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Effects of dietary levels of essential oil (EO) extract from bitter lemon (*Citrus limon*) fruit peels on growth, biochemical, haemato-immunological parameters and disease resistance in Juvenile *Labeo victorianus* fingerlings challenged with *Aeromonas hydrophila*

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Abstract

Essential oils (EOs) are used in the food industry because of their biological activity. We evaluated the effects of administration of essential oil (EO) extracted from bitter lemon (*Citrus limon*) fruit peels on the growth performance, biochemical, haemato-immunological parameters and possible disease resistance in fingerlings (4 weeks old) *Labeo victorianus* challenged with *Aeromonas hydrophila*. Fish were divided into five groups and fed diets supplemented with *C. limon* fruit peels EO extract at 1%, 2%, 5% and 8% [as fed basis] and treatment compared with control group fed diet without *C. limon* fruit peels EO extract. The experiment was executed in triplicate. Concentration of plasma cortisol, glucose, triglyceride and cholesterol decreased while that of total protein and albumin increased as dietary inclusion of the EO extract of *C. limon* fruit peels was increased from 2% to 5%. Meanwhile haemato-immunological parameters including red blood cell (RBC), white blood cell (WBC) counts, haematocrit (Htc), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC) and neutrophiles increased with increasing dietary inclusion from 1% to 5% inclusion of *C. limon* fruit peels EO extract. Serum immunoglobulins, lysozyme activity and respiratory burst increased with increasing dietary levels up to 5% inclusion of EO extract of *C. limon* fruit peels. We demonstrate that formulation of feeds by incorporating upto 5% the EO extract from *C. limon* fruit peels significantly improved biochemical, haematological and immunological response in juvenile fish resulting to lower mortality than the untreated groups and appear to be effective antibacterial against *A. hydrophila*.

Keywords: diseases, essential oil, serum biochemistry, serum haematology, immunological parameters, immunity

Introduction

Due to increased intensification of aquaculture, occurrence of microbial agents (bacteria, virus, fungi etc.) may cause outbreaks of disease in the cultured organisms resulting in massive mortality and losses of cultured organisms. Among the microbial agents, bacterial infections are most frequently reported (Sanmukh, Meshram, Paunikar & Swaminathan 2012). The broad genus of bacteria that contaminate fish are present in micro-flora such as: *Photobacterium*, *Vibrio*, *Streptoccocus* and *Aeromonas*. Reports of the occurrence of *Aeromonas hydrophila* (an opportunistic pathogen, facultative anaerobic, chemo-organotrophic gram-negative bacteria) a widespread microorganisms causing motile aeromonas septicaemia (Gonzalez, Santos, Garcia Lopez & Otero 2002; Austin & Austin

2007) are frequent (Laith & Najiah 2013). Such outbreaks routinely cause massive mortality of fish in the culture units (Hayes 2005). Therefore, one of the main aims of healthy aquaculture is maintenance of healthy stock, through prevention and/or control of the outbreak of diseases. Many fish farmers attempt to control bacterial infection through prophylactic and other chemotherapeutic treatments (Holmström, Gräslund, Wahlström, Poungshompoo, Bengtsson & Kautsky 2003). Most antimicrobial agents in aquaculture are toxic, non-biodegradable, are environmental pollutants and are resistant to pathogen (McPhearson, DePaola, Zywno, Motes & Guarino 1991; DePaola, Peeler & Rodrick 1995; Shak, Whitaker, Ribner & Burd 2011). Alternatives to antibiotics in aquaculture are therefore urgent.

Plant extracts with antimicrobial and/or immunostimulant properties have been used as therapeutic and/or prophylactic agents against fish pathogens (Newaj-Fyzul & Austin 2015). Against bacteria, they disrupt the bacterial cell wall, block the synthesis of proteins and DNA, inhibit enzyme secretion and hinder the bacterial signalling mechanism via *quorum* sensing (Citarasu 2010). Against outbreaks of *A. hydrophila*, several plant extracts as alternative to antibiotics have been tested. These include *Origanum heracleoticum* in *Ictalurus panctatus* (Zheng, Tan, Liu, Zhou, Xiang & Wang 2009), *Astragalus radix* and *Ganoderma lucidum* in *Cyprinus carpio* (Yin, Ardo, Thompson, Adams, Jeney & Jeney 2009), *Ocimum sanctum* in *Labeo rohita* (Das, Raman, Saha & Singh 2013), *Morus alba* foliage methanolic extract in *Clarias gariepinus* (Sheikhlar, Alimon, Daud, Saad, Webster, Meng & Ebrahimi 2014) and *Allium sativum* in *Oncorhynchus mykiss* (Nya & Austin 2009). The essential oil (EO) extract have exhibited a wide spectrum of biological activity, thus elicit varying physiological, biochemical and haemato-immunological response in fish.

Bitter lemon (*Citrus limon*), belong to Rutaceae (citrus family) originating from Asia (likely India and Pakistan) and is now grown commercially worldwide in tropical, semi-tropical and warm temperate countries (http://eol.org/pages/582200/details). Although the fruit juice is extracted for commercial purpose, the EOs are exploited in food industry as aroma flavour enhancers for soft and alcoholic beverages and in pharmaceutical industries. The *C. limon* EO mainly exists in fruit peels which are usually discarded as waste. Although the EO derived from other citrus fruit peels possess antibacterial activities against *Bacillus cereus*, *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes Salmonella typhimurium*, *Staphylococcus aureus* and *Vibrio vulnificus* (Kim, Marshall, Cornell, Preston & Wei 1995; Fisher & Phillips 2006), we have limited knowledge on the effects of EO extract from citrus fruit peels EO on *A. hydrophila.* This knowledge gape continues despite the fact that *C. limon* contain chemical compounds like other citrus fruits such as oranges (Fisher & Phillips 2006; Janati, Beheshti, Feizy & Fahim 2012; Acar, Kesbiç, Yılmaz, Gültepe & Türker 2015).

