyme are as shown in Table 5. A significant difference in serum lysozyme activity was detected on days 2 and 4 when fish were treated 0.5 g/kg extract.

Moreover, administered of 2.5 g/kg extract feeding caused an increase in serum lysozyme activity on day 14, and reach the highest value among all treatment groups.

Table 5. Lysozyme activity (µg/ml) in *E. fuscoguttatus* leukocytes fed different doses of *C. zedoaria*.

Treatments	Dosage (g/kg)	Days					
		0	1	2	4	7	14
Control	0,0	23,72±2,11	29,65±2,54 ^{ab}	18,86±3,12 ^a	23,39±0,78 ^{bc}	25,34±0,91 ^c	23,41±3,25 ^{bc}
<i>C. zedoaria</i>	0,5	3,24±0,11	27,52±0,81 ^{bc}	30,27±2,06 ^{ab}	40,71±2,99 ^a	26,35±2,71 ^b	31,25±1,22 ^b
<i>C. zedoaria</i>	1,0	3,24±0,11	34,92±3,22 ^a	21,39±3,26 ^c	25,42±0,97 ^b	18,21±2,17 ^b	17,52±2,33 ^a
<i>C. zedoaria</i>	2,5	3,24±0,11	24,35±2,39 ^{ab}	17,36±2,32 ^{bc}	36,17±3,47 ^b	37,64±3,27 ^a	45,82±5,51 ^c

*Data in the same column with different letters show significant differences (P<0.05) between different treatments.

In the present study, herbal plant (*C. zedoaria*) was tested for its ability to increase non-specific immunity after incubation using leukocytes derived from the head kidney of *E. fuscoguttatus* and then examined through the respiratory burst activity of superoxide anion (O₂⁻). After the test, the herbal extract was mixed into fish diets. According to Sakai (1999) giving the extract into feed and given orally does not cause stress and allows mass administration regardless of fish size.

The main purpose of giving immunostimulants is to increase the ability of macrophages to inhibit and stop the activity of pathogens. This macrophage ability noticed after the immunostimulant was consumed orally. The phagocytic activity of leukocytes also reported increase in rainbow trout after giving extract containing 1% *Zingiber officinale* (Düğenci *et al.*, 2003). The effect of giving herbal plant extracts was also observed in tilapia after being given a feed containing 0.1% of *Astragalus* extract (Ardó *et al.*, 2008). This present

experiment, also noticed a significant increase in the phagocytic rate, whereas the lowest dosage of *C. zedoaria* 0.5 g/kg showed the highest increment on day 2 to 4. This study detected that increasing the dose of *C. zedoaria* can shorten the induction time of head kidney phagocyte rate of tiger grouper.

It is essential to know the phagocytic activity and respiratory burst activity that produces superoxide anion in studying about bacterial framework in fish (Sharp and Secombes, 1993). Moreover, chemiluminescent and NBT assay is also needed to detect the pathway of macrophages to prevent diseases and to stop infection (Sakai, 1999). Data on this present study informed that respiratory burst activity in leukocytes of *E. fuscoguttatus* treated with *C. zedoaria* was significantly increased. Low dosage of *C. zedoaria* at 0.5 g/kg extract can stimulate production of superoxide anion (O_2^-) and this increase in activity occurred from day 4 to 7. *Astragalus* extract 0.1% in *Oreochromis niloticus* feed (Ardó *et al.*, 2008) showed a significant increase in respiratory burst activity caused by phagocytosis of zymosan particles.

In another study demonstrated the chemiluminescent response of *Rainbow trout* fed with vitamin C mixed with glucans (Verlhac *et al.*, 1996), whereas chemiluminescent response enhanced at 2 and 4 weeks after feeding. The same result was also reported by Lee *et al.* (2002), whereas chemiluminescent increased in *Paralichthys olivaceus* after received 1% of *Paecilomyces japonica*. In this present study, respiratory burst activity seen to be increased significantly in groups treated. Data showed that, the groups treated 1 g/kg extract were significantly triggered the chemiluminescent response and achieved the highest value on day 4 but detected an inhibitory on day 7.

