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Full Length Article

Effects of Holy Basil (*Ocimum sanctum*) Extract on the Growth, Immune Response and Disease Resistance against *Streptococcus agalactiae* of Nile Tilapia (*Oreochromis niloticus*)

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Abstract

This study was conducted to investigate the effects of supplementing the diet of Nile tilapia (*Oreochromis niloticus*) with holy basil (*Ocimum sanctum*) extract at the concentrations of 0 (control), 100, 200 and 400 mg/kg of feed for 30 days. The treated fish fed the holy basil extract at the 200 mg/kg level showed the significantly (P<0.05) greatest increases in specific growth rate and the greatest improvements in feed conversion ratio. The immune response, phagocytic activity and superoxide anion production were also stimulated the most on day 7 of the feed supplementation. In a streptococcal challenge experiment, the fish fed the holy basil extract at the 100 and 200 mg/kg levels showed 0% cumulative mortality, whereas the control group and the fish fed with extract at the 400 mg/kg level showed 100 and 40% mortality, respectively. The greatest stimulation of the immune response occurred in the fish fed the holy basil extract at the level of 200 mg/kg of diet. Thus, the analysis of the relative expression levels of three cytokine genes, which included interleukin-1β (IL-1β), interleukin-8 (IL-8) and transforming growth factor-β (TGF-β), were compared between a control group and a fish group fed the holy basil extract at the 200 mg/kg level. The expression of all the genes was significantly (P<0.05) greater in the treated than in the control fish, especially on day 7 of the feed supplementation. These results indicated that the addition of holy basil extract to fish feed at the rate of 200 mg/kg of diet could enhance the growth, immune response and disease resistance against *S. agalactiae* of Nile tilapia during a 30-day supplementation period.

Keywords: Holy basil; *Oreochromis niloticus*; *Streptococcus agalactiae*; Dietary Supplement; Cytokine genes

Introduction

Nile tilapia (*Oreochromis niloticus*) is one of the economically important freshwater fish in Thailand. This fish species is easy to manage and grows rapidly, reaching a harvest maturity of 1 to 1.5 kg in 8 to 10 months. In 2012, the production and value of this fish was approximately 236,500 tons and 11,162.1 million baht, respectively (Department of Fisheries, 2014). However, the culture of this fish is limited because of disease outbreaks. Streptococcosis is a disease caused by infection with the bacterium *Streptococcus agalactiae*. The symptoms of this disease, which causes more than 50% fish mortality in 3–7 days (Yanong and Floyd, 2002), are abnormal behavior, including erratic-spiral swimming, combined with the external clinical signs of exophthalmia, darkening body and swollen abdomen (Areechon *et al*., 2005). Farmers routinely mix antibiotics with their fish feed because antibiotic treatment is an effective method to control streptococcal infection. However, the overuse of antibiotics may lead to the development of antibiotic-resistant bacteria and the accumulation of antibiotics in fish can be dangerous to consumers. Therefore, the alternative methods including such approaches as feed supplementation with plant extracts are needed to manage streptococcal disease in aquaculture (Cheunbarn and Cheunbarn, 2015).

Holy basil (*Ocimum sanctum*) is a medicinal plant belonging to the family Lamiaceae. The leaves of holy basil contain water-soluble phenolic compounds and various components, such as eugenol, methyl eugenol and caryophyllene that act as immunostimulants (Bairwa *et al*., 2012). In aquaculture, the supplementation of feed with holy basil extract was studied in several fish, including *Oreochromis mossambicus* (Logambal *et al*., 2000), *Epinephelus tauvina* (Sivaram *et al*., 2004), *Catla catla* (Chitra and Krishnaveni, 2011), *Cyprinus carpio* (Pavaraj *et al*., 2011) and *Labeo rohita* (Das *et al*., 2013). Moreover, holy basil also affects the resistance to pathogens, such as *Vibrio harveyi* (Sivaram *et al*., 2004) and *Aeromonas hydrophila* (Logambal *et al*., 2000; Chitra and Krishnaveni,

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2011; Pavaraj *et al*., 2011; Das *et al*., 2013).