Growth performance, survival, haematological, biochemical and immunological variables are among the most significant physiological indicators of fish health (Campbell 2004). Thus, the objectives of the present study was to determine the effects of dietary *C. limon* fruit peels extract on growth, biochemical, haemato-immunological response in *Labeo victorianus* following experimental challenge with *A. hydrophila*. The *L. victorianus* is a high value, fast-growing, bottom-feeding omnivorous freshwater cyprinid endemic to Lake Victoria basin (Mokoro, Oyoo-Okoth, Ngugi, Njiru, Rasowo, Chepkirui-Boit & Manguya-Lusega 2014) and references therein). We recently showed that outbreak of *A. hydrophila* caused up to 100% mortality of *L. victorianus* when no prophylaxis was used in the diet of this species (Ngugi, Oyoo-Okoth, Mugo-Bundi, Orina, Chemoiwa & Aloo 2015).

Materials and methods

Laboratory setup and fish culture

This study was conducted in controlled hatchery conditions at Mwea AquaFish Farm, Kenya. The procedure for broodstock collection, larval production and rearing followed similar protocols detailed in (Ngugi *et al.* 2015). Three mature female broodstock (mean weight = 474.5 ± 12.9 g) and two mature males (mean weight = 461 ± 11.1 g) were netted from the rearing tanks and transferred to the hatchery. Prior to the experiment, fish were fed a formulated diet with a crude protein of 30.2% and crude lipid 8.0% (control diet, Table 1). Larvae were obtained through induced breeding and semi-natural spawning. Initially about 3000 larvae were hatched. The larvae were cultured for a period of 14 days to an initial mean weight of 21.0 ± 2.4 g in a flow-through raceway-type 2500 L open water tanks, supplied with filtered dechlorinated tap water

Table 1 Ingredients and proximate composition of the experimental diets (g kg^{-1}) used during the *Labeo victorianus* culture

	***Citrus limon* prepared diet (g kg^{-1})**				
Ingredients	**0**	**10**	**20**	**50**	**80**
Sardine fish meal*	360.0	360.0	360.0	360.0	360.0
Wheat floor	240.0	240.0	240.0	240.0	240.0
Corn starch	200.0	200.0	200.0	200.0	200.0
Perch oil	35.0	35.0	35.0	35.0	35.0
Binders (Cassava)	20.0	20.0	20.0	20.0	20.0
Vitamin premix†	20.0	20.0	20.0	20.0	20.0
Mineral premix‡	20.0	20.0	20.0	20.0	20.0
Cellulose	85.0	75.0	65.0	25.0	5.0
Salt (Nacl)	20.0	20.0	20.0	20.0	20.0
C. limon peel EO extract	0.0	10.0	20.0	50.0	80.0
Proximate composition (%)					
Dry matter	92.3	92.5	92.1	92.5	91.2
Crude protein	28.9	28.9	28.9	28.9	28.9
Crude lipid	7.6	7.6	7.6	7.6	7.5
Ash	6.2	6.2	6.1	6.2	6
Crude fibre	5.8	5.7	5.9	5.7	5.6
NFE	43.8	44.1	43.6	44.1	43.2
Gross energy (MJ kg^{-1} diet)§	1725.9	1731.0	1722.5	1731.0	1712.6

*Obtained locally.

†Commercial formula (mg premix kg^{-1} diet). Vitamins (mg): retinol, 1000; thiamine, 1200; riboflavin, 2000; pyridoxine, 1000; cyanocobalamine, 200; ascorbic acid (Stay C), 5000; cholecalciferol, 2400; a tocopherol, 1000; pantothenic acid, 400; choline chloride, 1600; folic acid, 2500; nicotinic acid, 1800; biotin, 1200; inositol, 3000; paraminobenzoic acid, 3200.

‡Minerals (mg): cobalt, 400; copper, 2100; iron, 2000; iodine, 1600; manganese, 4000; zinc, 2000; selenium, 400.

§Gross energy (MJ kg^{-1}) calculated according to 23.6 kJ g^{-1} for protein, 39.5 kJ g^{-1} for lipid and 17.0 kJ g^{-1} for NFE.

at a rate of approximately 50 L h^{-1}. During the culture period, the larvae were fed *Artemia* nauplii. The water was continuously aerated, and temperature controlled thermostatically at 26.0 ± 1.5°C. After acclimation period, juvenile fish were netted transferred to each of the 20 fibreglass open flow-through tanks (capacity 500 L) at ~500 fish. The tanks contained tap water previously stocked for 48 h in an intermediate holding tank. Aeration was done using electric pump. Water salinity, NO_3^-, NH_3 and pH were similar to our earlier study (Ngugi *et al.* 2015). The larvae were held in the tanks for 7 days before the experiments. We recorded about <2% mortality of fish during the acclimation period. Natural hatchery condition was 12 h light:12 dark which was then maintained during experiment. Temperature was controlled using thermostat heaters and ranged 25.1°C to 26.5°C. Water flow rate was maintained at 25 L^{-1}. The fish were then cultured for 28 days until they reached fingerling stage ready for stocking.