The fish group treated with 0.5 g/kg extract showed a sharp increase in superoxide anion production on days 4 and 7. However, in the same group, when tested with chemiluminescent, it only showed a slight increase at the same time.

The difference between NBT and chemiluminescent reduction can be explained that in NBT reduction the reduction potential for cell binding was measured, however in chemiluminescent analysis, the product of the extracellular space was also determined.

From the results above, it was shown that the activity of superoxide dismutase (SOD) was slightly higher on day 14. The highest activity appeared on day 2 after received *C. Zedoaria* with a dosage of 0.5 g/kg extract. Metaxa *et al.* (2006) mentioned that superoxide dismutase is a metalloenzyme which withhold a major role in protection the cells against oxidative damage. SOD activity has also been investigated in *E. fuscoguttatus* (Chiu *et al.*, 2008) and *E. coioides* (Yeh *et al.*, 2008) which were tested using sodium alginate, both of which showed significant activity compared to controls.

CONCLUSION

The conclusion of this study is that *Curcuma zedoaria* extract has the ability to enhance the immunity responses in *Epinephelus fuscoguttatus*. Supplementation of this medicinal plant extract had a positive effect on non specific immunity and it is recommended to use 0.5 g/kg of *C. zedoaria* extract in aquaculture.

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REFERENCES

- Ardó, L., Yin, G., Xu, P., Váradi, L., Szigeti, G., Jeney, Z. and Jeney, G., 2008. Chinese herbs (*Astragalus membranaceus* and *Lonicera japonica*) and boron enhance the non-specific immune response of Nile tilapia *Oreochromis niloticus* and resistance against *Aeromonas hydrophila*. *Aquaculture*, 275(1-4), pp.26–33. <https://doi.org/10.1016/j.aquaculture.2007.12.022>

- Bafna, A.R. and Mishra, S.H., 2005. Immunomodulatory activity of methanol extract of roots of *Cissampelos pareira* Linn. *Ars Pharmaceutica*, 46(3), pp.253-262. <https://revistaseug.ugr.es/index.php/ars/article/view/5078>
- Bricknell, I. and Dalmo, R.A., 2005. The use of immunostimulants in fish larval aquaculture. *Fish & Shellfish Immunology*, 19(5), pp.457-472. <https://doi.org/10.1016/j.fsi.2005.03.008>
- Cheng, A.C., Tu, C.W., Chen, Y.Y., Nan, F.H. and Chen, J.C., 2007. The immunostimulatory effect of sodium alginate and iota-carrageenan on orange-spotted grouper *Epinephelus coioides* and its resistance against *Vibrio alginolyticus*. *Fish & Shellfish Immunology*, 22(3), pp.197-205. <https://doi.org/10.1016/j.fsi.2006.04.009>
- Chiu, S.T., Tsai, R.T., Hsu, J.P., Liu, C.H. and Cheng, W., 2008. Dietary sodium alginate administration to enhance the non specific immune responses and disease resistance of the juvenile grouper *Epinephelus fuscoguttatus*. *Aquaculture*, 277(1-2), pp.66-72. <https://doi.org/10.1016/j.aquaculture.2008.01.032>
- Citarasu, T., Babu, M.M., Sekar, R.R.J. and Marian, M.P., 2002. Developing Artemia enriched herbal diet for producing quality larvae in *Penaeus monodon*, Fabricius. *Asian Fisheries Science*, 15, pp.21-32. <https://doi.org/10.33997/j.afs.2002.15.1.003>
- Düğenci, S.K., Arda N. and Candan, A., 2003. Some medicinal plants as immunostimulant for fish. *Journal of Ethnopharmacology*, 88(1), pp.99-106. [https://doi.org/10.1016/s0378-8741\(03\)00182-x](https://doi.org/10.1016/s0378-8741(03)00182-x)
- Ellis, A.E., 1990. Lysozyme Assay. In: Stolen, J.S., Anderson, B.S., Robertson, B.S (eds.), *Techniques in Fish Immunology*. Fair Heaven, NJ: SOS Publication.
- Esteban, M.A., Mulero, V., Cuesta, A., Ortuño, J. and Meseguer, J., 2000. Effects of injecting chitin particles on the innate immune responses of gilthead seabream (*Sparus aurata* L). *Fish & Shellfish Immunology*, 10(6), pp.543-554. <https://doi.org/10.1006/fsim.2000.0271>
- Fujiki, K. and Yano, T., 1997. Effect of sodium alginate on the non-specific defense system of Common carp (*Cyprinus carpio* L). *Fish & Shellfish Immunology*, 7(6), pp.417-427. <https://doi.org/10.1006/fsim.1997.0095>
- Gopalakannan, A. and Arul, V., 2006. Immunomodulatory effects of dietary intake of chitin, chitosan and levamisole on the immune system of *Cyprinus carpio* and control of *Aeromonas hydrophila* infection in ponds. *Aquaculture*, 255(1-4), pp.179-187. <https://doi.org/10.1016/j.aquaculture.2006.01.012>
- Harikrishnan, R., Kim, J.S., Kim, M.C., Balasundaram, C. and Heo, M.S., 2011. *Prunella vulgaris* enhances the non-specific immune response and disease resistance of *Paralichthys olivaceus* against *Uronema marinum*. *Aquaculture*, 318(1-2), pp.61-66. <https://doi.org/10.1016/j.aquaculture.2011.05.020>
- Hou, J.P. and Jin, Y., 2005. The healing power of Chinese herbs and medical recipes. *The Haworth Press*. Inc. NY. pp. 565-567.
- Kirubakaran, C.J.W., Subramani, P.A. and Michael, R.D., 2016. Methanol extract of *Nyctanthes arbortristis* seeds enhances non-specific immune responses and protects *Oreochromis mossambicus* (Peters) against *Aeromonas hydrophila* infection. *Research in Veterinary Science*, 105, pp.243-248. <https://doi.org/10.1016/j.rvsc.2016.02.013>
- Kuan, Y.C., Sheu, F., Lee, G.C., Tsai, M.W., Hung, C.L. and Nan, F.H., 2012. Administration of recombinant Reishi immunomodulatory protein (rLZ-8) diet enhances innate immune responses and elicits protection against nervous necrosis

