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The Effects of Dietary Supplementation of Methanolic Extracts of Herbal Medicine on Haematological Variable of Red Hybrid Tilapia (*Oreochromis* sp.)

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ABSTRACT

The most common strategy to treat in aquaculture disease is the use of antibiotics, however, such utilization has been accounted to have antagonistic impacts like accumulation of drugs in tissues, development of drug resistance and immunosuppression. One of the most promising methods of controlling diseases in aquaculture is strengthening the defence mechanisms through therapeutic administration. *Vitex trifolia*, *Strobilanthes crispus*, and *Aloe vera* have been reported to have better antimicrobial activity *in vitro* against *Streptococcus agalactiae*. However, there is no report on the application of the extracts on the treatment of *Oreochromis* sp. The objective of the study was to assess the effectiveness of diet supplementation of selected plant extract for 14 days as disease treatment. In red blood cell (RBC), haemoglobin (Hb), mean corpuscular volume (MCV), white blood cell (WBC), alanine transaminase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) showed significant differences between treatment and control. RBC, Hb and WBC levels of the infected group were significantly higher ($P<0.05$) than those of the control group indicating improve defence system in the fish fed with *V. trifolia*, *S. crispus*, and *A. vera*. These results suggested that of methanolic mixed herbal to applying *S. agalactiae* infected *Oreochromis* sp had a synergistic restorative effect on the haematological variables.

Key Words: *Vitex trifolia, Strobilanthes crispus, Aloe vera, Streptococcus agalactiae, Oreochromis* sp.

INTRODUCTION

The use of antibiotics and chemotherapeutics for treatment in intensive aquaculture has been widely criticized for its negative impact (Cristea et al. 2012) and research on interactions between immunity, growth and development of eco-friendly alternatives to antibiotics that may keep fish healthy such as probiotics and plant based immunostimulants has increased. Indigenous technological knowledge for treating diseases is receiving attention in fish health and disease management.

Vitex trifolia Linn belonging to the family of *Verbenaceae* is commonly known as lemuni hitam (Malay). Rajan et al. (2012) reviewed on phytochemical and pharmacological properties of *V. trifolia*. They have several pharmacological properties like antipyretic, antibacterial, against asthma, allergic diseases, arthritis, inflammations, lumbago, headache, indigestion, colic, dysentery, wounds, ulcers, bronchitis, cough, haemorrhoids, dysmenorrhea, colds, migraine, eye pain and general frailty. *Aloe vera* (*Xanthorrhoeaceae*) is a succulent, almost sessile perennial herb. It possess healing properties such as glucomannan, a mannose-rich polysaccharide, and gibberellin, a growth hormone, interacts with growth factor receptors on the fibroblast, thereby stimulating the

growth activity and proliferation, which in turn significantly increases collagen synthesis (Vogler & Ernst 1999). *Strobilanthes crispus* (L) Bremek or *Saricocalyx crispus* (L) Bremek (Acanthaceae) is native from countries like, Madagascar and Indonesia (Sunarto 1977). Afrizal (2008) reported the presence of secondary metabolites in *S. crispus* leaves included α-sitosterol, campesterol, phytol and stigmasterol.

The objectives of the study was to assess the efficacy of diet supplementation of selected plant extract for 14 days as *S. agalactiae* treatment.

MATERIAL AND METHODS

Vitex trifolia, *Aloe vera* and *Strobilanthes crispus* were collected, grounded and keep at room temperature. The plants were extracted using 2 methods *i.e.* aqueous and methanolic extraction. After that, on day 0, the fishes were injected with bacteria. Prepare for the diet and feeding for 14 days. The survival and behavior were observed every day. The blood of the fishes was collected in day 14 to measure complete haemogram and blood enzyme.