Preparation of citrus fruits, extraction and analysis of EO

The *C. limon* fruits were purchased from Karatina open air market in Karatina Town, Kenya. The fruits were washed and peeled within a day. The *C. limon* fruit peels were diced into 0.5 × 0.5 cm pieces and stored at −20°C before extraction. The peel pieces were vacuum dried and finally ground into powder. A mixture of EO were extracted from ~100 g of the peel using Microwave hydrodiffusion and gravity (Bousbia, Vianc, Ferhata, Meklatia & Chemat 2009).

The EO constituents were analysed using gas chromatographer (Shimadzu GC17; Shimadzu Corporation, Kyoto, Japan) coupled with mass spectrometer. Helium was used as the carrier gas. Separation was performed using a Scientific DB-5 fused capillary column measuring 30 m × 0.25 mm × 0.25 μm. The oven temperature was gradually increased from 60–105°C, 105–190°C and 190–280°C at a sequence of 5°C min^{-1}, 10°C min^{-1} and 20°C min^{-1} respectively. The injector was maintained at 260°C and detector at 280°C. The concentrations of the extracted compounds were quantified from the GC peak area. n-Alkanes were used as reference points in the calculation of relative retention indices.

Bacterial species culture and incubation

A strain of *A. hydrophila* (B2/12) was obtained from the Department of Microbiology, Karatina University, incubated and used for the challenge test. The *A. hydrophila* was stored at −80°C in tryptic soy broth (TSB) with 15% glycerol. Before testing, the bacteria stocks were streaked onto tryptic soy agar (TSA) for colonies to develop. A single colony was selected and incubated for 24 h in TSB at 37°C. After incubation, the bacteria were centrifuged at 3000 *g* for 10 min at 4°C and then suspended in phosphate buffered saline (PBS, pH 7.4). The bacteria species was confirmed using a light microscope. The optical density (OD) of the bacterial suspension was adjusted to 0.5 at

456 nm corresponding to 1×10^7 and kept in the water bath at 30°C for 2 h.

Proximate analysis, dietary formulation and analysis

Proximate composition was conducted on the ingredients and experimental diets using methods detailed in (AOAC 1995). As for the ingredients, the proximate analysis was essential to evaluate the quantity of ingredient needed for the dietary formulation to achieve the desired chemical composition of the diets. To determine the dry matter (DM), ingredients and feeds were oven dried at 105°C for 24 h (GCA, Model 18EM; Precision Scientific Group, Chicago, IL, USA). Kjelhdahl method following acid digestion (Labconco, Kansas, MO, USA) was used to determined the crude protein (CP) (N × 6.25). Crude lipids (CP) were extracted using an ASE-200 Accelerated Solvent Extractor (Dionex®, Thermo Fisher Scientific, Sunnyvale, CA, USA) and analysed using Gas Chromatography-mass spectrometry (GC-MS; Hewlett Packard 6890 Series II Plus Gas Chromatograph, San Diego, CA, USA). Ash content was determined by incineration in a muffle furnace at 550°C for 24 h and crude fibre (CF) by digestion with 1.25% H_2SO_4 and 1.25% NaOH. Nitrogen-free extracts (NFE) were calculated from the differences as: 100-[CP+CL+Ash+CF+(100-DM)]. Gross energy (GE) was calculated using conversion factors for protein, lipids and carbohydrates provided in Tacon (Tacon 1990).

Five isonitrogenous and isolcaloric experimental diets containing 28.9% crude protein, 7.6% crude lipid and 17.3 MJ kg^{-1} were formulated in the current study by mixing the ingredients in proportions shown in Table 1. The *C. limon* fruit peels EO extract was added to the feed ingredients [as fed basis] at 10 g kg^{-1}, 20 g kg^{-1}, 50 g kg^{-1} and 80 g kg^{-1} representing 1%, 2%, 5% and 8% dietary levels respectively. The control diet contained no *C. limon* fruit peels EO extract. The ingredients were ground and passed through a 500 μm mesh sieve, homogenized for 10 min in a blender (Hobart M-600; Hobart, Troy, OH, USA) and finally dried in an air oven at 40°C for 4 h. The experimental diets were stored at −4°C until used.

Feeding experiments

The feeding experimental was conducted in a Completely Randomized Design (CRD) in the fibreglass tanks after acclimation. Each fibre glass contained up to a 500 fish. Five fish group represented the five varying dietary levels with varying EO extract. The feeding experiments were executed in triplicate. The fish were fed at estimated 4% of the biomass (based on unpublished data on feeding trials of fingerlings) of each tank divided into two feedings regime (08:00 hours and 17:00 hours) 7 days a week. Any uneaten feed was then collected from the tank after the feeding experiment.

Sample collection and analysis

Growth performance

The feeding trial was carried out for 28 days. Fish in each aquarium were individually weighed at the beginning of the experiment and end of the experiment. Growth performance was calculated as:

$$\text{Weight gain (\%)} = 100 \times (\text{final body weight} - \text{initial body weight}) / \text{initial body weight}$$

$$\text{Specific growth rate (SGR\%)} = 100 \times \text{Ln}(\text{final body weight} / \text{initial body weight}) / \text{number of experimental days}$$

$$\text{Feed conversion ratio (FCR)} = \text{feed consumed (g dry weight)} / \text{weight gain (g)}$$

Blood samples and analyses

After 28 days, 20 fish were randomly selected from each tank, anaesthetized using 10 μg L^{-1} of phenoxyethanol and blood collected from the caudal vein using sterilized hypo-dermal syringe rinsed in 2.7% EDTA solution (Hi Media Laboratories, Mumbai, Maharashtra, India). The blood samples were divided two portions; the first portion was transferred to microtube containing heparin anti-coagulant and used for respiratory burst assay and immunological examination. The second portion was transferred to non-heparinized micro tube, placed at room temperature and allowed to clot for 2 h and the serum separated by centrifugation at 1500 *g* for 20 min at 4°C. After centrifugation, sera were collected with a micropipette and stored at −20°C until used.