- virus in grouper *Epinephelus coioides*. *Fish & Shellfish Immunology*, 32(6), pp.986-993. <https://doi.org/10.1016/j.fsi.2012.02.008>
- Lee, C.H., Paek, N.S., Kim, D.S. and Kim, K.H., 2002. Effects of a *Paecilomyces japonica* supplemented diet on the chemiluminescent response of phagocytes and growth in juvenile olive flounder (*Paralichthys olivaceus*). *Aquaculture*, 208(1-2), pp.51-57. [https://doi.org/10.1016/S0044-8486\(01\)00799-2](https://doi.org/10.1016/S0044-8486(01)00799-2)
- Manfield, C., Trotter, C. and Barber, A., 2005. Spices: Recipe to delight the senses. *Periplus edition* (HK) Ltd. Singapore. p.252.
- Metaxa, E., Deviller, G., Pagand, P., Alliaume, C., Casellas, C. and Blancheton, J.P., 2006. High rate algal pond treatment for water reuse in a marine fish recirculation: water purification and fish health. *Aquaculture*, 252(1), pp.92-101. <https://doi.org/10.1016/j.aquaculture.2005.11.053>
- Murray, A.L., Pascho, R.J., Alcorn, S.W., Fairgrieve, W.T., Shearer, K.D. and Roley, D., 2003. Effects of various feed supplements containing fish protein hydrolysate or fish processing by-products on the innate immune functions of juvenile coho salmon (*Oncorhynchus kisutch*). *Aquaculture*, 220(1-4), pp.643-653. [https://doi.org/10.1016/S0044-8486\(02\)00426-X](https://doi.org/10.1016/S0044-8486(02)00426-X)
- Nargis, A., Khatun, M. and Talukder, D., 2011. Use of medicinal plants in the remedy of fish diseases. *Bangladesh Research Publication Journals*, 5(3), pp.192-195.
- Rottmann, R.W., Francis-Floyd, R. and Durborow, R., 1992. *The role of stress in fish disease* (p.474). Stoneville, MS: Southern Regional Aquaculture Center.
- Sagdiç, O. and Özcan, M., 2003. Antibacterial activity of Turkish spice hydrosols. *Food Control*, 14(3), pp.141-143. [https://doi.org/10.1016/S0956-7135\(02\)00057-9](https://doi.org/10.1016/S0956-7135(02)00057-9)
- Sahu, S., Das, B.K., Mishra, B.K., Pradhan, J. and Sarangi, N., 2007. Effect of *Allium sativum* on the immunity and survival of *Labeo rohita* infected with *Aeromonas hydrophila*. *Journal of Applied Ichthyology*, 23(1), pp.80-86. <https://doi.org/10.1111/j.1439-0426.2006.00785.x>
- Sakai, M., 1999. Current research status of fish immunostimulants. *Aquaculture*, 172(1-2), pp.63-92. [https://doi.org/10.1016/S0044-8486\(98\)00436-0](https://doi.org/10.1016/S0044-8486(98)00436-0)
- Samad, A.P.A., Santoso, U., Lee, M.C., and Nan, F.H., 2014. Effects of dietary katuk (*Sauropus androgynus* L. Merr.) on growth, non-specific immune and diseases resistance against *Vibrio alginolyticus* infection in grouper *Epinephelus coioides*. *Fish & Shellfish Immunology*, 36(2), pp.582-589. <https://doi.org/10.1016/j.fsi.2013.11.011>
- Sharp, G.J.E. and Secombes, C.J., 1993. The role of reactive oxygen species in the killing of the bacterial pathogen *Aeromonas salmonicida* by rainbow trout macrophages. *Fish & Shellfish Immunology*, 3(2), pp.119-129. <https://doi.org/10.1006/fsim.1993.1013>
- Taukhid, Suharni, I. and Supriyadi, H., 2007. Efektivitas ekstrak daun sambiloto (*Andrographis paniculata*) bagi pengendalian penyakit Koi Herpes Virus (KHV) pada ikan mas (*Cyprinus carpio*). *Jurnal Riset Akuakultur*, 2(3), pp.411-418. <http://dx.doi.org/10.15578/jra.2.3.2007.407-414>
- Verlhac, V., Gabaudan, J., Obach, A., Schüep, W. and Hole, R., 1996. Influence of dietary glucan and vitamin C on non-specific immune responses of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, 143(2), pp.123-133. [https://doi.org/10.1016/0044-8486\(95\)01238-9](https://doi.org/10.1016/0044-8486(95)01238-9)
- Yeh, S.P., Chang, C.A., Chang, C.Y., Liu, C.H. and Cheng, W., 2008. Dietary

