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The effects gotu kola (*Centella asiatica*) powder on growth performance, skin mucus, and serum immunity of Nile tilapia (*Oreochromis niloticus*) fingerlings

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ABSTRACT

The present study was conducted to assess the possible effects of gotu kola (*Centella asiatica*) powder (GKP) on skin mucus and serum immune response, as well as growth performance of Nile tilapia, *Oreochromis niloticus*. Three hundred twenty Nile tilapia fingerlings (average weight of 17.84 ± 0.08 g) were divided into four treatments and fed four levels of gotu kola powder (GKP) as following 0, 5, 10, and 20 g kg^{-1} diet for 61 days. Completed Randomised Design with four replications was applied. The results showed that fish fed 5 g kg^{-1} GKP significantly improved skin mucus lysozyme (SMLA) and skin mucus peroxidase activities (SMPA) ($P < 0.05$). However, no significant differences in SMLA and SMPA were observed in fish fed 10 and 20 g kg^{-1} GKP compared to the control group ($P > 0.05$). For serum immunity, dietary administration of GKP showed significantly improved serum lysozyme and serum peroxidase activities compared to control group ($P < 0.05$). The highest value was found in fish fed 5 and 10 g kg^{-1} GKP ($P < 0.05$). Similarly, a significant increase in alternative complement (ACH50), phagocytosis, and respiratory burst activities were recorded in fish fed 5 and 10 g kg^{-1} GKP compared to the control ($P < 0.05$). However, no significance was observed in fish fed 20 g kg^{-1} GKP compared to the control. Similarly, no significant difference in growth performance, feed conversion ratio, and survival rate was observed in fish fed GKP compared to the control. In summary, diets supplemented with GKP (10 g kg^{-1}) increased serum and mucosal immunity. However, GKP supplementations had no effects on Nile tilapia growth and survival rate.

1. Introduction

Aquaculture has been considered as one of the most rapidly animal food-producing industries that provide to the world's well-being and wealth (Edwards et al., 2019). The rapid development of aquaculture and intensification has led to the stressful condition and consequence of the outbreak of diseases (Kennedy et al., 2016). Bacterial infections have been considered as a significant obstacle in intensive aquaculture farming because they cause considerable loss of production resulting in sizeable economic impact (Ahmadifar et al., 2019; Ngajilo, Jeebhay, 2019). Antibiotics and chemotherapeutics are common agents used to handle the outbreak of those diseases in aquaculture. However, the application of these prophylactics leads to the emergence of antimicrobial resistant bacteria and adverse impacts on the water environment (Done et al., 2015; Santos, Ramos, 2018). In the last decades, the scientific community has paid great attention to the use natural immunostimulants, such as prebiotics, probiotics, and medicinal plants in aquaculture (Dawood et al., 2018; Song et al., 2014; Van Hai, 2015; Wang et al., 2017; Zorriehzahra et al., 2016). Supplementation of natural prophylactics is considered as a promising preventive practice

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which assists in maintaining fish welfare, and a healthy environment (Bruce, Brown, 2017; Guardiola et al., 2016; Pohlenz, Gatlin Iii, 2014). Among them, medicinal plants have been considered as a promising one. Plant products have been widely applied in aquaculture to enhance growth performance, immune system and to provide antioxidant effects, due to their biological compounds, such as alkaloids, terpenoids, saponins, and flavonoid elements (Reverter et al., 2017). Moreover, dietary inclusion of plant products can reduce the risks associated with antibiotics and chemotherapeutic, and be considered as one of the most effective means for diseases prevention in aquaculture (Nayak, 2010). Thus, there is a rising trend in use of natural products in recent decades with a focus on medicinal plants as an alternative to antibiotics.

As alternatives to antibiotics, natural, environmentally friendly, and cost-effective, medicinal plants have been widely applied in aquaculture (Abdel Rahman et al., 2018; Kaleo et al., 2019). One of these feed additives is Gotu Kola (*Centella asiatica*), a herbaceous, frost-tender perennial plant, is a capable compound applied in conventional asiatic medicine and is known to cure human diseases (Roy et al., 2013). As approved in recent pharmacology, it displays multiple pharmacological properties, such as an antitumor, antimicrobial, and anti-inflammatory (Vaishali et al., 2016). In fish and shellfish, it has currently turned into an essential antibiotic for inhibiting enteritis and other diseases via the co-administration with other medical products (Haniffa, Kavitha, 2012; Rattanachaikunsopon, Phumkhachorn, 2010). Gotu kola can be used as immunomodulators for infectious diseases in aquatic animals. However, no data is available about using gotu kola on the Nile tilapia's growth performance and immune response.

Tilapia is one of the most important farmed fish worldwide and its production has increased fourfold over the last decades due to of its well-adapted for intensive farming, high commercial value, and unfluctuating market prices (Wang, Lu, 2016). Tilapia's world production was evaluated to be 6.532 million metric tons in 2018 (GOVL, 2017) and forecasted to touch 7.3 million metric tons by 2030 (Behera et al., 2018). The expansion and intensification of Nile tilapia farming makes the fish more susceptible to infectious diseases, consequently results in vast economic loss from the fish mortalities and the cost of antibiotics (Guoliang et al., 2001; Sakai, 1999). So, harmless and cost-effective alternatives to antibiotics are necessary to protect fish from the harmful effects of antibiotics (Reverter et al., 2014). Thus, this study aimed to assess the effects of Gotu kola (*C. asiatica*) powder on growth performance, skin mucus, and serum immunity of Nile tilapia (*O. niloticus*).