Collection of plants

Vitex trifolia, *A. vera* and *S. crispus* were obtained from University Agriculture Park, University Putra Malaysia, Selangor, Malaysia. Fresh healthy leaves, stems and including flowers were collected in morning and washed under running tap water to remove dirt particles. They were allowed to dry in a draught oven at a temperature of 65°C for 48 hours. They were then chopped into small pieces and grounded into powder using mechanical grinder (Panasonic, MY333). The powdered plant materials were kept in airtight bottles prior to extraction in room temperature.

Preparation of extraction

For aqueous extraction, a 100 g of the powdered plant were mixed into 1 l of deionised distilled water in a 2 l of conical flask. While, for methanolic extract was prepared by adding 100 g of plants powder into 1 l of 70% of methanol. The mouth of conical flasks were kept covered using aluminium foil and agitated using shaking incubator for 3 days at room temperature. Then, the mixture was filtered through 11 µm membrane filter paper (Whatman® No. 1). After that, the extracts were evaporated to dryness using rotary evaporator at 40°C. The methanolic extracts were dissolved again in deionised distilled water (aqueous extracts) and 70% methanol (methanolic extracts) to make a 0.5 g/ml stock solution and stored at -20°C until further use.

Experimental design

This study was to evaluate the effect of dietary supplementation of methanolic extracts of *V. trifolia* (VTE), *A. vera* (AVE) and *S. crispus* (SCE) on haematological and biochemical indices in *Oreochromis* sp. as disease treatment.

Pathogenic bacteria culture

The culture and maintenance of *S. agalactiae* by injection to fish for 4 times to make sure the virulence of bacteria. The isolate was routinely checked for purity by growing on

TSA agar and micro morphology was determined at a magnification of ×1000 on light microscope using gram stain.

Diets and experimental design

The experiments were conducted at an Aquatic Animal Health Unit, Faculty of Veterinary Medicine, Universiti Putra Malaysia. *Oreochromis* sp. fingerling were obtained from a local supplier. According to the method of Rawling et al. (2009) with some modification, they were allowed 2 weeks adaptation before allotted to the different treatments. They were given a commercial starter diet fed to satiation twice a day. To determine whether the VTE, AVE and SCE were protected against *S. agalactiae*, 450 tilapia with mean weight of 10±0.5 g were divided into 45 tanks of 10 fishes each, including uninfected controls and infected controls. On day 0, all fishes were injected by intraperitoneal administration with 0.1 ml *S. agalactiae*. Infected control *Oreochromis* sp. were with *S. agalactiae* and uninfected control injected with normal saline at the same volume. The experimental was conducted for 14 days with daily monitor for clinical signs and mortality. Infected tilapia were observed for behavioural and pathological signs of erratic swimming. Dead fishes were removed twice a day and bacterial samples were obtained aseptically from the brain, kidney, liver, and intestine of 20% of morbid and dead fish to confirm the presence of *S. agalactiae*. Samples were cultured onto TSA, and Gram-stained positive coccus colonies were identified as *S. agalactiae*. The mean percent mortality and mean percent cumulative mortality of infected and uninfected fish for each trial was determined over a 14 day period. A non-stop aeration to maintain the dissolved oxygen to the optimal level was provided. All fishes were fed two times daily at 4% of body weight and the daily ration was adjusted accordingly. The feed consumption in each aquarium was recorded daily. Dead fishes from each aquarium were collected daily and weighed.