The purposes of the present research were to investigate the effects of the supplementation of fish feed with holy basil extract at different concentrations on Nile tilapia growth, immune response and disease resistance against the bacterial pathogen *S. agalactiae*. The relative expression levels of cytokine genes, including interleukin-1β (IL-1β), interleukin-8 (IL-8) and transforming growth factor-β (TGF-β), were also determined using semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). The holy basil extract in fish feed may be an alternative practice for disease management. The holy basil extract is a natural material that may replace the use of antibiotics in aquaculture. Although antibiotics effectively control diseases, they can remain in the environment and in consumers. Thus, the replacement of antibiotics with the plant extract can potentially reduce the cost of production and increase the sustainability of aquaculture.

Materials and Methods

Plant Extract

Holy basil was purchased from a local market in Phayao Province, Northern Thailand. The leaves were washed and dried at 50–60°C for 2 days and then ground to a powder. A 100 g aliquot of the holy basil powder was extracted with 300 mL of absolute ethanol (1:3) at room temperature for 48 h. The slurry was filtered, and insoluble fraction was removed by centrifugation at 4,800 rpm at 4°C for 20 min. The upper phase was transferred and condensed at 35°C by rotary evaporator until the solvent had evaporated (Sivaram *et al*., 2004). The crude extract was stored at 4°C until used.

Animals and Experimental Design

Healthy juvenile Nile tilapia with an average weight of 40±5 g were purchased from a commercial farm in Chiang Mai province, northern Thailand and stocked in 500 L tanks. The fish were acclimatized for 2 weeks and fed a commercial diet (32% protein). After acclimatization, the fish were divided into four experimental groups, which were provided feed supplemented with the holy basil extract at concentrations of 0, 100, 200 and 400 mg/kg of diet. The crude extract was dissolved in absolute ethanol at various concentrations and mixed with the commercial diet. Each experimental group contained 90 fish, which were divided among three tanks and fed one of the experimental diets twice daily for 30 days.

Immune Responses

Aliquots of the blood of nine fish from each experimental group were collected on days 1, 3, 7, 15 and 30 of the feed supplementation period. The fish were randomly sampled and anesthetized with MS-222 (Sigma-Aldrich, Saint Louis, MO, USA) suspension in water. The blood was collected from the caudal vein with a 1 mL plastic syringe rinsed with anticoagulant (10% EDTA). For leukocytes, 1 mL of the collected blood from each fish was suspended in 2 mL of RPMI 1640 medium (PAA Laboratories GmbH, Pasching, Austria) and then separated by density gradient centrifugation. The 3 mL of diluted blood was gently rinsed into 3 mL of Lymphoprep (Axis-Shield, Oslo, Norway) and centrifuged at 2,500 rpm for 30 min at 25°C. The leukocyte phase was pipetted, resuspended in 2 mL of PBS and centrifuged twice for 10 min, each time at 1,500 rpm and 25°C.

The leukocytes were adjusted to 2 x 10^7 cells/mL and deposited on a cover slip before they were incubated at room temperature for 2 h. The non-adherent cells were removed, and the cells adhering to the cover slip were washed three times with PBS. Baker's yeast (2×10^7 cells/mL) was added to these leukocytes and the mixture was incubated at room temperature for 1 h. After incubation, the cover slip was washed three times with PBS and stained with the Wright rapid stain set (RVL Supply, Bangkhen, Bangkok, Thailand). The percentage of phagocytic activity (PA) was calculated from the number of phagocytotic cells per 100 leukocytes.

The superoxide anion production was determined using the nitroblue tetrazolium (NBT) test. The adjusted leukocytes were deposited on a cover slip and incubated in a moisture chamber at room temperature for 30 min. After incubation, the cells were washed three times with PBS to remove the non-adherent cells. NBT (0.2%) was added to the adherent leukocytes, which were then incubated in the moisture chamber at room temperature for another 30 min. The cover slip was washed three times with PBS, air dried and placed under a microscope to count the number of deep blue cells per 100 leukocyte cells.