2. Materials and methods

2.1. Gotu kola powder preparation

The gotu kola (*Centella asiatica*) leaves and stems were collected from Chiang Mai local market. They were oven-dried for 48 h at 50 °C, then ground into a fine powder (0.2-mm) for diet preparation.

2.2. Fish diets

The basal diet was formulated based on the previous investigation of Van Doan et al. (2018). This formulation had been demonstrated its suitable for Nile tilapia (*O. niloticus*). An extruder was used to produce fish pellets. The pellets were dried in the oven for 48 h at 50 °C and consequently reserved in plastic bags at 4 °C. Ingredients and chemical compositions (g kg^{-1}) of the basal diets are given in Table 1.

2.3. Study design

Oreochromis niloticus (mono-sex) fingerlings were purchased from the Chiang Mai Pathana Farm Co., Ltd., Chiang Mai. Upon arrival, fish were released into 5 × 5x2 m cages and given a commercial feed (CP, 9950 -Charoen Pokphand Foods PCL company) for two months. Before the experiment, fish were fed the control diet for two weeks. A total of 320 fingerlings (average weight of 17.84 ± 0.08 g $fish^{-1}$) were distributed into 16 glass tanks (150 liters), consisting of 20 fish $tank^{-1}$. A Completely Randomised Design (CRD) with four replications was employed for 61 days. Growth rate, skin mucus, and serum immune responses of the *O. niloticus* were computed after 61 days post-feeding. Fish in each treatment was given the diet *ad libitum* at 8:30 a.m. and 5:30 p.m., the water temperature was ranged from 26.59 ± 1 °C, and pH preserved at 7.79 ± 0.70. The dissolved oxygen was maintained as a minimum of 5 mg $litre^{-1}$.

Table 1
The formulation and proximate composition of the experiment (g kg^{-1}).

Ingredients	Diets (g kg^{-1}) Diet 1
Fish meal	300
Corn meal	145
Soybean meal	270
Wheat flour	60
Rice bran	150
Cellulose	30
Soybean oil	30
Premix[a]	10
Vitamin C[b]	5
Proximate composition of the experimental diets (g kg^{-1} dry matter basis)	
Crude protein	322.06
Crude lipid	74.75
Fibre	52.48
Ash	106.68
Dry matter	817.80
GE (cal/g)[c]	4,105

[a] Vitamin and trace mineral mix supplemented as follows (IU kg^{-1} or g kg^{-1} diet): retinyl acetate 1,085,000 IU; cholecalciferol 217,000 IU; D, L-a-tocopherol acetate 0.5 g; thiamin nitrate 0.5 g; pyridoxine hydrochloride 0.5 g; niacin 3 g; folic 0.05 g; cyanocobalamin 10 g; Ca pantothenate 1 g kg^{-1}; inositol 0.5 g; zinc 1 g; copper 0.25 g; manganese 1.32 g; iodine 0.05 g; sodium 7.85 g.
[b] Vitamin C 98% 8 g.
[c] GE = gross energy.

2.4. Immunological assays

2.4.1. Samples preparation

Serum was obtained from blood from 4fish $tank^{-1}$. Briefly, 1 mL was withdrawn from the caudal vein of each fish through a 1 mL syringe. Collected blood was immediately transferred into a 1.5 mL Eppendorf tube with no anticoagulant. The blood was left at 25 °C for 1 h and kept at 4 °C for 4 h. The clotted blood was then centrifuged at 10,000 RPM for five minutes at 4 °C. After centrifugation, anticipated serum was collected and retained at - 80 °C for further assays.

Isolation of leucocyte from fish's blood was followed by the method of Chung and Secombes (1988). Briefly, collected blood (1 mL) from each fish (4 fish $tank^{-1}$) was mixed with 2 mL RPMI 1640 (Gibthai) in a 15 mL. The mixture was then carefully loaded into a 15 mL tube, consisting of 3 mL of *Histopaque* (Sigma, St. Louis, MO, USA). The tube was centrifuged at 400 *g* for 30 min at 25 °C. After centrifugation, buffy coat of leucocytes cells drifted to the top of the Histopaque was carefully gathered and transferred into a sterile 15 mL tube. Then, a phosphate buffer solution (PBS: Sigma-Aldrich, USA) was added to each tube (3 mL) and gently aspirated. This tube was then centrifuged twice at 250 *g* for 10 min at 25 °C, in order to remove any residual *Histopaque*. The achieving cells were re-suspended in the PBS and adjusted to the desired cells number for measurement of phagocytic and respiratory burst activities.