In this experiment, the commercial starter diet were used. The methanolic extract of *V. trifolia*, *A. vera* and *S. crispus* were added into commercial feed. The control diets do not contain any herbs. Control diet was formulated according to NRC (1993) recommendation and contained 35% crude protein and 3493 kcal of digestible energy kg^{-1} of dietary dry matter (DM). Additional diets were the basal diet supplemented with *V. trifolia*, 2 g VTE kg^{-1} DM of diet (VTE-2), 5 g VTE kg^{-1} DM of diet (VTE-5), 7 g VTE kg^{-1} DM of diet (VTE-7) and 9 g VTE kg^{-1} DM of diet (VTE-9). Next on *A. vera*, 2 g AVE kg^{-1} DM of diet (AVE-2), 5 g AVE kg^{-1} DM of diet (AVE-5), 7 g AVE kg^{-1} DM of diet (AVE-7) and 9 g AVE kg^{-1} DM of diet (AVE-9), and for *S. crispus*, 2 g SCE kg^{-1} DM of diet (SCE-2), 5 g SCE kg^{-1} DM of diet (SCE-5), 7 g SCE kg^{-1} DM of diet (SCE-7) and 9 g SCE kg^{-1} DM of diet (SCE-9), prepared according to Zilberg et al. (2010). The diets were air-dried at ambient temperature for 72 h, packed in air-tight containers, labelled and stored. The herbs extracts were given by oral method.

Haematological assessment

On day 14^{th}, five fish were randomly chosen from each tank for each experimental and control groups and were anaesthetized with tricane methane sulfate (MS222) at 150 mg/l prior to blood collection after 24 hours of final feeding. The blood samples were collected by puncturing the caudal vein by using a 25G X 1 syringe and transferred into lithium heparin tubes. The collected blood samples were immediately subjected to haematological analysis. Evaluation of the haemogram involves the determination of the red blood count (RBC), haematocrit (Hct), hemoglobin concentration (Hb), white blood cell count (WBC),

mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC). The blood enzyme measurement in alkaline phosphatase (ALP), alkaline transeaminase (ALT), and aspartate transeaminase (AST). An automatic blood enzyme analyzer (Hitachi 704) was used for the following determinations: Alkaline phosphatase (ALP, U/L), alkaline transeaminase (ALT, U/L), and aspartate transeaminase (AST, U/L). The apparatus is based upon dry chemical technology and colorimetric reaction. Kits obtained PLIVA-Lachema and DIALAB®, were used for the determination of all indices. Also for controls, same kits were used. The hematological and biochemical parameters are expressed in international units (SI).

Statistical analysis

All the results were subjected to analysis of variance (ANOVA). Duncan multiple range test was further used to evaluate the mean differences at 0.05 significant levels.

RESULTS AND DISCUSSION

Treatment study on the effect of the single herb was conducted on *Oreochromis* sp. to determine the haematological, biochemistry and the survival rate. The experiment conducted with *S. agalactiae* infected on day 0, followed by feeding for 14 days. The summary for haematological and biochemical parameters are presented in Table 1. Fish survival after the diseases challenge range from 72 to 100% with significant difference ($P<0.05$) among the different all treatments compared to control infected group (Table 2). In the control infected fed with OTC, there are no significant difference with VTE-7, AVE-2 and AVE-9.

For the present study, three control group were compared, first control uninfected, second control infected and the third one control infected fed with Oxytetracycline. For the control, red blood cell (RBC) and white blood cell (WBC) were no significant differences between the infected and the uninfected but there were significant differences between OTC. Haemoglobin (Hb) and packed cell volume (PCV) were significant different between the control group. However for mean corpuscular volume (MCV), there was no significant difference between the infected and OTC but there were differences with uninfected group. However mean corpuscular haemoglobin concentration (MCHC) and plasma protein exhibited no significant difference. For the biochemical profile, alkaline phosphatase (ALP), alkaline transeaminase (ALT) and aspartate transeaminase (AST) were significant difference compared to control.

In general data showed significant differences ($P<0.05$) in RBC, PCV, WBC, MCHC, ALT, AST and ALP of fish fed VTE, AVE and SCE in certain concentration compared to infected control. There were no significant difference in RBC data for VTE-7, VTE-9 and SCE-7 compared to infected control. Nevertheless, the VTE-5 showed no significant difference with Control OTC, suggesting the comparable capacity in diseases resistance. Fishes fed on diets supplemented with VTE had significantly higher ($P<0.05$) white blood cell counts than the control infected, with others herbs showing significant difference. Again VTE-7 exhibited same reaction with Control OTC IN WBC, Hb value.