Growth

After the 30-day dietary treatment, the weights of the individual fish from each experimental group were measured to calculate the specific growth rate (SGR). The total amount of diet consumed by each experimental group was weighed to calculate the feed conversion ratio (FCR). The SGR and FCR were calculated according to Ricker (1979) as follows:

$$SGR = \frac{\ln(\text{final weight}) - \ln(\text{initial weight}) \times 100}{\text{Total duration of the experiment}}$$

$$FCR = \frac{\text{Total amount of feed}}{\text{Weight gain}}$$

Disease Resistance

S. agalactiae was isolated from diseased Nile tilapia and

obtained from the Department of Aquaculture, Kasetsart University. A suspension of *S. agalactiae* was prepared in sterile 0.85% NaCl and diluted to 2×10^7 cells/mL. After the 30-day feed supplementation, thirty fish from each experimental group were intraperitoneally injected with 0.1 mL of bacterial suspension containing 2×10^7 cells/mL. The control group was divided into two groups, a positive control was injected with the bacterial suspension and a negative control was injected with the PBS solution. Dead fish were removed and recorded daily for 15 days to calculate the cumulative mortality. Bacteria were isolated from the head kidney to confirm the cause of mortality.

Expression of Cytokine Genes using Semi-quantitative RT-PCR

The head kidney tissues were collected from nine fish that fed on the 200 mg/kg level of holy basil extract and nine untreated control fish on days 1, 3, 7, 15 and 30 of feed supplementation. The tissues were extracted for total RNA using TRIzol reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions. The tissues were first homogenized in 1 mL of TRIzol reagent using glass gliders. The homogenate was incubated at room temperature for 5 min. Two hundred microlitres of chloroform was added and vigorously shaken for 15 s. Then, the mixture was centrifuged at 12,000 rpm at 4°C for 15 min. After the color less upper phase was transferred to a new 1.5 mL microcentrifuge tube, the total RNA was precipitated by adding 500 µL of isopropanol followed by centrifugation. The quantity and quality of the total RNA were determined by measuring the OD at 260 nm and by electrophoresis on 1.0% formaldehyde-agarose gel, respectively.

The first-strand cDNAs were synthesized with 1 µg of total RNA and 1 µL of oligo (dT_{18}) primer using the iScript™ Select cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The reaction was incubated at 65°C for 5 min and chilled on ice for 1 min. One microlitre of Moloney murine leukemia virus (MMLV) reverse transcriptase, 4 µL of 5× first-strand buffer and 2 µL of 10 mM dNTPs were added and incubated first at 42°C for 90 min and then at 85°C for 5 min. The first-strand cDNAs were stored at -20°C until used.

The expression levels of three cytokine genes, including IL-1β, IL-8 and TGF-β, were determined using β-actin as an internal control. Primers were designed from expressed sequence tags (ESTs) sequences obtained from a head kidney library (Wangkahart *et al*., 2008) (Table 1).

A standard PCR reaction was performed in a 25 µL reaction containing 0.5 µL of cDNA template, 0.2 mM of each dNTP, 1× *Taq* buffer, 1 U of *Taq* DNA polymerase and 1 µM of forward and reverse primers. The cycling conditions included an initial denaturation at 95°C for 5 min, denaturation of 95°C for 30 s and annealing at 55°C for 30 s, with an extension at 72°C for 30 s followed by 72°C for 5 min. The $MgCl_2$ concentration and cycle numbers were optimized for the analysis of relative gene expression. The optimal concentration between 1 to 4 mM $MgCl_2$ and the cycle numbers, including 22, 25, 28, 32 and 35, were examined using the standard PCR conditions.

Ten microlitres of each PCR product was electrophoresed on a 1.5% agarose gel, stained with ethidium bromide and visualized with UV light. The intensity of the resulting bands was measured with a gel documentation system (SynGene, Frederick, MD, USA) and subsequently analyzed with GeneTools software. The percentage of the relative expression of each cytokine gene was calculated from the ratio between the relative expression of the gene and that of β-actin.

Data Analysis

Data were presented as means ± standard deviation. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by a post hoc test (Duncan's new multiple range test). Significance was set at $P<0.05$.

Results

Immune Responses

Compared with the control group, the phagocytic activity in all the experimental groups whose diets were supplemented with the holy basil extract was significantly ($P<0.05$) increased during the 30-day supplementation (Fig. 1A). The fish treated with any concentration of the extract showed the highest phagocytic activity on day 7, especially at the 200 mg/kg level. However, the feeding of holy basil extract at the highest test concentration of 400 mg/kg of diet resulted in a significant ($P<0.05$) decline in phagocytic activity.