Skin mucus was collected from 4 fish $tank^{-1}$ according to the method of Hoseinifar et al. (2016). The anesthetized fish was put into the plastic bag containing 10 mL of 50 mM NaCl. The fish was gently rubbed inside the plastic bag for 2 min. Obtaining mucus solution was immediately released into 15 mL sterile tube and centrifuged at 1500 *g*

at 4 °C for 10 min (5810R Eppendorf, Engelsdorf, Germany). The supernatant was gathered and kept at -80 °C for further assays.

2.4.2. Lysozyme activity of serum and skin mucus

Serum and mucus lysozyme activities were determined following the method of Parry et al. (1965). Briefly, 25 µL of serum and 100 µL of skin mucus from each fish was loaded into 96 well-plates in triplication. *Micrococcus lysodeikticus* (175 µL, 0.3 mg mL^{-1} in 0.1 M citrate phosphate buffer, pH 5.8; Sigma-Aldrich, USA) solution was loaded into each well and gently mixed. The changes in turbidity were recorded every 30 s for 10 min at 540 nm, 25 °C using a microplate reader. The sample's equivalent unit of activity was determined and compared with the standard and expressed as µg mL^{-1} serum.

2.4.3. Peroxidase activity of serum and skin mucus

The peroxidase activity was performed following the protocol of Quade, Roth (1997); and Cordero et al. (2016) protocol. Shortly, 5 µL of serum or skin mucus from each fish was loaded into flat bottomed of 96 well-plates in triplicate. Then, 45 µl of *Hank's Balanced Salt Solution* (without Ca^{+2} or Mg^{+2}) and 100 µL of solution (contains 40 ml of distilled water +10 µL of H_2O_2, 30%; Sigma Aldrich + one pill of 3,3′,5,5′-tetramethylbenzidine, TMB; Sigma Aldrich) were added into each well. Once the reaction color turned blue (30–60 seconds), 50 µl of 2 M H_2SO_4 was added to each well right away. The optical density was read at 450 nm by a microplate reader (Synergy H1, BioTek, USA). Samples not containing serum or skin mucus were considered to be blanks. A single unit was defined as the amount which produces an absorbance change, expressed as units (U) mL^{-1} of serum or mucus.

2.4.4. Phagocytic activity

The phagocytic rate was performed following the method of Yoshida and Kitao (1991). In brief, 200 µL of leucocyte cells (2×10^6 cells mL^{-1}) were placed on a coverslip in duplicate and incubated at 25 °C for 2 h. Afterward, the coverslips were washed with 3 mL of RPMI-1640 to remove any non-adherent cells. Then, a solution of 200 µL of fluorescence latex beads (2×10^7 of beads mL^{-1}) (Sigma-Aldrich, USA) was loaded into each coverslip and re-incubated at 25 °C for 1.5 h. After incubation, the coverslips were then rewashed with 3 mL of RPMI- 1640 and then fixed with methanol following by staining with Diff-Quik (Sigma-Aldrich, USA) for 10 s per solution. After staining, the coverslips were cleaned by PBS (pH 7.4) and allowed to dry at 25 °C, and then attached to the slides using Permount (Merck, Germany). The number of phagocyted cells was later counted microscopically (300 cells per coverslip). The phagocytic index (PI) was created through the following equation: PI = average number of beads per cell divided by the number of phagocytizing cells.

2.4.5. Respiratory burst

We calculated the respiratory burst activity of blood leucocytes following the protocol of Secomebs (1990). Briefly, 175 µL PBS cells suspension at a concentration of 6×10^6 cells mL^{-1} were loaded into the 96 well plates in triplication. We then added 25 µL of nitro blue tetrazolium (NBT) at a concentration of 1 mg mL^{-1} to each well and incubated the solution for two hours at room temperature. Later, we carefully discarded the supernatant in each well, and 125 µL of 100% methanol was then added into each well for five minutes, in order to fix the cells. After that, 125 µL of 70% methanol well^{-1} were added into each well, twice, for clean-up. We then dried the plates for thirty minutes at room temperature. The second solution of 125 µL of 2 N KOH and 150 µL of DMSO were added to each well. Afterward, the plates were measured at 655 nm via microplate-reader, according to the following: Spontaneous O2- production = (absorbance NBT reduction of the sample) – (absorbance of blank).

2.4.6. Alternative complement pathway activity

We analysed the alternative complement pathway activity (ACH50) employing the 1/2 scale technique of Yanno (1992). The degree of hemolysis and the volume of serum producing 50% hemolysis was calculated to determine the ACH50 via the following formula ACH50 (units/ml) = $1/K \times r \times 1/2$. Where K is the amount of serum giving 50% hemolysis, r is the reciprocal of the serum dilution, and 1/2 is the correction factor.

2.5. Growth performance

After 60 days post-feeding, growth performance and survival rate of the fish were measured using the following equations: Specific growth rate (SGR%) = 100 x (ln final weight - ln initial weight)/total duration of experiment; Feed conversion ratio (FCR) = feed given (dried weight)/weight gain (wet weight); Survival rate (%) = (final fish number/initial fish number) x 100.

2.6. Statistical analysis

We analyzed the least significant differences (LSD) among treatment (given the application of Duncan's Multiple Range Test) via the SAS Computer Program (SAS, 2003). Significant different mean values ($P < 0.05$) and other data are displayed as means ± standard deviation.