Determination of serum biochemical parameters

Serum cortisol was measured using a commercial kit for radioimmunoassay, "Coat-a-Count Cortisol®" – DPC (Diagnostic Products, Los Angeles, CA, USA). Serum glucose quantified using colorimetric method described in Trinder (Trinder 1969). Serum total protein was determined using a micrometer as described in Wootton (Wootton 1964). Albumin, triglyceride and cholesterol were estimated using biochemical auto analyser instrument (Eurolyser, Leuven, Belgium) (Shahsavani, Mohri & Gholipour 2010).

Determination of haemato-immunological parameters

The red blood cell (RBC: 10^6 mm^{-3}) and white blood cells (WBC: 10^4 mm^{-3}) were counted using haemocytometer. Levels of hematocrit (Htc %) was measured in microhematocrit tube reader (Hawkley and Sons, Lancing, UK). The haemoglobin concentration in blood (Hb g dL^{-1}) was measured by cyanomethaemoglobin method. Aproximately 20 μL of blood was mixed with 5 mL of Drabkin's working solution and the absorbance measured using spectrophotometer (Therrmo Electron; Merck, Rahway, NJ, USA) at a wavelength of 540 nm. The mean corpuscular volume (MCV; fL), mean corpuscular haemoglobin (MCH; pg) and mean corpuscular haemoglobin concentration (MCHC; %) were calculated according to the following equations (Haney, Hursh, Mix & Winton 1992).

$$\text{MCV (fL)} = \text{Hct(\%)} \times 10/\text{RBC(million/mm}^3\text{)}$$

$$\text{MCH (pg)} = \text{Hgb (gm/dl)} \times 10/\text{RBC(million/mm}^3\text{)}$$

$$\text{MCHC (\%)} = \text{Hgb (gm/dl)} \times 100/\text{Hct(\%)}$$

Neutrophiles and leucocyte counts were obtained using peripheral blood smears stained by Giemsa (Beutler, Lichtman & Coller 2001).

Modified Anderson and Siwicki (Anderson & Siwicki 1995) protocols guided the estimation of total immunoglobulin in plasma. Lysozyme activity was measured using turbidimetric method described by Ellis (Ellis 1990) and the respiratory burst activity measured by Chemiluminescent assay (CL) as described by Khoshbavar-Rostami, Soltani & Hassan (2006) [see detailed description in (Ngugi *et al.* 2015)].

Challenge tests with *A. hydrophila*

After 28 days, 12 fish from each experimental fish batch were challenged by injecting 0.10 mL of *A. hydrophila* suspension intraperitoneally and the fish transferred in different tanks. The fish were then monitored for 18 for mortality. Tissues were extracted from the fish carcass and used for the bacteriological culture. A group of five fish per groups were sampled to monitor biochemical and haemato-immunological parameters as already described.

Survival was calculated as:

$$\text{Relative percentage survival (RPS)} = \text{Number of surviving fish after challenge} / \text{Number of fish injected with bacteria} \times 100$$

Statistical analysis

Results of the assays were presented as average (± SEM) for fish per treatment group. Significant differences between dietary experimental groups were determined using one-way analysis of variance (ANOVA). Survival data (%) was arcsine transformed before statistical analysis. Duncans Multiple Range Test (DMRT) was used for multiple comparisons of means. Differences in measured parameters before and after challenge test were determined using paired t-test. Data were analysed using STATISTICA (ver. 7) (StatSoft, Tulsa, OK, USA). Analysis of the differences were done up to 99.9% confidence levels, however, results were considered as significant at $P < 0.05$.

Results

GC analysis of essential oil components

Extracted phytochemicals and their relative percentages in the EO extract and their retention times are provided in Table 2. Ten major compounds were detected by GC in the EO of bitter lemon fruit peels. The most abundant chemical constituent was limonene (81.4%) followed by β-pinene (9.23%). Other compounds, except for α-terpineol, α-pinene, sabinine and p-cymene, were all less than 1% of the total composition.

Growth performance

Parameter of growth performance following feeding with our experimental diets is shown in

Table 2 Chemical constitution of bitter lemon (*Citrus limon*) fruit peels essential oil determined by GC

No. of peaks	Chemical	Retention time (min)	Concentration	
			Relative ratio (%)	Absolute concentration (mg g^{-1})
1	α-terpineol	4.53	1.39	4.76
2	α-pinene	18.9	2.09	7.15
3	β-pinene	7.65	9.23	31.58
4	Sabinine	9.19	1.55	5.30
5	p-cymene	10.91	1.66	5.68
6	Limonene	13.42	81.40	278.47
7	Neryl acetate	19.31	0.93	3.18
8	Citral	22.34	0.67	2.29
9	γ-terpinene	32.10	0.35	1.20
10	Geranyl acetate	33.90	0.31	1.06

Table 3 Stocking weight, harvest weight, weight gain (%), SGR (% day^{-1}) and FCR of *Labeo victorianus* fed with varying levels of *Citrus limon* fruit essential oil extract in the formulated diets

