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Effects of traditional Chinese medicines (TCM) on the immune response of grass carp (*Ctenopharyngodon idellus*)

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Abstract The use of traditional Chinese medicine (TCM) in aquaculture has been widely investigated. It is believed that herbal supplements could enhance the immunity and promote fish growth. The powdered TCM or its decoction can be easily applied in the form of feed supplements by direct incorporation into fish feeds and side effects are seldom found. In this study, *Radix scutellaria* (Rs) and *Rhizoma coptidis* (Rc) showed strong bacterial inhibition on *Aeromonas hydrophila* out of the sixteen selected herbal extracts. Four TCMs, *R. scutellaria*, *R. coptidis*, *Herba andrographis* (Ha), and *Radix sophorae flavescentis* (Rsf) were selected to form a compound formulation in the ratio of 1:1:2:3. A feeding experiment on grass carp was conducted with addition of the above TCM formulation (0.5, 1 and 2 % w/w) in the fish feed, and bactericidal activity, total protein and immunoglobulin in blood plasma, and nitroblue tetrazolium activity in the whole blood were measured. 2 % supplementation significantly improved ($p < 0.05$) the bactericidal activity and total immunoglobulin in the plasma after feeding for 21 days. Compared to control, 2 % TCM feeding groups showed a significantly lower mortality after *A. hydrophila* challenge at the end of experiment and the same result was archived in field trial. In the cost evaluation, the use of the TCM formulation in grass carp culture was insignificant and could be compensated by improved yield. TCM formulation contained different active ingredients including some immuno-stimulant and antimicrobial agents and therefore could be applicable to multiple diseases. Application of this TCM formulation would be a cost-effective and prophylactic approach for disease control in aquaculture, replacing the use of antibiotics for treating enteritis and even other general diseases.

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Introduction

Grass carp is the second largest production (after silver carp) in freshwater aquaculture worldwide, which is more than 3 million tonnes, and China contributed 95.7 % of the total global production in 2002 (FAO 2009). Enteritis is one of the most common diseases in grass carp, especially for young fish, with mortality around 50–90 % (Yang 2008). *Aeromonas* spp in the intestine proliferated rapidly and caused dysfunction of capillaries on the intestine and released endotoxin leading to septicemia, the major symptom of enteritis (Xu et al. 1988). Due to the high stocking density, fish diseases have a crucial effect on aquaculture yield. Antibiotics are commonly used to control and treat diseases in aquaculture, with sulphaguanidine and furazolidone commonly used for treating enteritis in grass carp (FAO 2009).

The frequent occurrence of antibiotic-resistant bacteria near aquaculture site is probably due to the abuse of veterinary antibiotics and sub-therapeutic doses applied in aquaculture (Smith et al. 2002). As a consequence, more and more antibiotic-resistant fish pathogens have been detected around aquaculture sites (Sørum 2000; Furushita et al. 2005; Cabello 2006; Sørum 2006). Terrestrial veterinary pathogens and even human pathogens may gain antibiotic resistance determinants through horizontal gene transmission from bacteria in the aquaculture environment (Angulo and Griffin 2000; Heuer et al. 2009). Due to the various threatening effects of antibiotics, the European Union has banned its use as feed additives in food-producing animals after January 2006. Other feed supplements which are environmentally safe and cost-effective should be used, with herbal medicines one of the popular options.

The possible use of traditional Chinese medicine (TCM) for aquaculture has been explored in recent years with Direkbusarakom (2004) confirming that its usage plays an important role in the Asian aquaculture industry. Herbal medicine can serve as antimicrobial agents or immunostimulants to prevent fish disease and could be a potential alternative to replace vaccines (Anderson 1992; Secombes 1994), and as alternatives to antibiotics (Galina et al. 2009). Besides, herbs are also known to exert positive effects on growth, combating viral infection, appetite stimulation, and stress relief (Francis et al. 2005; Citarasu et al. 2006; Venketramalingam et al. 2007; Ardó et al. 2008). Therefore, the use of TCMs may greatly reduce the disease outbreak in aquaculture production and replace the use of antibiotics as an effective measure for disease control in the future.

The principle of TCM is quite different from the western pharmacological and therapeutic principles which target on diseases or pathogens directly. The rationale of TCM is mainly based on the theories, such as the five elements theory and the Yin-Yang balance, which are considering the overall balance in the human body (Cheng 2000). In other words, TCM is aimed at maintaining and restoring the balance in human body or enhancing the ability of body to defense diseases.

The therapeutic effects of active ingredients in TCM have been validated in some animal models such as mice, fish, and human cells. *Scutellaria* extracts exhibited good antibacterial and antiviral effects and boosted phagocytic activity in mice at low dose (Cai et al. 1994), and with similar activity found in fish at low dose (Yin et al. 2006). *Andrographis paniculata* extracts were especially important for treating inflammation by modulating macrophage and neutrophil activity in mouse (Chiou et al. 2000) and human blood cells (Shen et al. 2002). These basic medicinal values of herbs exhibited in other animals advocate the use of TCMs in fish farming.