3. Results

3.1. Skin mucus immune response

Variation in skin mucus immune response was observed in fish fed

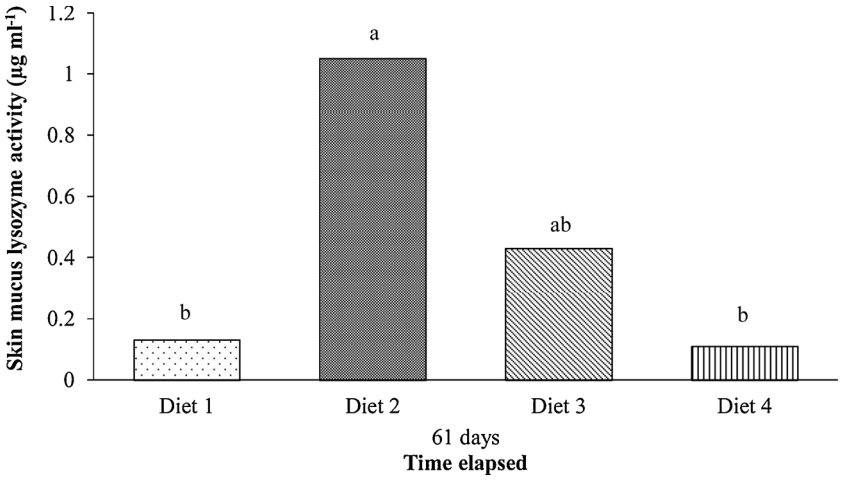

Fig. 1. Skin mucus lysozyme activity of *O. niloticus* after 61 days feeding trial fed different concentrations of dietary gotu kola powder (GKP) (mean ± S.E., n = 4): Diet 1 (0 - control), Diet 2 (5 g kg^{-1} FWP), Diet 3 (10 g kg^{-1} FWP), and Diet 4 (20 g kg^{-1} FWP). Columns sharing the same superscript letter are not significantly different ($P < 0.05$) (by Duncan's Multiple Range Test).

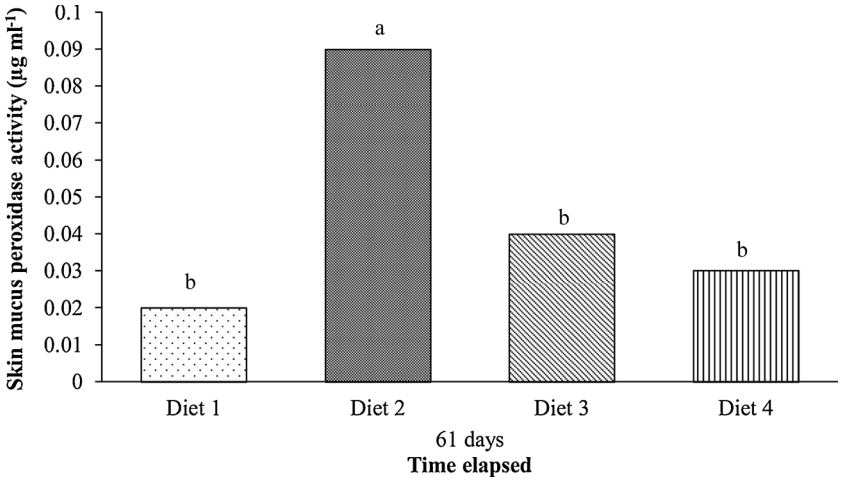

Fig. 2. Skin mucus peroxidase activity of *O. niloticus* after 61 days feeding trial fed different concentrations of dietary gotu kola powder (GKP) (mean ± S.E., n = 4): Diet 1 (0 - control), Diet 2 (5 g kg^{-1} FWP), Diet 3 (10 g kg^{-1} FWP), and Diet 4 (20 g kg^{-1} FWP). Columns sharing the same superscript letter are not significantly different ($P < 0.05$) (by Duncan's Multiple Range Test).

gotu kola powder (GKP) for 61 days (Figs. 1 and 2). Significant increase in skin mucus lysozyme activity (SMLA) was recorded in fish 5 g kg^{-1} GKP ($P < 0.05$). However, no significant difference was observed in fish fed 5 and 10 g kg^{-1} GKP, and between 10 and 20 g kg^{-1} GKP with the control group (Fig. 1). Significant enhance skin mucus peroxidase activity (SMPA) was also recorded in fish fed 5 g kg^{-1} GKP (Fig. 2; $P < 0.05$). However, no significant difference was observed between fish fed 10 and 20 g kg^{-1} GKP compared to the control ($P > 0.05$).