Growth parameters	Dietary inclusion levels of *C. limon* peels EO extract				
	Control (0.0%)	1.0%	2.0%	5.0%	8.0%
Initial weight (g)	25.1 ± 1.2	24.6 ± 1.6	25.3 ± 1.7	24.3 ± 1.8	24.7 ± 1.5
Final weight (g)	38.1 ± 3.6^{a}	47.1 ± 4.3^{b}	56.1 ± 4.8^{c}	64.3 ± 4.6^{d}	64.9 ± 4.5^{d}
Weight gain (%)	51.8 ± 4.9^{a}	91.5 ± 8.4^{b}	121.7 ± 13.2^{c}	164.6 ± 17.9^{d}	162.8 ± 15.2^{d}
SGR	1.49 ± 0.13^{a}	2.32 ± 0.24^{b}	2.84 ± 0.25^{c}	3.48 ± 0.21^{d}	3.45 ± 0.26^{d}
FCR	1.48 ± 0.05^{d}	1.24 ± 0.06^{c}	0.91 ± 0.06^{b}	0.72 ± 0.04^{a}	0.69 ± 0.05^{a}

Values are means ± SEM, SGR, specific Growth Rate; FCR, food Conversion Ratio. Values with different letters differ significantly ($P < 0.05$).

Table 3. The final fish weight, weight gain and SGR were significantly higher in fish fed with diets containing *C. limon* fruit peels EO extract compared with the control diet ($P < 0.05$) and increased with increasing levels of *C. limon* EO in the diet up to 5%. The FCR significantly reduced with increasing dietary inclusion levels of dietary *C. limon* EO up to 5%.

Biochemical variables

Biochemical response of *L. victorianus* fed with varying dietary extract of EOs of *C. limon* is shown in Fig. 1. Plasma glucose and cortisol were significantly ($P < 0.05$) higher in fish groups fed diets containing *C. limon* EO extract than the untreated groups and reduced systematically following increased level of citrus in the diet until 5% of the EOs. The value of these parameters were similar in fish fed at 5% EO extract in the diet, Total protein and albumin in blood of healthy and pathogen challenged fish were higher in fish groups fed diets containing citrus EOs and increased in tandem with increasing dietary level of dietary *C. limon* EOs up to 5% levels. Triglyceride and cholesterol values were lower in the treated groups and subsequently reduced with increasing dietary *C. limon* EO up to 5% level; the bacteria challenged fish groups contained higher concentration of these biochemical parameters when fed diets with dietary EO below 5%.

Haemato-immunological parameters

The effects of varying dietary *C. limon* EO on the haemato-immunological parameters of *L. victorianus* at the end of the experiment is provided in Fig. 2. The RBC count, WBC, Hb, Htc, MCV, MCH and MCHC were higher in fish fed diets treated with *C. limon* peel EO extract and these values increased with increasing dietary *C. limon* being highest at 2% and 5% level. Lymphocyte was higher in bacteria challenged fish only in the control treatment. In both healthy and bacteria challenged fish, the levels of lymphocytes reduced after feeding on dietary *C. limon* fruit peels EO extract.

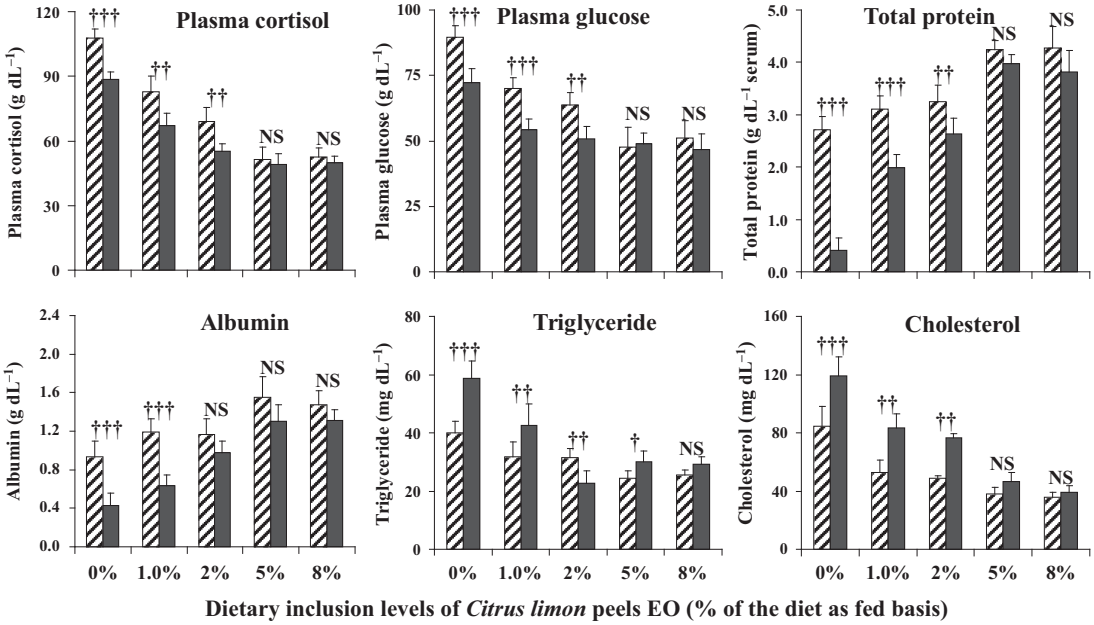

Figure 1 Mean (± SD) of the serum biochemical indices for pre-challenged (diagonal dashed) and post challenged (dark shaded) *Labeo victorianus* fed with 0%, 1%, 2%, 5% and 8% dietary *Citrus limon* fruit peels EO extract for 28 days. $n = 20 \times 3$ replicates; †, †† and ††† denotes that the treatments are significantly different before and after bacteria challenge at $\alpha = 0.05$, $\alpha = 0.01$ and $\alpha = 0.001$ respectively. NS denotes no significant differences in the measured biochemical parameter before and after bacteria challenge.