The medicinal values of TCMs in fish have also been studied, but they mainly focused on the non-specific immune responses and disease resistances of fishes to bacterial infections. Common carp (*Cyprinus carpio*) fed with *Astragalus* root extract showed better survival rates after challenged with *A. hydrophila* (Yin et al. 2009). A mixed use of *Astragalus* and *Lonicera* extracts in feed also enhanced phagocytic and respiratory burst activity of blood phagocytic cells and improved disease resistance against *A. hydrophila* in Nile tilapia (*Oreochromis niloticus*) (Ardó et al. 2008). Rohu fed with *Achyranthus* showed enhanced superoxide anion production level, lysozyme, and serum bactericidal activity (Rao et al. 2006). Mortalities in TCM fed fish due to bacterial infections, e.g., *Aeromonas hydrophila* in Nile tilapia and common carp (Ardó et al. 2008; Yin et al. 2009), *Vibrio harveyi* in grouper (Punitha et. al 2008), were significantly reduced. Better disease resistance was shown in different studies using herbal mixture rather than single herb. In the study on *A. radix* and *Ganoderma lucidum*, the vaccinated common carp fed with mixture of both herbal extracts showed the highest survival rate compared with non-vaccinated, vaccinated control, and vaccinated groups fed with either herb (Yin et al. 2009). A herbal formulation containing *Ocimum basilicum*, *Cinnamomum zeylanicum*, *Jugpans regia,* and *Mentha piperita* enhanced the non-specific immunity and disease resistance to *A. hydrophila* in common carp (Abasali and Mohamad 2010). In general, herbal medicine has shown antimicrobial and immuno-stimulatory effects in the treated fish.

In TCM theory, TCM compound formulation involves a complex interaction between drugs. It may have better effect on immune stimulation or improvement on disease resistance of fish, compared with the use of single herb, and hence is a more cost-effective approach. The major components showed chief therapeutic effects to balance the disharmony in the body, while others such as adjuvant and minister constituents (second component) assisted the therapeutic action of major components, and the messenger constituent (component making the formula prescription targeting pathological tissues) functioned as a "guider" to modulate the formulation to targeted organ or to eliminate the disharmony caused by other medicines in the formulation (Cheng 2000; Gao and Wu 2008). Therefore, TCM formulas are not just an addition of the herbs, but with many interactions between the herbs. There is a lack of information on the use of compound formulation containing more than three types of TCM as feed supplements. Investigation on the compound formulations of TCMs may be beneficial to aquaculture industry in combating fish diseases.

Chinese herbs enhanced immunity of fish, ideally replacing the use of antibiotics. The application of TCMs as feed supplements may be the best option for disease prophylactic; although a substantial number of studies on the use of TCM have been conducted in past decade, there is limited research investigating the feasibility of its application in real aquaculture practice, especially on its cost-effectiveness. In this study, a compound formulation of TCMs, in a ratio of 1:1:2:3 with *Rhizoma coptidis*: *Radix scutellaria*: *Herba andrographis*: *Radix sophorae flavescentis*, was concocted. Two herbs (*R. coptidis* and *R. scutellaria*) which showed strong antibacterial activities on *A. hydriphila* were combined with another two herbs (*H. andrographis*: *Radix sophorae flavescentis*) which showed immuno-stimulating properties. The effects of this formulation (in a form of TCM supplemented fish feed) on immune parameters of grass carp, i.e., neutrophil oxidative activity in blood, total protein and total immunoglobulin and bactericidal activity in plasma, were investigated. Finally, an evaluation on the cost-effectiveness of applying the TCM feed in aquaculture was performed as other similar feeding studies only focus on the fish immunity, but not on application of TCM feed (Yin et al. 2006, 2009). The major objectives of this study were (1) to investigate the effect of this TCM on grass carp's immunity and disease resistance and (2) to evaluate its effectiveness in aquaculture both biologically and economically via laboratory experiments and field trial.

Materials and methods

Traditional Chinese medicine (TCM)

Sixteen Chinese medical herbs were obtained from the School of Chinese Medicine, Hong Kong Baptist University for testing the antimicrobial activity of herbal extracts on *A. hydrophila*, pathogen of enteritis in grass carp. *R. coptidis, Radix astragali, H. andrographis, Herba houttuyniae, R. scutellaria, Radix angelicae sinensis, Herba astemisia capillaris, Frucutus cnidii, Radix isatidis, Folium isatidis, Radix et rhizome glycyrrhizae, Rhizoma rhei, Cortex phellodendri, Semen sinapis*, *Fructus forsythiae*, and *Radix sophorae flavescentis* were used in the disk agar test.

Screening of suitable TCM for treating enteritis in grass carp

The herbs were dried at 30 °C for 24 h and then pulverized to powder using a mechanical blender. Chinese herbs powder was soaked for 30 min in deionized water (1:10 w/v) and boiled for 30 min. The extract was removed and another portion of deionized water was added and boiled for another 30 min. The extracts from two extractions were filtered through Whatman No. 1 filter papers. The herb extracts were pooled and dried at 50 °C, and the stock solution (240 mg mL^{-1}) was autoclaved before storage at 4 °C.

The screening of TCM for fish feeding experiment was performed by standardized disk agar method (NCCLS 1999). The bacteria strain *A. hydrophila* was successfully isolated from infected grass carp, with the assistance from Institute of Hydrobiology, Jinan University at Guangzhou, and the strain was identified by Analytical Profile Index (API) 20NE kit (Biomerieux, Lyon, France). *A. hydrophila* and ATCC 25922 *Escherichia coli* (used as control) were inoculated in Luria–Bertani (LB) broth and then incubated at 28 and 37 °C for 18 h, respectively. The cultures were centrifuged at 850*g* for 15 min. The supernatant was removed and the pelletized bacteria were washed twice in sterile 0.9 % saline. The concentration of bacteria was adjusted to the McFarland 0.5 turbidity standard. The adjusted inoculum (50 µL) was spread on the LB agar thoroughly; three sterilized 6-mm-diameter paper discs soaked with herbal extract (240, 120, 60, 30, 15 mg mL^{-1}) were put on each LB agar plate; the inverted agar plates were incubated for 18 h at 28 °C. Antibiotic discs Amikacin (30 µg) and Gentamicin (10 µg) (BBL Microbiology, Voigt Global Distribution, Kansas, USA) were used as positive control and sterile deionized water was (replacing herbal extracts) used as negative control.