3.2. Serum immune response

The variations in serum immunity activities were recorded between the control and the supplemented GKP groups (Figs. 2–7). Dietary inclusion of GKP resulted in significantly higher serum lysozyme (SL) and serum peroxidase activities (SP) compared to control group ($P < 0.05$) after 61 days post-feeding. The highest value was found in fish fed 5 g kg^{-1} GKP ($P < 0.05$). Nonetheless, no significant ($P > 0.05$) differences were revealed between 5 and 10 g kg^{-1} (Figs. 3 and 6). Similarly, significant increase alternative complement activity (ACH50), phagocytosis, and respiratory burst activity (RB) were observed in fish fed 5 or 10 g kg^{-1} GKP (Figs. 4, 5 and 7). However, no significant differences in ACH50, phagocytosis, and RB were detected in fish fed 20 g kg^{-1} GKP compared to the control group ($P > 0.05$).

3.3. Growth performance

After 61 days of feeding, dietary administration of GKP (5 and 10 g kg^{-1}) resulted in higher the specific growth rate (SGR), weight gain (WG), and final weight (FW); compared with the control treatment (Table 2). However, there were no statistically significant differences between these diets compared to the control treatment. Similarly, no significant ($P > 0.05$) differences in FCR and survival rate were found among treatments after eight 61 days post-feeding (Table 2).

4. Discussion

The environmental stressors and the infectious diseases are among the major obstacles for the expansion of the aquaculture industry (Mishra et al., 2018; Wang, Lu, 2016). Throughout the last decades, the aquaculture industry was heavily dependent on antibiotics and chemotherapeutics to control the infectious diseases (Dawood et al., 2019; Doan et al., 2018; Van Doan et al., 2019a). There are significant concerns on the detrimental effects of antibiotics on the environment and human health by residual antibiotic-related issues. So, call for a reliable, environmentally friendly, and health safety methods, such as medicinal herbs intervention to protect against stressors, reduce and possibly eliminate disease occurrence is in needed (Chakraborty, Hancz, 2011). Medicinal herbs employ fewer complex approaches most likely to prevent early and late onset of disease without the risk of drug resistance in animals (Dawood et al., 2017). They confer several benefits including growth and immune enhancement to host against pathogens while sustaining health and environmental stability in fish generally and tilapia particularly. This study revealed that gotu kola resulted in enhanced serum and mucosal immunity, which may help Nile tilapia to resist the infection for the first time.

Skin mucus is a vital molecule of the non-specific immune system

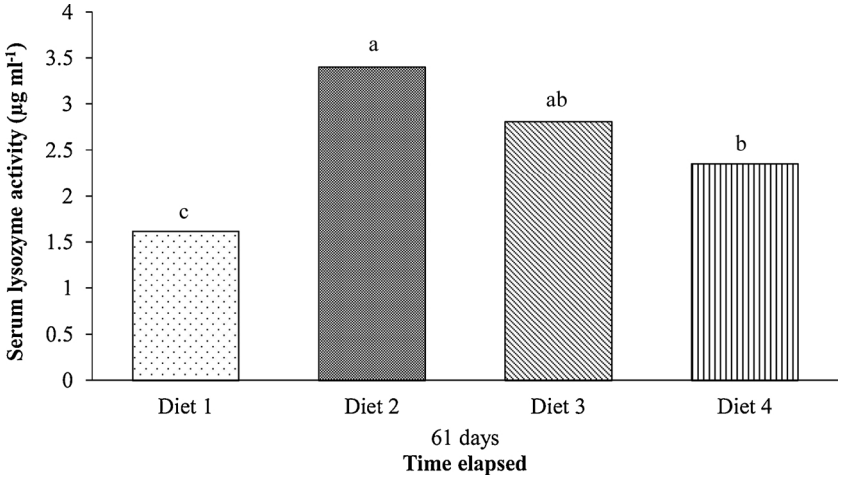

Fig. 3. Serum lysozyme activity of *O. niloticus* after 61 days feeding trial fed different concentrations of dietary gotu kola powder (GKP) (mean ± S.E., n = 4): Diet 1 (0 - control), Diet 2 (5 g kg^{-1} FWP), Diet 3 (10 g kg^{-1} FWP), and Diet 4 (20 g kg^{-1} FWP). Columns sharing the same superscript letter are not significantly different ($P < 0.05$) (by Duncan's Multiple Range Test).

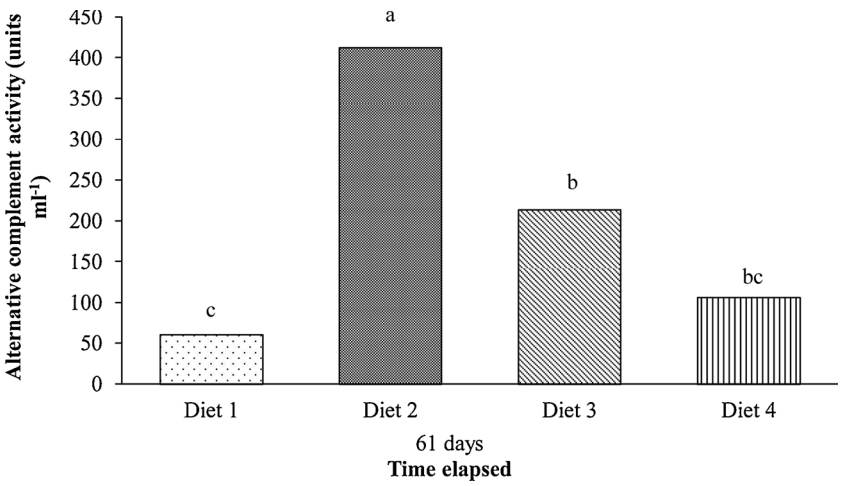

Fig. 4. Alternative complement activity of *O. niloticus* after 61 days feeding trial fed different concentrations of dietary gotu kola powder (GKP) (mean ± S.E., n = 4): Diet 1 (0 - control), Diet 2 (5 g kg^{-1} FWP), Diet 3 (10 g kg^{-1} FWP), and Diet 4 (20 g kg^{-1} FWP). Columns sharing the same superscript letter are not significantly different ($P < 0.05$) (by Duncan's Multiple Range Test).