Neotrophiles was higher in challenged fish in control group and in the group treated using 1% EO but was lowest at dietary administration of *C. limon* EO of 2% and 5%. In healthy fish, there was no significant difference in the level of neutrophiles observed. The bacteria challenged fish had lower concentration of serum immunoglobulin, lysozyme activity and respiratory burst activity. These latter parameters were consistently higher in treated fish group than in the in untreated control and increased until 2% dietary *C. limon* fruit peels EO extract in healthy fish and up to 5% in bacteria challenged fish.

Bacteria challenge test

After 28 days of feeding, fish were challenged with *A. hydrophila* and survival recorded for 18 days (Fig. 3). All treated groups showed higher survival than control groups ($P < 0.05$). 100% mortality was observed in fish challenged with *A. hydrophila* and fed control diet. RPS significantly ($P < 0.05$) increased in bacteria challenged fish at increasing dietary inclusion levels. The first observation of mortality was recorded at 3 day post infection and increased to 100% in control group after 15 days. Mortality in the treated group started on the sixth day and only exceeded 80% in the diet with 1% *C. limon* fruit peels EO. In bacteria unchallenged groups, significantly ($P < 0.05$) higher RPS compared with control group occurred at 2% and 5% dietary inclusion levels of *C. limon*.

Discussion

The EOs extracted in *C. limon* was dominated by hydrocarbon (lemonine, α and β-pinene, γ-terpinene), with lower proportion of monoterpenes (e.g., α-terpineol, sabinene, ρ-cymene, neryl and geranyl acetates) consistent with previous studies (Lota, de Rocca Serra, Tomi, Jacquemond & Casanova 2002; Fisher & Phillips 2008). These are secondary metabolites and possess antimicrobial properties (Tajkarimi, Ibrahim & Cliver 2010), antibacterial properties (Oussalah, Caillet & Lacroix 2006), antiparasitic (George, Smith, Shiel, Sparagano & Guy 2009), antiviral (Schnitzler, Astani & Reichling 2011) and antifungal properties (Fitzgerald, Stratford & Narbad 2003; Silva, Ferreira, Duarte, Mendonça & Domingues 2011). However, the EOs derived from the plant materials may have different efficacies on antimicrobial agents. The