The diameters of the clear zones were measured and recorded in millimeters (mm). The inhibition zones of the tested extracts were represented by excluding clear zones in the negative control (subtracting diameter of paper disc). The minimum inhibitory concentration (MIC) of each herbal extract (if any) was the minimum test herb extract concentration showing inhibition zone. Inhibition zones >11 mm were stated as strong, from 9 to 11 mm as moderate, and <9 mm as weak inhibitions.

TCM fish feed preparation

The herbal medicines were grounded and mixed according to the following ratio: *R. coptidis*: *R.* s*cutellaria*: *H. andrographis*: *Radix Sophorae flavescentis* = 1:1:2:3. 0.5, 1, and 2 % w/w of TCM powder (1:10 w/v, TCM powder: Water) was weighed and boiled for 30 min with 200 mL of deionized water and the aqueous extracts were filtered through Whatman No. 1 filter paper. The TCM residues were boiled with another 200 mL of deionized water. The extracts and the TCM residues were pooled and mixed with powdered commercial grass carp feed thoroughly. The commercial fish feed used was Jinfeng®, 601 Grass carp formulated feed, with 33.7 % protein, 4.2 % crude fat, and 7.7 % ash. Deionized water was added and the fish feed dough was pelletized with a meat grinder and dried at 50 °C for 24 h. The fish feed containing no TCM was used as control.

Fish feeding experiment and blood sampling procedure

Five hundred healthy grass carp fingerlings (27.1 ± 4.2 g) were bought from a fish farm in Hong Kong, with 20 fish per tank (65 L, 12 tanks in total), and remaining fish were cultured in 3 stock tanks (~200 L). The fish tank was continuously aerated and the water temperature was maintained at 22 ± 2 °C. The water temperature, values of pH, and dissolved oxygen were measured three times a week using a portable Hanna pH meter and a YSI digital Dissolved Oxygen (DO) meter and the values were 21.9 ± 1.7 °C, 6.5 ± 0.28, and 6.7 ± 0.48 mg/mL. The fish were fed with experimental control feed, by 1 % of body weight (g) per meal and two meals per day.

The fish were acclimatized for 2 weeks and adapted to the experimental control feed, and the experimental fish were evaluated by a careful examination of physical appearance and behavior (e.g., rapid responses to light, and active feeding behavior) and showing no sign of infection (body lesion, scratching, lethargy). After 2 weeks acclimation in laboratory condition, the fish were redistributed into the 12 tanks (20 fish per tank, 4 treatments in triplicates) randomly. Dead fish were discarded and replaced by qualified fish from the stock tanks, and the fish were acclimated for further 2 weeks (without any mortality during this period) before start of the experiment. Three different dosages of the mentioned TCM formulation were used in the experiment: 0.5, 1.0, and 2.0 % (w/w). A treatment without TCM added in fish feed was used as control. There were triplicates for each treatment, i.e., 12 tanks in total. The fish blood samples (two fish per tank) were collected by caudal venous puncture at Day 0, 7, 14, and 21 after euthanized by 100 mg L^{-1} of Anesthetic Tricaine, MS-222 (Sigma-Aldrich, St. Louis, Missouri, USA). The nitroblue tetrazolium (NBT) assay, bactericidal activity and protein and immunoglobulin assays were investigated. No mortality was observed during the 21 days of experiment.

Disease resistance to *A. hydrophila*

Aeromonas hydrophila was inoculated in LB broth overnight at 28 °C. The cultures were centrifuged at 850*g* for 15 min. The supernatant was removed and the bacteria pellet was washed twice in sterile 0.9 % saline. The suspension was adjusted to 1×10^8 cfu mL^{-1}, based on the optical density of suspension with sterile saline (~0.13 absorbance at 625 nm). Suspended bacteria (0.1 ml) was injected into the peritoneal cavity of fish (12 fish for each tank) at day 21 of the feeding experiment. The mortality rate was recorded in the following 7 days after infection.

Plasma total protein and total immunoglobulin assay

The total immunoglobulin in plasma was determined using the method described in Siwicki et al. (1994) with modifications, and the protein concentration of the plasma was determined according to the modified colorimetric method based on Bradford protein assay (Bradford 1976). Briefly, 5 μL of plasma and 250 μL of Bradford solution (Sigma-Aldrich, St. Louis, Missouri, USA) were added to 96-well microtiter plates (Iwaki, Tokyo, Japan). After 20 min of incubation at 22 ± 1 °C, the absorbance was measured with a microplate reader (Infinite 200, Tecan Austria GmbH, Grődig, Austria) at 595 nm. The protein concentration (g L^{-1}) was calculated from the standard curve.

For the total immunoglobulin, plasma (50 μL) and polyethylene glycol (50 μL) (10 % w/v, PEG, Bioultra) (Sigma-Aldrich, St. Louis, Missouri, USA) were incubated at 22 ± 1 °C for 2 h and the mixture was centrifuged at 1,000*g* for 15 min. The protein content of the supernatant was determined by the assay mentioned above. The total immunoglobulin concentration (Total IgI) of the plasma (g L^{-1}) was subtracted from the total protein (TP) level.

Bactericidal activity of blood plasma

The bactericidal activity of blood plasma was conducted based on the method of Abidov and Mirismailov (1979). *A. hydrophila* was inoculated in LB broth at 28 °C for 18 h. The cultures were centrifuged at 850*g* for 15 min. The supernatant was removed and the bacteria pellet was washed by sterile 0.9 % saline twice. The concentration of bacteria was adjusted to 1×10^8 cfu mL^{-1} based on the optical density of suspension and diluted to 10^{-4} by sterile saline. Equal volumes (80 μL) of diluted bacterial suspension and plasma (0.9 % sterile saline for growth control) were mixed and incubated at 28 °C for 30 min. After incubation, 50 μL of mixed solution was poured onto the LB agar plate and the dishes were incubated for 18 h at 28 °C. The bactericidal activity was represented by the percentage decreased of colony counts in the sample compared to control.