and acts the first protective layer stand against pathogens infection (Esteban, 2012). The present study showed that the administration of gotu kola created remarkable boosts of skin mucus lysozyme (SMLA) and skin mucus peroxidase activities (SMPA). Similar to the present study, significant increase SMLA and SMPA have been reported in gilthead seabream fed enriched diets with fenugreek seed and dehydrated lemon peel (Bahi et al., 2017; García Beltrán et al., 2017; Guardiola et al., 2018b); in Nile tilapia fed spent mushroom substrate, corncob-derived xylooligosaccharide, and orange peels derived pectin (Van Doan et al., 2017, 2019a; Van Doan et al., 2019b); in common carp fed with *Psidium guajava* and bioactive substance from turmeric (Giri et al., 2019; Hoseinifar et al., 2019). A significant increase in skin mucus lysozyme and peroxidase activities may be due to the immunostimulant effect of gotu kola (Belwal et al., 2019). It is well-documented that the mucosal immune system of fish could be boosted by dietary administration of prebiotics, probiotics, and medicinal plants (Caipang, 2015). Several mechanisms, such as skin-associated lymphoid tissues (SALT), gill-associated lymphoid tissues (GIALT), and gut-associated lymphoid tissues (GALT) were triggered a powerful immunological response combat infectious disease (Bagni et al., 2005; Caipang, 2015; Sahlmann et al., 2013; Strand, Dalmo, 1997). At an immunological level, GALT is gathered of leukocytes, plasma cells, as well as T and B cells. These cells, together with epithelial cells, goblet cells, and neuroendocrine cells can activate and regulate gut immunity (Parra et al., 2015; Vallejos-Vidal et al., 2016). Nonetheless, a significant increase in skin mucus immune response of the fish fed gotu kola diets needs further investigations.

The fish innate immune response comprises of many elements that play a crucial role in protecting the fish from disease infection (Rebl, Goldammer, 2018). Lysozyme represents a critical defense element which is in charge of the lysing of pathogens (Saurabh, Sahoo, 2008). In this study, fish fed with gotu kola demonstrated significantly enhanced lysozyme activity. Similar to several studies in which lysozyme activity was enhanced in Indian major carp, *Labeo rohita* fed aloin (Srivastava et al., 2018); common carp, *Cyprinus carpio* fed rosemary leaf powder (Yousefi et al., 2019); zebrafish, *Danio rerio* fed ginger powder (Ahmadifar et al., 2019), and in rainbow trout, *Oncorhynchus mykiss* fed *Aloe vera*, *Stachys lavandulifolia,* and *Coriandrum sativum* (Mehrabi et al., 2019; Naderi Farsani et al., 2019; Sarvi Moghanlou et al., 2018). Peroxidase is also an essential enzyme which plays a crucial microbicidal substance that efficiently eradicated $H_2O_2$and retains the redox balance of immunological cells and systems (Guardiola et al., 2014). It is well-established that the dietary intake of *Origanum vulgare* leaf extracts and fenugreek seeds stimulates the serine peroxidase activity in gilthead seabream (Beltrán et al., 2018; Guardiola et al., 2018a,b). Similarly, the present work indicated the peroxidase activity was significantly improved in fish fed gotu kola administrated diets compared to the control. In contrast, this activity was not affected by dietary supplementation of fenugreek seeds after four weeks of feeding in seabream (Awad et al., 2015). Phagocytic activity is one of the crucial mechanisms of the non-specific immune system in fish (Esteban et al., 2015). The phagocytic process is to eliminate infectious pathogens, such as bacteria, viruses, and parasites, which has been comprehensively investigated in fish, particularly in teleosts (Vallejos-Vidal et al., 2016). In the present study, dietary administration of 5 and 10 g kg^{-1} gotu kola powder was significantly stimulated the phagocytic ability in