PVC showed no significant difference between SCE-5, SCE-7 and AVE-9 compared the uninfected control. For the MCV, no significant difference was exhibited for SCE-2, SCE-5 and AVE-7 compared to uninfected control. On the other hand, MCHC showed significant differences between herb supplementation with all control groups. For thrombocyte, there were no significant differences encounter with supplementation SCE-5 and AVE-2, AVE-9 compared to the infected control group. Plasma protein showed no

significant difference with all control groups for supplementation of VTE-7, SCE-7 and AVE 9. Biochemical profile for the prophylaxis study showed comparative significant difference. For the ALT study with comparable Control OTC, SCE 5 and 9 showed significant difference, SCE-5 also exhibit ALP. For AST, only SCE-2 showed no significant difference with infected control group.

Diagnosis of bacterial diseases was based on the isolation and identification of the etiological agents. Fish diseases diagnosis is complicated because issues in the taxonomy of bacteria are still not being resolved and similar to other diseases, such as septicemias, could be caused by different bacterial species (Roberts 1993). Antisera against specific bacterial pathogens of fish are available but only for a few species. A wide range of bacterial pathogens is known to infect tilapia such as Streptococcosis.

Infected *Oreochromis* sp showed behavioral and pathological signs of erratic swimming. Abnormal behaviour due to the tropism of the bacteria for the central nervous system, swirling behaviour, lethargy, bent bodies and disorientated fish are commonly observed. Also in eye lesions, sick fish often have eye lesions such as endophthalmia or exophthalmia. The classical clinical signs reported with *S. agalactiae* infections in tilapia include erratic swimming (such as spiralling or spinning), uni- or bi-lateral exophthalmia also known as "pop-eye", corneal opacity, and haemorrhages in the eye, at the base of the fins and in the opercula. Also, internally, the disease appears to affect the liver, spleen, kidney, heart, eyes and brain, where abnormalities are visible grossly. The affected fish show congestion and haemorrhage of the liver, spleen, kidney and brain. The spleen and liver are often enlarged and the liver is pale in colour, inflammation around the heart and kidney has been reported as well as brain softening and the accumulation of fluid within the abdominal cavity or also known as ascites.

The vast majority of antibiotic treatments in aquaculture is administered to populations. Antibiotic treatments could be classified as prophylactic if the populations treated do not contain any infected individuals. This kind of prophylaxis, therefore, is likely to be effective if and only if the occurrence and especially the timing of the infection and, hence, the disease hazard, could be accurately foretold. Provided these conditions are satisfied, however, specific risk prophylaxis could be seen as prudent and may be economically justifiable. Despite the fact that there are few studies of the efficacy and long-term consequences of this form of prophylaxis, the prudence of antibiotic use in these situations must be seriously questioned. Therefore, it is highly recommended to invention a better option than continuous reliance on antibiotics such usage herbs.

The RBC levels were highest in SCE-2 (2.36×10^{12}/l) and lowest in control infected with *S. agalactiae* (2.03×10^{12}/l). The highest WBC concentrations were observed in SCE-5 (9.59×10^{9}/l) followed by SCE-7 (4.72×10^{9}/l) and the lowest levels were recorded in control without challenge with bacteria (1.26×10^{9}/l). The present study showed that fish fed with VTE, AVE and SCE significantly restored the altered haematological and immunological parameters and triggered the innate immune system against *S. agalactiae*. This is supported by study on, *Clarias gariepinus*, fingerlings enhances the hematological parameters even at a low level (0.5%) incorporation of garlic peel in feed and resulted in highly immunecompetent and more resistant to infection by *A. hydrophila* (Thanikachalam et al. 2010). Moreover, decaffeinated green tea in lower doses (20mg s/kg feed) of administration could be optimum to enhance the immunity of rainbow trout (Sheikhzadeh et al. 2011).