All the dietary concentrations of holy basil extract stimulated the nitrobluetetrazolium staining of cells when compared with the cells of the control group (Fig. 1B). The effect was strongest and significant ($P<0.05$) after 7 days of supplementation, especially at the concentration of 200 mg/kg of diet. However, the fish fed the holy basil extract at 100 rather than 200 mg/kg of diet showed a significantly ($P<0.05$) lower NBT.

Growth

The fish fed the holy basil extract at a concentration of 200 mg/kg of feed showed an enhanced specific growth rate when compared with that of the control fish and that of the experimental groups fed the extract at the rate of 100 or 400 mg/kg of diet; however, only the difference between the 100 and 200 mg/kg concentrations was statistically

Table 1: Gene-specific primers used for semi-quantitative RT-PCR

Gene	Accession number	Primer sequence 5' to 3'	Product length (bp)
IL-1β	FF279762	F: GTCACTGACAGCCAAAAGAG R: GACAGACATGAGAGTGCTGA	339
IL-8	FF279523	F: CTGTCATGGTCTGCATCTC R: GAGGAAGTGGTCTTCTGCT	256
TGF-β	FF280142	F: CATAAGCCAACGGGTTAC R: CCCCATGTCCACATTATC	359
β-actin	FF280738	F: CGTGACCTCACAGACTACCT R: CTGTGATCTCCTTCTGCATC	409

Table 2: Specific growth rate and feed conversion ratio of fish fed four different concentrations of holy basil extract (control, 100, 200 and 400 mg/kg of diet) for 30 days

Concentration of the extract (mg/kg diet)	Average weight gain (g)	Specific growth rate (%)	Feed Conversion Ratio
0	18.28 ± 2.28^{ab}	1.18 ± 0.12^{ab}	1.02 ± 0.14^{b}
100	17.04 ± 1.73^{b}	1.12 ± 0.11^{b}	0.64 ± 0.07^{a}
200	25.78 ± 6.40^{a}	1.58 ± 0.31^{a}	0.55 ± 0.12^{a}
400	19.18 ± 4.00^{ab}	1.23 ± 0.23^{ab}	0.68 ± 0.15^{a}

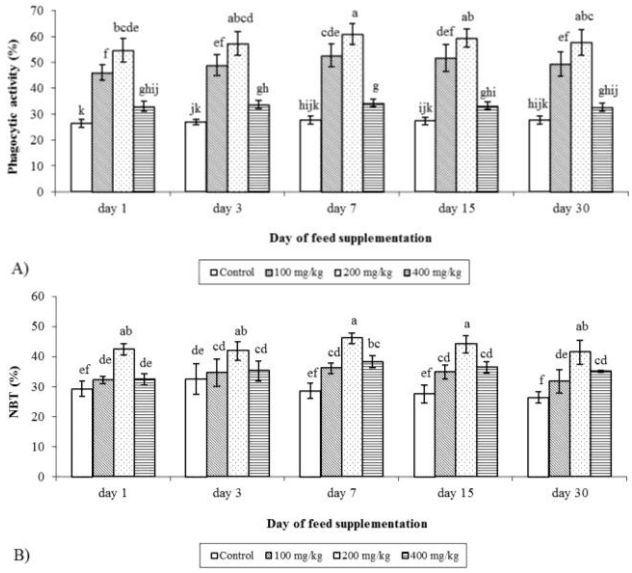

Fig. 1: Immune responses measured as A) leukocyte phagocytic activity and B) superoxide anion production in fish fed four different concentrations of holy basil extract (control, 100, 200 and 400 mg/kg of diet) for 30 days

significant (P<0.05) (Table 2). Moreover, the holy basil extract also influenced the feed conversion ratio (FCR). The experimental groups that included the fish fed any concentration of the holy basil extract showed a significantly (P<0.05) better FCR than that of the control fish (Table 2).

Disease Resistance

Oral administration of the holy basil extract to the fish at the concentrations of 100 and 200 mg/kg of diet enhanced their disease resistance against *S. agalactiae* and led to 0% mortality (Fig. 2). The positive control (injected with bacterial suspension) and the fish fed the holy basil extract at the 400 mg/kg level showed 100 and 40% mortality, respectively, at day 15. The negative control (injected with PBS) also showed 0% mortality.