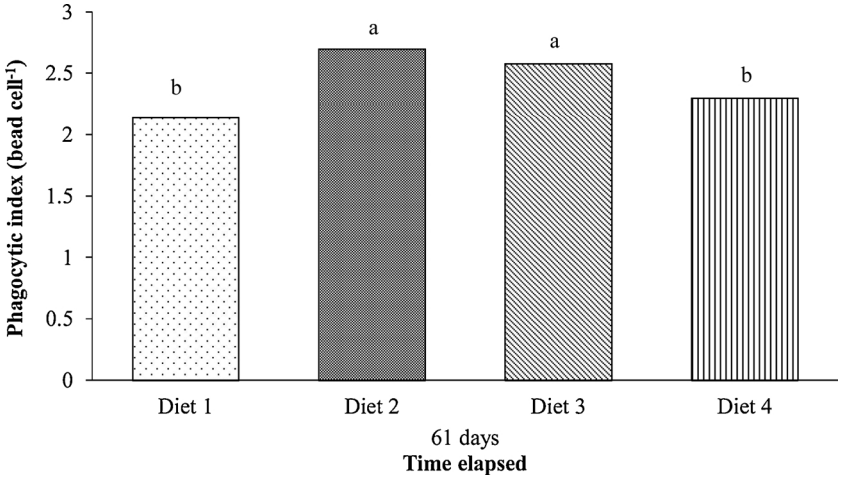

Fig. 5. Phagocytosis activity of *O. niloticus* after 61 days feeding trial fed different concentrations of dietary gotu kola powder (GKP) (mean ± S.E., n = 4): Diet 1 (0 - control), Diet 2 (5 g kg^{-1} FWP), Diet 3 (10 g kg^{-1} FWP), and Diet 4 (20 g kg^{-1} FWP). Columns sharing the same superscript letter are not significantly different ($P < 0.05$) (by Duncan's Multiple Range Test).

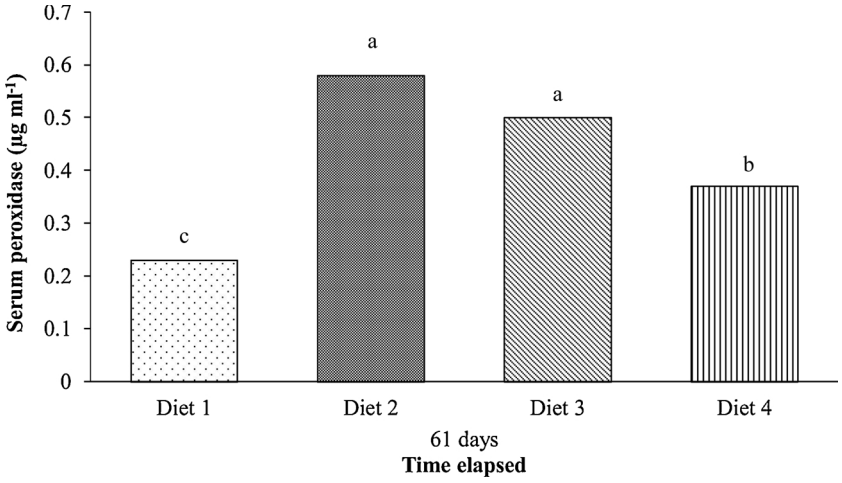

Fig. 6. Serum peroxidase activity of *O. niloticus* after 61 days feeding trial fed different concentrations of dietary gotu kola powder (GKP) (mean ± S.E., n = 4): Diet 1 (0 - control), Diet 2 (5 g kg^{-1} FWP), Diet 3 (10 g kg^{-1} FWP), and Diet 4 (20 g kg^{-1} FWP). Columns sharing the same superscript letter are not significantly different ($P < 0.05$) (by Duncan's Multiple Range Test).

comparison with the control treatment. This was consistent with previous studies reported in sea bream, *Sparus aurata* and European Sea bass, *Dicentrarchus labrax* fed tetra (*Cotinus coggygria*) and common mallow (*Malva sylvestris*) plant extracts (Bilen et al., 2019) and Nile tilapia fed elephant's foot, *Elephantopus scaber* extract (Doan et al., 2019). Respiratory burst in fish is related to the secretion of cytokines and inflammatory responses (Neumann et al., 2000; Rieger et al., 2010). The present study recorded an increase in a respiratory burst in fish fed 5 g kg^{-1} gotu kola compared to the control and other supplemented groups. Similarly, an increase in respiratory burst was found in sea bream, *Sparus aurata* and European sea bass, *Dicentrarchus labrax* (Bilen et al., 2019; Fazio et al., 2017) and Nile tilapia (Doan et al., 2019). The complement system is an essential element of the non-specific immune system composed of 35 proteins in serum with considerably close and controlled inter-relationships and with other immune system molecules (Sunyer et al., 1997). The present study has found that alternative complement activity was increased gotu kola inclusion diets after eight weeks post-feeding. This result is in agreement with previous investigations reported in rainbow trout, *O. mykiss* (Mehrabi et al., 2019; Naderi Farsani et al., 2019; Sarvi Moghanlou et al., 2018); in common carp, *C. carpio* (Yousefi et al., 2019); Indian major carp, *Labeo rohita* (Srivastava et al., 2018); Nile tilapia (Doan et al., 2019), and striped catfish, *Pangasianodon hypophthalmus* (Nhu et al., 2019).

Despite the fact that the mode of action to which *C. asiatica* powder boosted fish immunity is not clarifies yet, it may be owing to the existence of several bioactive substances viz. pentacyclic triterpenes (Puttarak et al., 2017). It has been revealed that asiatic acid (a naturally occurring of pentacyclic triterpenes) has many pharmacological properties, which including antioxidant, anti-inflammatory, and control apoptosis that attributes its remedial impacts in various diseases. It also displayed effective antihypertensive, nootropic, neuroprotective, cardioprotective, antimicrobial, and anticancer activities in preclinical studies (Nagoor Meeran et al., 2018).