In the control infected fed with OTC, there were no significant differences with VTE-7, AVE-2 and AVE-9. The supplemented feeds reduced the mortality rate of fish experimentally infected with *S. agalactiae* and had no apparent adverse effect on the fish. Nya & Austin (2011) reported the prophylactic effect of dietary garlic application to

rainbow trout, infected with *Aeromonas hydrophila*. Fourteen days after the cessation of feeding with garlic, mortality rates of 12% were recorded in groups which received 0.5 g and 1.0 g of garlic 100 g^{-1} of feed, respectively, compared to 84% mortalities in the controls.). Sahu et al. (2007) reported that long term dietary administration of mango kernel led to considerably increases immunity and survival of fingerlings of rohu. The group fed with 5 g kernel/kg dry diet showed highest percentage survival (98%).

Bilen et al. (2011) showed the highest values of the non-specific immune parameters observed in the group of rainbow trout fed with 1% tetra (*Cotinus coggyria*) against *A. hydrophila* infection. Harikrishnan et al. (2010), suggested that 8 weeks intraperitoneal administration of the leaf extracts of *P. granatum* at 50 or 100 mg/kg dose enhanced the innate immune responses and disease resistance after against natural LVD (lymphocystis viral disease) infection.(Yilmaz et al. 2012) reported high values of hemoglobin, hematocrit, lysozyme and myeloperoxidase activity, with the addition of thyme, rosemary and fenugreek in fish feed. The author, suggested these herbal plants could be considered as feed additives for improving haematological and immune status and so the fish welfare in aquaculture.

Table 1. Haematological characteristic of *Oreochromis* sp fed with *V. trifolia*, *S. crispus*, and *A. vera*