Table 3: Optimal $MgCl_2$ concentrations and cycle numbers for amplifying the expression level of cytokine genes

Gene	$MgCl_2$ concentration (mM)	cycle number
IL-1β	4	28
IL-8	2	28
TGF-β	1.5	28
β-actin	1.5	28

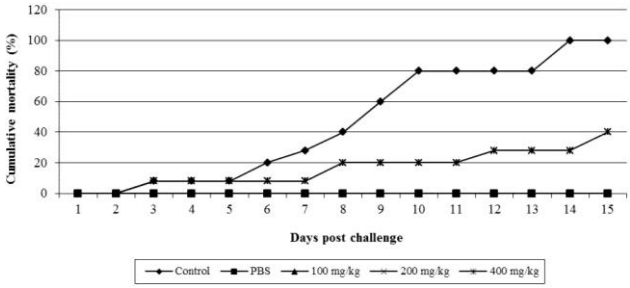

Fig. 2: Cumulative mortality (%) of *S. agalactiae*-challenged fish during 15 days following injection with the bacterium and the completion of the 30-day dietary administration of holy basil extract at four concentrations (control, 100, 200 and 400 mg/kg of diet) for 30 days

Expression Levels of Cytokine Genes

The optimal $MgCl_2$ concentration for each primer was determined using a range of concentrations (1–4 mM). The concentrations that gave the highest yields were chosen (Table 3). The optimal cycle numbers were determined similarly, and for each gene, the cycle number that generated the highest yield before the product reached the plateau phase was chosen (Table 3).

The relative expression levels of the IL-1β, IL-8 and TGF-β cytokine genes were determined by normalization with the expression of β-actin. The expression patterns of all the cytokine genes were similar (Fig. 3). After feeding the holy basil extract at 200 mg/kg of diet for 30 days, the expression of all the cytokine genes was significantly

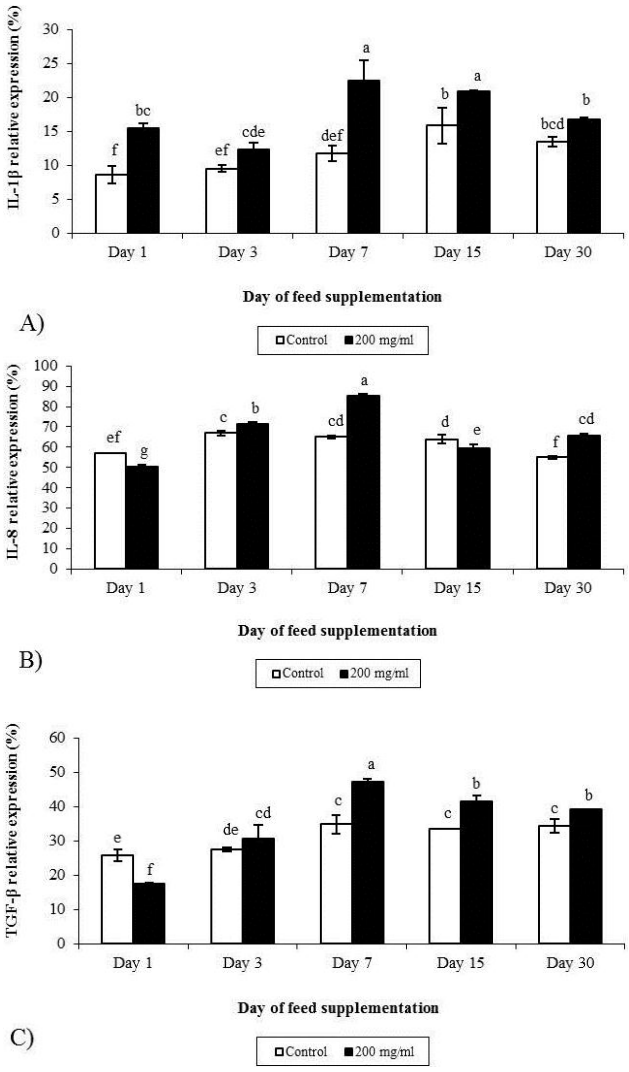

Fig. 3: Relative expression of the A) IL-1β, B) IL-8 and C) TGF-β genes of fish fed holy basil extract at concentration of 0 (control) and 200 mg/kg of diet for 30 days

(P<0.05) increased compared with that of the control group. The expression of the genes was stimulated to a significantly (P<0.05) higher degree in the treated than in the untreated fish. The transcription levels gradually increased until reaching their highest on day 7; then, the levels decreased by day 15 and 30.