Nitroblue tetrazolium (NBT) assay

The nitroblue tetrazolium (NBT) assay was carried out based on the method described in Anderson and Siwicki (1995). Heparinized fish blood (100 μL) was added to an equal volume of 0.2 % NBT (Sigma-Aldrich, St. Louis, Missouri, USA) solution, and the mixture was then incubated at 22 ± 1 °C for 30 min. The resultant suspension (50 μL) was added into a glass tube 1.0 ml *N*,*N*-dimethyl formamide (Sigma-Aldrich, St. Louis, Missouri, USA) and centrifuged at 3,000*g* for 5 min. The optical density (OD) of the supernatant was measured by a spectrophotometer (UV-1601, Shimadzu, Tokyo, Japan) at 540 nm.

Field trial in Yuen Long and cost evaluation on the application of TCM feed

The production cost of TCM feed for field trial experiment was estimated, based on the cost of control feed same as the laboratory experiment, and compared with the cost of the TCM added feed. Both the control fish feed and the two types of TCM feeds (1.0 and 2.0 %) were processed by a commercial fish feed manufacturer in Lau Fau Shan, Hong Kong. The feeding costs using the 2 % TCM feed in grass carp culture were estimated with the following assumptions; the feeding period of 2 % TCM feed was 60 days at 2 % daily feeding rate on young grass carp (~0.028 kg, mean weight with 1 % daily weight gain) at two stocking densities (9,000 and 18,000 fish ha^{-1}) with the marketable grass carp size of

1.5 kg (FAO 2009). The wholesale price of grass carp was converted from the average wholesale prices (\$15.62 HKD kg^{-1}) in Hong Kong during 2006–2008 (CSD 2009), which was \$2.0 USD/kg (1 USD = 7.8 HKD). The profit of improved yield when using the TCM feed was also evaluated based on the reduced mortality observed in field trial.

Calculation on applying 2 % TCM feed.

$$\text{(i) Applying 2 \% TCM feed cost (USD ha}^{-1}\text{)}$$
$$= \text{DFR (\% of b.w.)} \times \text{MBW} \times \text{SD (fish ha}^{-1}\text{)} \times \text{FD} \times \text{TCM feed cost (USD kg}^{-1}\text{)}$$

$$\text{(ii) Profit of improved yield when using the TCM feed (USD ha}^{-1}\text{)}$$
$$= \text{SD (fish ha}^{-1}\text{)} \times \text{RD (\%)} \times \text{HFW (kg/fish)} \times \text{WP (USD kg}^{-1}\text{)}$$

where SD, stocking density = 9,000 or 18,000 fish ha^{-1} (FAO 2009); HFW, harvested fish weight = 1.5 kg/fish (FAO 2009); DFR, daily feeding rate = 2 % body weight per day; MBW, mean body weight in the 60 feeding days = 0.028 kg (calculated from 1 % specific growth rate); FD, feeding day = 60 days; 2 % TCM feed cost = \$1.02 USD kg^{-1}; RD, reduced mortality due to feeding TCM feed = 20 % (refer to field trial results); WP wholesale price = 2.0 USD kg^{-1} (CSD 2009);

A field trial on the effects of TCM formulation on the resistance of grass carp to *A. hydrophila* was conducted at the fish pond in Yuen Long, Au Tau Fisheries Centre of Agriculture, Fisheries and Conservation Department (AFCD), Hong Kong SAR Government. Two types of TCM feeds (only 1.0 and 2.0 % were tested, based on laboratory results) and the feed without adding TCM serving as control were used in this field trial. Fifty individuals of grass carp (~20 g) were cultured in a fish cage (~2 m × 2 m × 1.5 m) placed in a fish pond and acclimatized for 2 weeks before experiment.

The fish were fed about 1 % of body weight (g) per meal and twice per day (average weight of fish was about 20 g). The pond water temperature, pH, and dissolved oxygen levels were recorded 5 times a week by a handheld Multiparameter Instrument YSI 556MPS which were 19.5 ± 3.3 °C, 6.7 ± 0.31, and 6.2 ± 0.35 mg/mL, respectively. The same screening procedures for fish followed those of the laboratory study and the clinical signs in diseased fish were also examined.

Same as the laboratory experiment, *A. hydrophila* was administrated to grass carp after feeding for 60 days (12 fish for each cage). Fish mortality was recorded for the following 7 days.

Statistical analysis

The results were compared at each sampling days (day 0, 7, 14, 21) using one-way ANOVA and Duncan's multiple range tests (SPSS Statistics 17.0, Chicago, Illinois, USA). Significant differences between experimental groups were expressed at the significance level of $p < 0.05$.