sodium administration affects fingerling growth and resistance to *Streptococcus* sp. and iridovirus, and juvenile non-specific immune responses of the orange-spotted grouper, *Epinephelus coioides*. *Fish & Shellfish Immunology*, 25(1-2), pp.19-27. <https://doi.org/10.1016/j.fsi.2007.11.011>

Yin, G., Ardo, L., Thompson, K.D., Adams, A., Jeney, Z. and Jeney, G., 2009. Chinese herbs (*Astragalus radix* and *Ganoderma lucidum*) enhance immune response of carp, *Cyprinus carpio*, and protection against *Aeromonas hydrophyla*. *Fish & Shellfish Immunology*, 26(1), pp.140-145. <https://doi.org/10.1016/j.fsi.2008.08.015>

Yin, G., Ardo, L., Jeney, Z., Xu, P. and Jeney, G., 2008. Chinese herbs (*Lonicera japonica* and *Ganoderma lucidum*) enhance non-specific immune response of tilapia, *Oreochromis niloticus*, and protection against *Aeromonas hydrophila*, pp. 269-282. In Bondad-Reantaso, M.G., Mohan, C.V., Crumlish, M. and Subasinghe, R.P. (eds.). *Diseases in Asian Aquaculture VI*. Fish Health Section, Asian Fisheries Society, Manila, Philippines.