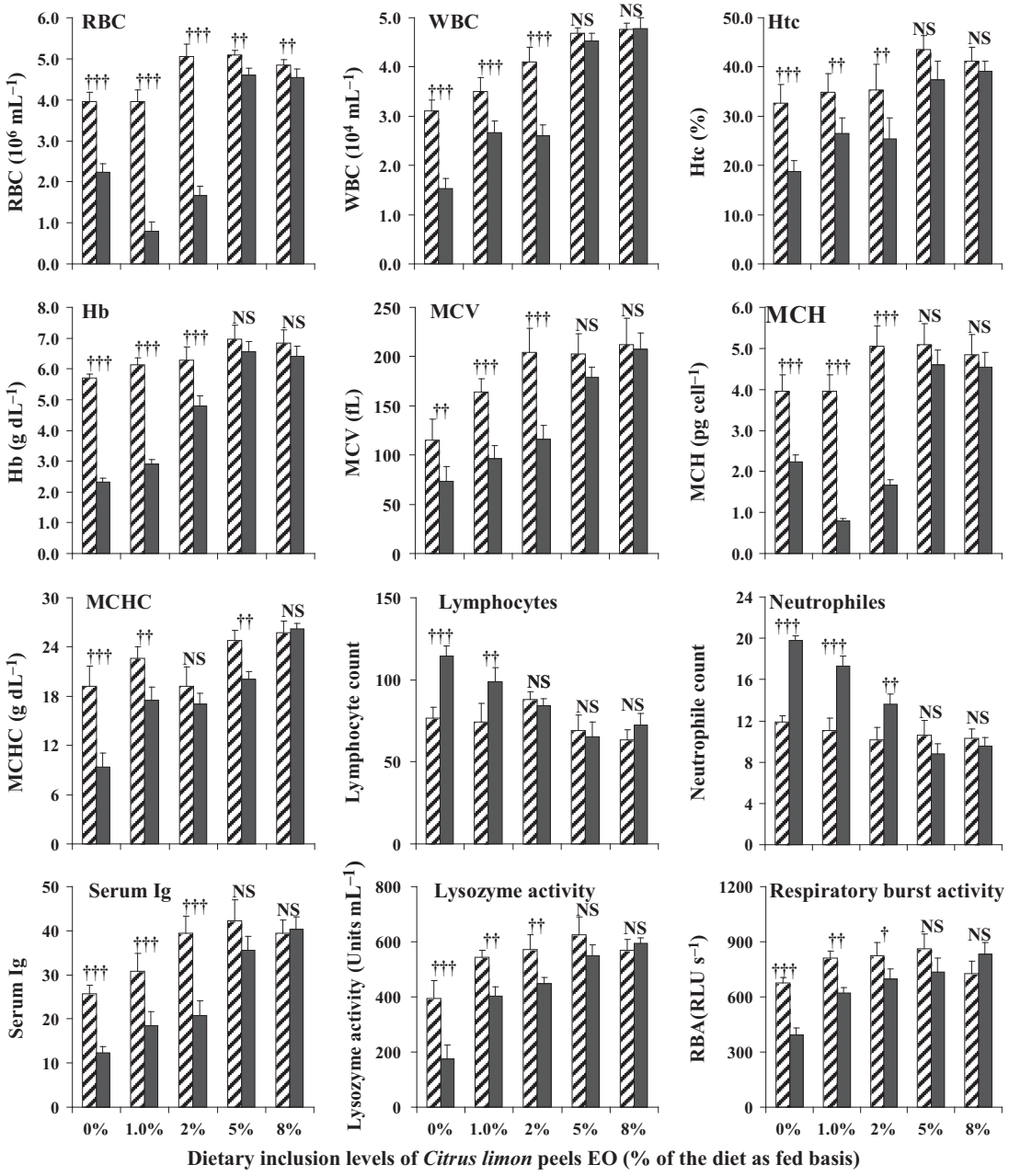

Figure 2 Serum haemato-immunological parameters of pre-challenged (diagonal dashed) and post challenged (dark shaded) *Labeo victorianus* juveniles fed diets containing 0%, 1%, 2%, 5% and 8% of *Citrus limon* fruit peels EO extract for 28 days. $n = 20 \times 3$ replicates; †, †† and ††† denotes that the treatments are significantly different before and after bacteria challenge at $\alpha = 0.05$, $\alpha = 0.01$ and $\alpha = 0.001$ respectively. NS denotes no significant differences in the measured biochemical parameter before and after bacteria challenge.

EOs in all groups of the citrus family is enriched in the peels which currently considered a waste in many countries. There is scant information on the EO extracted from the citrus, more specifically *C. limon* fruit peels on fish health management.

The results indicated that all parameters of growth performance such as weight gain, SGR and FCR increased with increasing levels of *C. limon* fruit peels EO in the diet and appeared to reach maximum levels at 5%. The current results

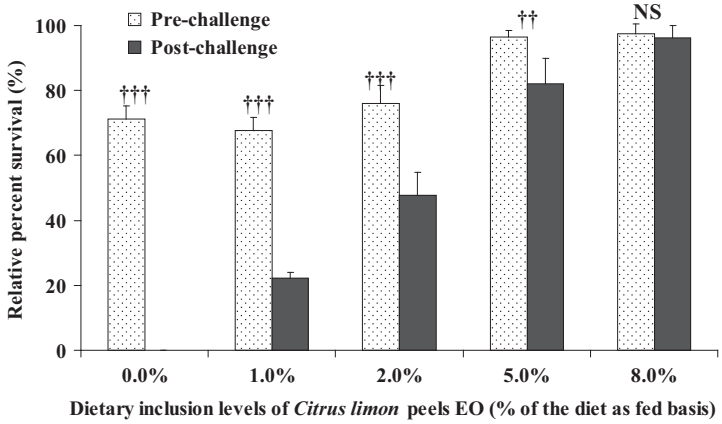

Figure 3 RPS (%) of *Labeo victorianus* under different experimental conditions. $n = 12 \times 3$ replicates; †, †† and ††† denotes that the treatments are significantly different before and after bacteria challenge at $\alpha = 0.05$, $\alpha = 0.01$ and $\alpha = 0.001$ respectively. NS denotes no significant differences in survival before and after bacteria challenge.

are consistent with results obtained for *Oreochromis mossambicus* when the EO extracted from *Citrus sinensis* peels was used in the diet (Acar *et al.* 2015). The improved growth performance and food conversion efficiency of fish at *C. limon* fruit peels EO extract at 5% may be attributed the antimicrobial and antioxidant properties (Milos *et al.*, 2000). The EO improve intestinal microflora (decrease growth of pathogenic bacteria and colonization by beneficial bacteria) (MacLennan, Wilson & Taylor 2002). These pathogenic bacteria increase the rate of passage and thickness of intestinal mucosa which reduces nutrient digestibility and absorption (Xia, Hu & Xu 2005). It has also been shown that EOs increase the digestive enzyme activities of the pancreas (trypsin and α-amylase) and intestines (maltase, alkaline phosphatase and leucine amino peptidase) (Jang, Ko, Kang & Lee 2007), which have the potential to enhance growth performance of the fish. Also a number of minerals such as Na, K, Ca, Cu, Fe, Mg, Zn, P and manganese have been reported in peels of most citrus fruits (Janati *et al.* 2012) and may play an important growth functions.