Results

Bacterial inhibition of TCM extracts

Strong inhibition on *A. hydrophila* was observed in *R. coptidis* at 240, 120, and 60 mg mL^{-1} and *R. scutellaria* at 240 mg mL^{-1}, while only a weak inhibition zone was

Table 1 Inhibitory effect of traditional Chinese medicines on *A. hydrophila* (Only the herbs that showed inhibitory effect were shown)

Herbs/antibiotics	Diameter of inhibition zones (mm)				
	A. hydrophila				*E. coli*
	Herb extract concentration (mg mL^{-1})				
	240	120	60	30	
R. coptidis	20.33 ± 0.58	14.00 ± 2.0	13.67 ± 1.53	6.5 ± 0.50	–
R. scutellaria	12.75 ± 0.42	9.33 ± 1.51	7.92 ± 0.66	7.33 ± 0.82	–
F. forsythiae	7.08 ± 0.12	–	–	–	–
Amikacin (30 μg mL^{-1})		22.21 ± 1.43			20.43 ± 1.54
Gentamicin (10 μg mL^{-1})		19.51 ± 1.14			18.06 ± 1.37

–, No inhibition in tested concentrations

found in *F. forsythiae* at 240 mg mL^{-1} (Table 1), but none of the selected herbal extracts showed inhibition on *E. coli*. The minimum inhibitory concentrations (MIC) of *F. forsythiae*, *R. scutellaria,* and *R. coptidis* were 240, 30, 30 mg mL^{-1}, respectively. *R. coptidis* and *R. scutellaria* which showed stronger inhibitory effects on *A. hydrophila* were chosen as the TCM formulation with another two herbs, *H. andrographis* and *R. Sophorae flavescentis,* for the fish feeding experiment of grass carp.

Immune parameters in grass carp blood feeding with TCM formulation

No significant difference in total protein was found among all TCM feed groups compared to control feed group at all sampling days ($p > 0.05$) (Fig. 1a). A significantly ($p < 0.05$) higher total immunoglobulin level (IgI) was observed in 1 and 2 % TCM feed groups than control feed group at day 21 ($p < 0.05$), and the total IgI of 1 % TCM group at day 14 was also significantly ($p < 0.05$) higher than the control group (Fig. 1b). All groups feeding the TCM formulation showed a higher bactericidal activity of plasma at day 21 as compared to control group, but only significantly higher ($p < 0.05$) bactericidal activity of plasma was shown in 2 % TCM group, compared to the control feed group at day 21, while the bactericidal activity of 2 % feed group was gradually increased since day 0, and significantly improved at day 21 compared to the value at day 0 (Fig. 2a). A higher NBT activity was found in 2 % TCM group at day 14 significantly, but no significant difference was found among all treatments for the neutrophil oxidative activity in the NBT assay ($p > 0.05$) at day 21 (Fig. 2b). In general, 2 % TCM feed enhanced the total immunoglobulin and bactericidal activity of plasma.

Fish growth and disease resistance to *A. hydrophila* in laboratory experiment

The mortality of fish was monitored for 7 days after bacterial infection. The diseased grass carps in laboratory and field studies showed lethargy, expanded abdomen with red blotches, and loss of appetite after infection; redden and swollen anus was observed, with yellow mucus released from the anus when slight pressure was applied to the abdomen. Hemorrhages in the intestinal wall and liver enlargement were observed in diseased fish. These signs matched with enteritis infection observed in grass carp (NACA 1989;

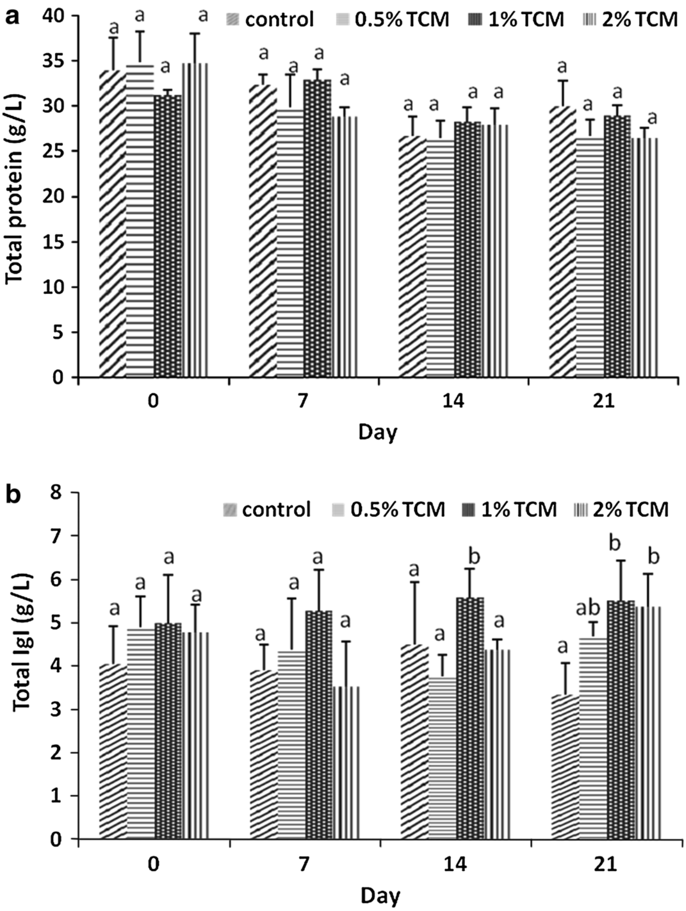

Fig. 1 **a** Total protein (g L^{-1}) (Mean ± SD) and **b** total immunoglobin (IgI) (g L^{-1}) (Mean ± SD) of grass carp plasma in control feed group and feeding various doses of formulated TCM feed group. Means in same sampling day with different *superscripts* are significantly different at $P < 0.05$

Li et al. 2007). Generally, all groups feeding with various doses of formulated TCM showed lower mortality of after feeding for 21 days (Fig. 3a), but significantly reduced mortality ($p < 0.05$) was only observed in 1 and 2 % TCM groups. The mortality of 2 % TCM group was halved when compared with control group. The TCM feeding groups, i.e., 0.5, 1, and 2 % also showed a higher weight gain rate and specific growth rate (Table 2) compared to control group, but the difference is not significant ($p > 0.05$).