Discussion

The previous studies of the effects of supplementing fish diets with plant extract mostly examined nonspecific immune responses, such as phagocytic activity and respiratory burst activity. The results of these studies showed that these parameters could be improved using extracts from several plants, such as *Spirulina platensis* (Watanuki *et al*., 2006), *Curcuma longa* (Swagatika *et al*., 2008) and *Echinacea purpurea* (Mohamad and Abasali, 2010). Additionally, the use of Holy basil extract as a feed supplement was especially effective in stimulating these immune responses (Logambal *et al*., 2000; Sivaram *et al*., 2004). In the present study, the holy basil extract at concentrations of 100 and 200 mg/kg of diet induced significant increases in the levels of phagocytic activity and superoxide anion production, and these increases were highest on day 7 of supplementation. However, increasing the concentration to 400 mg/kg of diet did not further increase the immune responses, as well as the results from Sivaram *et al*. (2004). Although eugenol, that is an important component in holy basil can stimulate immunity, but at the high concentrations can affect the toxicity to fish (Doleželová *et al*., 2011).

The feeding of holy basil extract effected growth of Nile tilapia. The specific growth rate (SGR) of the fish fed the holy basil extract at 200 mg/kg of diet was higher than that of the other groups but differed significantly only from that of the 100 mg/kg treatment group. Sivaram *et al*. (2004) suggested that greasy grouper fed of holy basil at the concentration of 200 mg/kg of diet also increased the highest SGR. The feed conversion ratio (FCR) is a measure of the efficiency of food use. The holy basil extract resulted in the treated fish could improve their (lower) FCRs at all the treatment concentrations.

The oral administration of holy basil extract for 30 days could enhance disease resistance against *S. agalactiae* in Nile tilapia. An extract of holy basil can play an important role in immunostimulant functions (Bairwa *et al*., 2012) and antibacterial activity (Singh *et al*., 2005; Aggarwal and Goyal, 2012). In the present study, fish fed any of the test concentrations of the holy basil extract showed stimulation of the immune system and reduced cumulative mortality. Feeding of holy basil at the concentration of 100 and 200 mg/kg of diet also significantly reduced the mortality from *V. harveyi* than control in greasy grouper (Sivaram *et al*., 2004). Moreover, dietary holy basil extract of 0.2% showed significantly higher protection relative percentage survival of *Labeo rohita* against *A. hydrophila* infection than control (Das *et al*., 2013).

The relative expression levels of cytokine genes, including IL-1β, IL-8 and TGF-β, were investigated using the semiquantitative RT-PCR technique. The optimal $MgCl_2$ concentration and cycle number for maximizing the yields of the amplified PCR products were determined. In the present study, the expression of three cytokine genes of the fish fed holy basil extract was increased significantly above that of the control group. IL-1β and IL-8 are two important proinflammatory cytokines and reliable markers of inflammatory responses (Corripio-Miyar *et al*., 2007). IL-1β, one form of IL-1, is induced in fish by extracts of plants, such as *Spirulina platensis* (Watanuki *et al*., 2006) and *Curcuma longa* (Panprommin *et al*., 2011). IL-8 is another member of the chemokine family, which is produced by

many cell types and plays an important defensive role against inflammation and infection (Laing and Secombes, 2004) in the innate immune system. Several plants also induce increased expression levels of the IL-8 gene. For example, the root of *Panax ginseng* (Sonada *et al*., 1998) and the rhizomes of *Curcuma longa* (Panprommin *et al*., 2011) stimulate the expression of IL-8 in human monocytic cell lines and the head kidney of Nile tilapia, respectively. TGF-β, another type of cytokine, plays a role in immunity and can be stimulated by plant extracts, as shown in the present study.

In conclusion, supplementation with 200 mg of holy basil extract per kg of diet could significantly enhance the growth and immune system function of Nile tilapia and protect the fish against *S. agalactiae* infection. In addition, the relative expression levels of three cytokine genes were also stimulated. However, the sources and harvest season of the holy basil plant are the major factors that will affect the quality of the extract.

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