Table 2
Growth performances and feed utilization (mean ± SE) of tilapia after 61 days feeding with experimental diets.

	Diet 1	Diet 2	Diet 3	Diet 4
IW (g)	17.84 ± 0.01	17.86 ± 0.01	17.88 ± 0.01	17.89 ± 0.01
FW (g)	130.57 ± 1.76	137.39 ± 0.58	131.04 ± 0.87	127.55 ± 0.89
WG (g)	112.73 ± 1.76	119.52 ± 0.57	113.25 ± 0.86	109.67 ± 0.88
SGR (%)	3.25 ± 0.02	3.35 ± 0.01	3.27 ± 0.01	3.22 ± 0.01
FCR	1.07 ± 0.02	1.01 ± 0.02	1.07 ± 0.02	1.05 ± 0.01
SR (%)	95.00 ± 0.46	91.25 ± 1.61	91.25 ± 1.15	97.50 ± 0.32

Diet 1 (0- control), Diet 2 (5 g kg^{-1} GKP), Diet 3 (10 g kg^{-1} GKP), and Diet 4 (20 g kg^{-1} GKP). Different letter in a row denote significant difference ($P < 0.05$). Different letter in a row denote significant difference ($P < 0.05$).

Growth rate and feed conversion ratio are critical indicators used for evaluating the effects of a medicinal plant in aquafeed (Hoseinifar et al., 2018; Rashidian et al., 2018). However, the present study revealed that the dietary incorporation of gotu kola had no effects on tilapia growth and feed utilization. Likewise, no effects on growth performance and feed utilisation were recorded in rainbow trout fed bay laurel, black cumin, and olive leaf (Baba et al., 2018; Bilen, Bilen, 2012; Celik Altunoglu et al., 2017); koi carp fed tetra extract (Bilen et al., 2013);

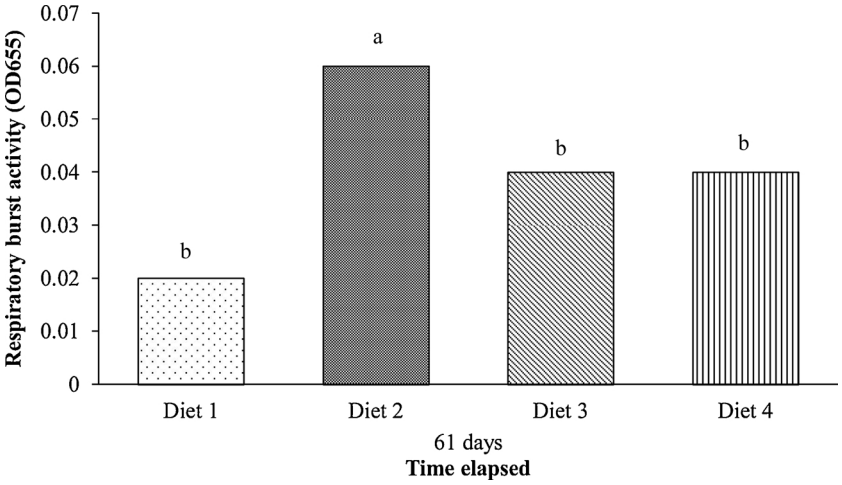

Fig. 7. Respiratory burst activity of *O. niloticus* after 61 days feeding trial fed different concentrations of dietary gotu kola powder (GKP) (mean ± S.E., n = 4): Diet 1 (0 - control), Diet 2 (5 g kg^{-1} FWP), Diet 3 (10 g kg^{-1} FWP), and Diet 4 (20 g kg^{-1} FWP). Columns sharing the same superscript letter are not significantly different ($P < 0.05$) (by Duncan's Multiple Range Test).

hybrid grouper fed dandelion extracts (Sun et al., 2019), and European sea bass fed tetra (Bilen et al., 2019). It is proven that proper use of medicinal plants relies on species, age, size, dietary concentration, and any stress conditions under the rearing period (Reverter et al., 2014). Medicinal plants typically contain a considerable fiber, which may adversely influence the fish's feed utilisation and growth rate accordingly (Cho et al., 2007). Li et al. (2012) indicated that fish could tolerate a dietary content up to 23% total dietary fibre before displaying a decrease in growth rate. Polyphenols were able to exert their impact on the emulsion interface, interacting with digestible enzymes, for decline feed consumption and weight gain (Bandyopadhyay et al., 2012). However, further works on the use of gotu kola in aquatic animals are necessary to understand this phenomenon.

In conclusion, the inclusion of dietary gotu kola can be an important choice for sustainable aquaculture. The present study revealed that gotu kola supplementation might potentially activate the humoral and mucosal immune mechanisms in Nile tilapia.

Ethical approval

The study was performed in accordance with the guidelines on use of animals for scientific purposes (Chiang Mai University Approved No. 2561/AQ-0005).

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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