Disease resistance to *A. hydrophila* in field trial at Yuen Long and cost evaluation on TCM feed application

The diseased grass carp showed same clinical signs of enteritis as observed in laboratory study. There were lower fish mortalities (77.8 and 69.4 %) in both 1 and 2 % TCM feeding groups, respectively, compared to control group (88.9 %), significant ($p < 0.05$) mortality reduction was only observed in 2 % TCM (Fig. 3b). The TCM feeding groups, i.e., 1 and 2 % also showed a higher weight gain rate and specific growth rate (Table 2) compared to

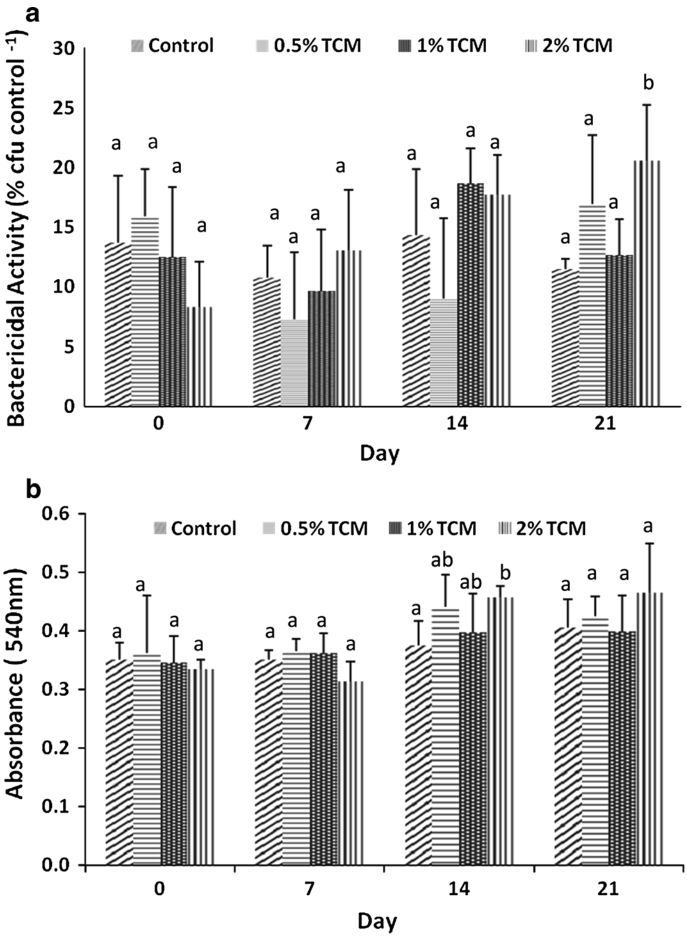

Fig. 2 **a** Bactericidal activity (%) (Mean ± SD) and **b** optical density of NBT assay (Mean ± SD) of grass carp plasma in control feed group and feeding various doses of formulated TCM groups. Means in same sampling day with different *superscripts* are significantly different at $P < 0.05$

control group, but the difference was not significant ($p > 0.05$). The production cost of the TCM feed is listed in Table 3. The TCM costs involved in the total feed production cost were 7.3 and 13.8 % for 1 and 2 % TCM feeds, respectively. The costs using the 2 % TCM feed are 308.4 and 616.9 USD ha^{-1}, and the profits of improved yields when using the TCM feed were 5,400 and 10,800 USD ha^{-1}, for low (9,000 fish ha^{-1}) and high (18,000 fish ha^{-1}) stocking density, respectively. The profits were based on 20 % of the reduced mortality in field trial when induced disease.

Discussion

There are a number of studies on adding one or two herbs in fish feeds, but there is a general lack of studies focusing on the use of compound formulation, which may be more effective in combating various diseases. Studies on compound formulation in aquatic

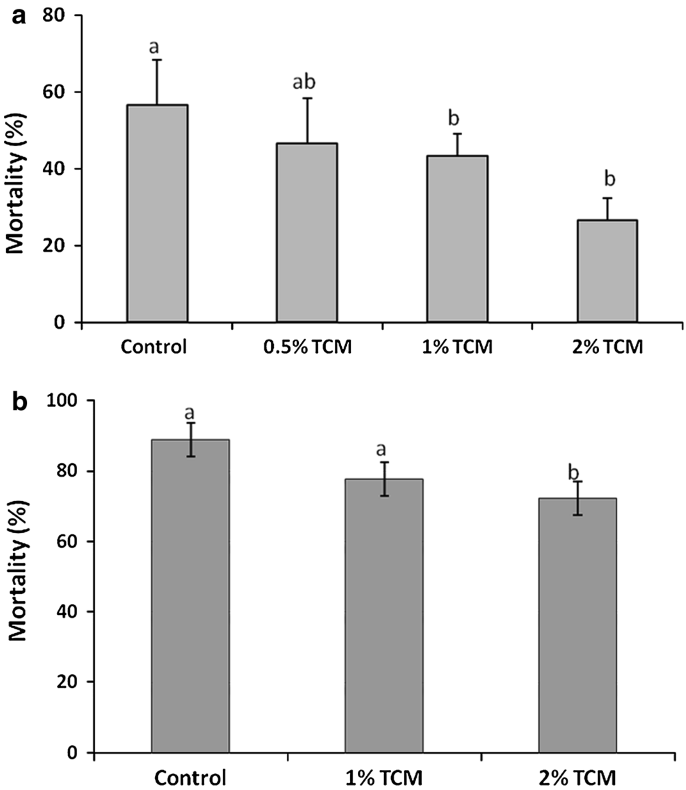

Fig. 3 Mortality (%) (Mean ± SD) of grass carp of different feeding groups after intra-peritoneal injection of *A. hytrophila*, **a** in laboratory experiment (control 0.5, 1, and 2 % formulated TCM) and **b** field trial (control 1 and 2 % formulated TCM). Means with different *superscripts* are significantly different at $P < 0.05$