Parameter	Control (uninfected)	Control (infected)	Control (OTC)	VTE-2	VTE-5	VTE-7	VTE-9	SCE-2	SCE-5	SCE-7	SCE-9	AVE-2	AVE-5	AVE-7	AVE-9
RBC	2.15±0.05^{a}	2.03±0.00^{a}	2.25±0.02^{b,c}	2.33±0.05^{d}	2.17±0.02^{b,c}	2.13±0.05^{a}	2.07±0.03^{a}	2.36±0.00^{d}	2.23±0.02^{c}	1.75±0.05^{a}	2.48±0.03^{e}	2.14±0.01^{b}	2.28±0.02^{d}	2.33±0.05^{d}	2.13±0.01^{b}
Hb	105.5±2.5^{e}	85.6±1.2^{a}	93.3±2.4^{c,d}	96.4±0.6^{d}	87.3±1^{a,b}	93.2±0.4^{c,d}	90.0±0.9^{b,c}	91.8±0.0^{b,c}	99.0±1.0^{d,e}	77.1±1.9^{a}	99.0±4.0^{d,e}	85.0±2.8^{b}	84.5±0.1^{b}	75.9±2.3^{a}	75.2±0.3^{a}
PCV	0.29±0.00^{c}	0.24±0.00^{a}	0.26±0.01^{b}	0.34±0.01^{e}	0.32±0.01^{d}	0.31±0.01d	0.31±0.01^{d}	0.30±0.01^{d}	0.29±0.00^{c}	0.28±0.01^{c}	0.33±0.01^{e}	0.31±0.01^{d,e}	0.34±0.01^{f}	0.32±0.01^{e}	0.30±0.01^{c,d}
MCV	133±1^{b}	116±2 ^{a}	114±1^{a}	144±1^{e}	145±0^{d}	147±1^{d}	150±0^{d}	127±0^{b}	124±1^{b}	157±2^{d}	133±2^{c}	143±3^{c,d}	147±3^{d}	135±1^{b}	138±2^{b,c}
MCHC	370±2^{d}	364±3^{d}	366±4^{d}	288±3^{b}	277±1^{a}	305±4^{c}	295±2^{b}	306±0^{b}	360±3^{c}	280±2^{a}	300±3^{b}	278±4^{c}	252±4^{a,b}	241±3^{a}	255±3^{b}
WBC	1.26±0.01^{a}	1.31±0.01^{a}	1.66±0.01^{b}	4.65±0.19^{e}	3.96±0.03^{d}	1.62±0.01^{b}	2.15±0.01^{c}	2.42±0.01^{c}	9.59±0.07^{f}	4.72±0^{e}	4.22±0.02^{d}	1.96±0.01^{c}	3.85±0.02^{e}	3.26±0.03^{d}	4.63±0.15^{f}
Plasma protein	34±1^{a}	37±1^{a}	36±1^{a}	48±1^{c}	52±2^{d}	36±1^{a}	41±1^{b}	43±1^{b}	56±2^{c}	37±1^{a}	43±1^{b}	41±1^{b}	46±1^{c}	42±0^{b}	36±2^{a}
ALT	31.2±0.4^{c}	51.4±0.2^{d}	26.3±0.6^{b}	12.5±0.1^{a}	30.4±0.3^{c}	15.2±1.7^{a}	13.1±2.0^{a}	40.0±0.7^{c}	32.3±0.9^{b}	38.7±0.8^{c}	32.9±0.1^{b}	22.7±0.3^{a}	34.1±0.8^{d}	30.5±0.1^{c}	38.4±0.3^{e}
ALP	28±3^{a}	44±2^{e}	38±1^{d,e}	33±1^{a,b,c}	34±2^{c,d}	32±2^{a,b}	34±1^{a,b,c}	35±1^{b,c,d}	39±1^{d,e}	32±1^{a,b}	33±1^{a,b,c}	32±2^{a,b}	31±1^{a,b}	33±4^{a,b}	35±3^{a,b}
AST	223.3±2.1^{b}	481.6±0.85^{e}	184.1±1.5^{a}	305.6±0.5^{c}	307.7±1.15^{c,d}	311.4±3.05^{d}	218.9±0.7^{b}	285.3±3.3^{e}	232.5±1.4^{c}	222.4±2.35^{b}	239.0±0.5^{d}	225.1±2.25^{c}	220.5±0.95^{b,c}	215.1±0.85^{b}	322.3±2.05^{d}

Table 2. Survival rate of *Oreochromis* sp after post-infection fed with *V. trifolia*, *A. vera*, and *S. crispus*

Growth performance	Diets														
	Control (uninfected)	Control (infected)	Control (OTC)	VTE-2	VTE-5	VTE-7	VTE-9	AVE-2	AVE-5	AVE-7	AVE-9	SCE-2	SCE-5	SCE-7	SCE-9
Survival (%)	100±0.00 ^{e}	72±5^{a}	95±^{5d,e}	87.5±2.5 ^{a,b,c}	92.5±2.5^{c,d,e}	95±5^{d,e}	82.5±2.5 ^{a,b,c}	95±5^{d,e}	92.5±2.5^{c,d,e}	90±0.00^{b,c,d,e}	95±5^{d,e}	83.5±1.5^{b,c,d}	83.5±3.5 ^{b,c,d}	89±1.0^{b,c,d,e}	81±1.00^{a,b}

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CONCLUSION

The ability of bacteria to become clinically resistant to antibiotics is a major threat to the continued therapeutic use of these agents in aquaculture. It has been argued that reductions in antibiotic use could be achieved with the usage of herbs. It could be concluded from the results of the present study that supplementation of methanolic herbal extract are able suppress the *S. agalactiae* infection on *Oreochromis* sp. with a possible synergistic restorative effect on the haematological variables. Comparable with all control group, *S. crispus* showed as promising agent as treatment against *S. agalactiae*. However, the exact bioactive compound responsible for the reaction is unknown and the results pattern is not dependent to the concentration.

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