Table 2 Weight gain rate (%) and specific growth rate (% day^{-1})

Treatment	Tank experiment ($n = 20$)		Pond trial ($n = 30$)	
	WGR (%)	SGR (% day^{-1})	WGR (%)	SGR (% day^{-1})
Control	12.66 ± 2.58^{a}	0.57 ± 0.11^{a}	17.20 ± 0.71^{a}	0.55 ± 0.08^{a}
0.5 % TCM	15.00 ± 1.14^{a}	0.66 ± 0.17^{a}	N.T	N.T
1 % TCM	13.42 ± 1.60^{a}	0.60 ± 0.07^{a}	18.69 ± 3.56v	0.60 ± 0.11^{a}
2 % TCM	13.58 ± 2.09^{a}	0.61 ± 0.09^{a}	18.47 ± 2.08^{a}	0.59 ± 0.12^{a}

Values in the same column with different superscripts are significantly different ($p < 0.05$)

Weight gain rate WGR = [(Final body weight−initial body weight)/initial body weight] × 100 %

Specific growth rate SGR = (Ln weight final−Ln weight initial) × 100/day

NT not tested

animals are more common in China, for example, the phagocytosis of white blood cell in carp was enhanced by the use of *Rheum officinale*, *A. paniculata*, *Isatis indigotica,* and *Lonicera japonica* (Chen et al. 2003). Applications of compound formulations in

Table 3 The product cost (in USD) of 1 and 2 % traditional Chinese medicine (TCM) feed

Item	Cost per kg ($USD kg^{-1})	1 % TCM feed			2 % TCM feed		
		Quantity (kg)	Cost		Quantity (kg)	Cost	
			$USD	(%)		$USD	(%)
Raw material and processing cost (control feed)	0.9	990	891	92.7	980	882	86.2
R. coptidis	27.69	1.43	39.60	4.1	2.86	79.19	7.7
R. scutellaria	6.79	1.43	9.67	1.0	2.86	19.33	1.9
H. andrographis	2.56	2.86	7.33	0.8	5.71	14.62	1.4
Radix sophorae flavescentis	3.21	4.29	13.77	1.4	8.57	27.51	2.7
Total	/	1,000	961.37	100	1,000	1,022.65	100

Control feed cost = $0.891 USD kg^{-1}

1 % TCM feed cost = $0.961 USD kg^{-1}

2 % TCM feed cost = $1.02 USD kg^{-1}

aquaculture are believed to be more acceptable and beneficial as different herbs with diverse mechanisms showed complementary effects between herbs in the formulation (Wang et al. 2005a).

Various herbal extracts selected in this study showed inhibition on several bacteria, e.g., *R. coptidis* extracts on *Staphylococcus aureus* (Yu et al. 2005) and *A. hydrophila* (Zhang and Yang 2006), *Andrographitis* extracts on *Bacillus cereus*, *E. coli*, and *Pseudomonas aeruginosa* (Singha et al. 2003) and *R. scutellaria* and *R. coptidis* extracts on *A. hydrophila* (Zhang and Yang 2006). In this study, there were only three out of 16 selected herbs that showed inhibitions on *A. hydrophila*: *R. coptidis*, *R. scutellaria,* and *F. forsythia*. The difference in inhibitory effects on bacteria may be due to the types of solvent for extraction such as methanol, chloroform, and water and bacteria strains, and similar variations of bacterial inhibitions of herbal extracts were also found in a recent study (Philip et al. 2009), where different active compounds were dissolved in different solvents and using different extraction methods.

The active ingredients in *Coptidis* such as palmatine, jateorrhizine, and berberine might be responsible for the antimicrobial activities (Kong et al. 2009). A study showed that *Andrographitis* extracts had an inhibitory effect on *B. cereus*, *E. coli,* and *P. aeruginosa* (Singha et al. 2003), but no inhibition on *E. coli* was found in the present study. This inhibitory effect of *Andrographitis* extract may be due to the presence of both arabinogalactan proteins and andrographolides (Singha et al. 2003).

The oxidative radicals produced from neutrophils and monocytes is an important defense system, indicating the competence of fish immunity (Anderson et al. 1992); hence, the greater NBT reduction by oxidative radicals usually indicates stronger immunity. The production of intracellular superoxide radicals by leukocytes was quantified by the NBT assay (Sahu et al. 2007a; Ardó et al. 2008). In this study, no significant change of NBT activity was noted in all TCM feeding groups. However, Yin et al. (2006) demonstrated that high doses of *Scutellaria* extract (0.5 and 1.0 %) reduced the phagocytic cell function in tilapia, while low dose (0.1 %) activated the function. A similar dose–response effect of baicalin was also shown in the study on phagocytosis of macrophages in mice with baicalin as one of the main active ingredients in *Scutellaria* (Cai et al. 1994).

Enhanced NBT activity was shown in 2 % TCM group after feeding for 14 days but not observed on day 21, which may be related to the optimal time and dose of feeding of *Scutellaria* (Yin et al. 2006). Although administration of feed supplement excreted both positive and negative effects on NBT activity with different feeding period and dose, the use of this TCM formulation at 2 % dose should be applicable as no inhibition of NBT activity was found after feeding for 21 days. In other studies, the respiratory burst activity of fish phagocytic cells was enhanced in large yellow croaker (*Pseudosciaena crocea*) and common carp (*Cyprinus carpio*) after feeding a mixture of *Astragalus membranaceus* and *Angelica sinensis* extracts (Jian and Wu 2003, 2004). Further investigation should be carried out in order to identify the causal relationship between these factors.

The bactericidal activity of plasma in fish had been enhanced after 21 days of feeding in 2 % TCM group, compared with control group. The stronger serum bactericidal activity indicated the stronger innate immune responses in fish (Das et al. 2009). It has also been noted that the activities were enhanced after feeding with some powdered dietary garlic (Sahu et al. 2006 and 2007b) and mango kernel (Sahu et al. 2007a) in rohu. A correlation between bactericidal activity of plasma and disease resistance against *A. hydrophila* was observed in a study on grass carp with cortisol injection (Wang et al. 2005b). Several studies also stated that higher than 50 % of bactericidal in fish activity indicates very good disease resistance, and 40–50 % shows good resistance (Mikriakov and Silkin 1978; Atanasova 2003; Atanasova et al. 2008). Although the bactericidal activity of grass carp was below 25 % in this study, the highest disease resistance and bactericidal activity ($\sim$25 %) were also found in 2 % TCM group. The level of bactericidal activity of plasma should be related to the disease resistances. The activation of bactericidal activity in this study may be due to the presence of berberine, an active ingredient of *R. coptidis*, and Ji et al. (2012) suggested berberine could enhance bactericidal activity through activating complement system.

In this study, the herbal extracts in feed did not show any effect on the protein levels in serum, but 1 and 2 % of TCM elevated the immunoglobulin levels at day 21 (Fig 1b). Serum proteins are responsible for innate immune response of fish and a higher level of serum protein provided stronger response (Wiegertjes et al. 1996; Sahu et al. 2007b), but generally, fish serum protein level was not altered by feed supplements like immunostimulants unless under nutritional deficiencies (Siwicki et al. 1994). The addition of feed supplement may have no effect or even adverse effect on plasma protein levels, e.g., rainbow trout fed with *Laurus nobilis* had no effect on the protein level (Bilen and Bulut 2010).

All TCM feeding groups showed lower mortalities compared to the control group. The highest disease resistance to *A. hydrophila* infection was observed in the highest tested dose (2 % w/w), whereas the highest serum bactericidal activity was noted in the same group at day 21 (Fig 2a). A study using dried powder of *A. paniculata* reduced the mortality of Nile tilapia (*Oreochromis niloticus*) after infected with *Streptococcus agalactiae,* and a dose-mortality-dependent relationship was also observed (Rattanachaikunsopon and Phumkhachorn 2009). A similar dose response was also observed in this study. However, further investigation should be conducted to verify the response.

A number of studies were conducted using herbal powders and/or extracts on disease prevention and treatment. However, there is a lack of information on the field application of herbal feed and its cost evaluation in real aquaculture situation. The field trial on this TCM formulation in Yuen Long demonstrated a better disease resistance to *A. hydrophila* in the grass carp fed with TCM, with the significant reduction of about 20 % of mortality ($p < 0.05$), when compared to control group in the field experiment (Fig. 3b). However, the mortality rates observed in the field experiment (69–89 %) were much higher compared to those in the laboratory experiment (27–57 %). This could be due to the fluctuation of environmental conditions such as temperature and dissolved oxygen encountered in the pond environment, but both the mortalities in the control groups were matched with previous mentioned mortality, about 50–90 % mortality for *A. hydriophila* infection (Yang. 2008). Zheng et al. (2012) demonstrated a superior effect of antibiotic treatment on grass carp enteritis, reducing 39–74 % mortality compared to control ($\sim$90 %) depending on dosages (1–2 g/kg feed) of norfloxacin. However, the use antibiotics for disease treatment in aquaculture also raised concerns on the development of drug resistance in pathogens which threatens human food security (Ibrahim et al. 2010; Zheng et al. 2012). Besides, in both laboratory and field experiments, no significant differences on the weight gain rate and specific growth rate were noted between control and TCM feeding groups ($p > 0.05$).

Based on the TCM feed cost evaluation for this field trial, the costs of control feed, and 1 and 2 % TCM feed were \$0.891, \$0.961, and \$1.02 USD kg^{-1}, respectively (Table 3); the TCM contributed 7.3 and 13.8 % to the total feed production cost for 1 and 2 % (w/w) TCM feed, respectively. The use of this formulation in aquaculture industry seems to be cost-effective based on the estimations in this study. It could be applied to young fish in hatchery farms and fish farms as the enteritis is prevalent in young fish when the water temperature is above 18 °C (Xu et al. 1988). The disease could also be prevalent in 1-year-old grass carp (NACA 1989); hence, the TCM feed should be applied to grass carp 1–2 months before spring.

When assuming the young grass carp (0.028 kg $fish^{-1}$) were fed with 2 % TCM feed for 2 months, the cost is only equivalent to approximately 83 kg of grass carp, which could be compensated by the higher survival rate and subsequently high yield. In addition, the cost of TCM feed in such short feeding period ($\sim$60 days) was insignificant, when compared to the overall feed used throughout the whole production period (>1 year) (FAO 2009). Therefore, the application of TCM feed may be profitable for grass carp culture, due to the low cost, which could be compensated by improved yield.

The optimal dose of the formulated TCM based on the laboratory experiment was 2.0 % (w/w) for the grass carp fingerlings, as reflected by promoted bactericidal activity and total immunoglobulin in plasma and the enhanced disease resistance of grass carp against *A. hydrophila* after 21 days of feeding. The field trial also demonstrated the practical use of TCM containing feed in aquaculture with cost-effective outcome based on reasoned estimation. However, further studies are required to show the effect of this formulation on the disease resistance against other bacterial diseases such as bacterial gill rot disease. TCM supplement could then be applicable to prevent and combat other diseases, replacing the usage of antibiotics in aquaculture.

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