Dietary administration of the EO of *C. limon* fruit peels extract resulted in significantly reduced plasma glucose and cortisol. The reduction in plasma cortisol and glucose concentrations is recognized as the main hormonal response to stressors and is widely used as a stress response indicator (Barton & Iwama 1991; Morgan & Iwama 1997). Previous studies have established that EO of *Lippia alba*, *Ocimum gratissimum*, *Lippia alba*, *Aloysia triphylla* and *Hesperozygis ringens* have resulted in reduced cortisol and glucose and mediated primary stress in fish (Heldwein, Silva, Reckziegel, Barros, Bürger, Baldisserotto, Malmann, Schmidt, Caron & Heinzmann 2012; Silva, Parodi, Reckziegel, Garcia, Bürger, Baldisserotto, Malmann, Pereira & Heinzmann 2012; Gressler, Riffel, Parodi, Saccol, Koakoski, Costa, Pavanato, Heinzmann, Caron, Schmidt, Llesuy, Barcellos & Baldisserotto 2014; Toni, Martos-SItcha, Baldisserotto, Heinzmann, de Lima, Martínez-Rodríguez & Mancera 2015). Both α- and β-pinene have been reported as sedatives (Mercier *et al.*, 2009) and analgesics (Erazo *et al.*, 2006) and the most likely ingredient that resulted in reduce cortisol and glucose in the current study. The results suggest that the EO of *C. limon* fruit peels contain compounds that reduce primary stress in fish. Increased blood total protein and albumin of healthy and pathogen challenged fish were higher in fish groups fed diets containing *C. limon* fruit peels EO extract and increased in tandem with increasing dietary level of dietary citrus up to 5%. This observation is similar to observed increase in these parameters in *O. mykiss* fed with diet containing 1%, 2% and 3% black cumin oil (*Nigella sativa*) (Awad, Austin & Lyndon 2013). Similar results were reported for *O. mossambicus* treated with EO extracted from *Citrus sinensis* (Acar *et al.* 2015), in *O. mossambicus* fed EO extracted from ginger oil (Immanuel, Uma, Iyapparaj, Citarasu, Punitra Peter, Babu & Palavesam 2009) and in *O. niloticus* when garlic oil was used (Metwally 2009). Citrus fruit peels may be more effective at lowering cholesterol than other citrus fruits because they contain, lemonin polymethoxylated flavones and a flavonoid hesperitin, which has been established to lower cholesterol and triglycerides in other animals (Kurowska & Manthey 2004; Youssef, Youssef & Mousa 2014) and currently seem to have the same effects in fish. The reduced level of cholesterol supports

the possibilities of the inhibition of *de novo* cholesterol biosynthesis by the aqueous EO extract of *C. limon*.

In the current study, addition of *C. limon* fruit peels EO extract to the diets increased RBC count, WBC, Hb, Htc, MCV, MCH, and MCHC. It has been established that the EO of *Eucalyptus globolus* and *Zataria multiflora* increased WBC in *O. mykiss* when supplied in the diet (Sheikhzadeh, Soltani, Ebrahimzadeh-Mousavi, Khosravi, Bagheri, Zargar & Fathi 2008; Soltani, Sheikhzadeh, Ebrahimzadeh-Mousavi & Zargar 2010). Also supplementation of EO of *Zataria multiflora* in *C. carpio*, increased WBC at concentration of 30 ppm and 60 ppm fed groups after 15 days (Sheikhzadeh *et al.* 2008). The increased values of these parameters point towards improved haematological status of fish as was previously determined in with other herbal plant extracts (Harikrishnan, Balasundaram & Heo 2010). Serum immunoglobulin as a component of teleost humoral immune system, has the main function of neutralizing foreign microbial antigens. In the current study, feeding fish with diets containing EO extract of *C. limon* fruit peels resulted in statistically higher levels of Ig in normal and bacteria challenged fish compared with the untreated control. Moreover, higher Ig occurred in fish fed increasing levels of EOs. The significant increase in serum Ig levels may be due to the fact that EO of *C. limon* can actively stimulate the secretion of Ig. Lysozyme is an important defense molecule of the innate immune system. Its role in leucocyte respiratory burst activity as an indicator of innate immunity was observed first in mammals as directly linked with elevated oxygen consumption (Biller-Takahashi, Takahashi, Saita, Gimbo & Urbinati 2013). Respiratory burst activity was previously associated with cytokines release and inflammatory response in fish. Presently the EO of *C. limon* fruit peels increased the reparatory burst activity.

After challenge with *A. hydrophila*, all treated groups showed increased survival as compared with the infected untreated groups suggesting that compounds from the EO extract of *C. limon* fruit peels had antibacterial efficacy against *A. hydrophila*, in agreement with (Sutili, de Lima Silva, Gressler, Gressler, Battisti, Heinzmann, de Vargas & Baldisserotto 2015). The composition of limonene, α-pinene, γ-terpinene, geranyl acetate, neryl acetate, thymol, β-pinene, sabinene, β-myrcene, β-citral (Neral) and α-terpineol of several species of citrus plant species has been demonstrated with different sensitivity against various microbes (Soković, Glamočlija, Marin, Brkić & van Griensven 2010; Espina, Gelaw, de Lamo-Castellví, Pagán & García-Gonzalo 2013; Jing, Lei, Li, Xie, Xi, Guan, Sumner & Zhou 2014). Antimicrobial activity also is dependent on volatility stability and hydrophobicity of compounds. Limonene has high volatility, easy oxidation, and low solubility in water which indicate that it cannot be absorbed by agar. Therefore, high contents of limonene may not result in high antimicrobial activity (Inouye, Takizawa & Yamaguchi 2001; Ou, Liu, Sun & Chan 2015). Previous reports have also demonstrated that the most active antimicrobial ingredients of EOs are aldehyde, phenol and alcohol followed by ketone, ether, and hydrocarbon (Jing *et al.* 2014; Inouye *et al.* 2001). There the presence of potentential antimicrobial composition, α-citral, β-citral, γ-terpinene and α-terpineol exhibited antimicrobial activities. Also the antioxidative activity limonene, α-terpinine, β-caryophyllene, β-pinene,and myrcene and geraniol of citrus oils has been demonstrated (Sarrou, Chatzopoulou, Dimassi-Theriou & Therios 2013; Jing *et al.* 2014), further evidence suggesting that these compounds decrease or inhibit the production of certain virulence factors in the bacteria (Yu, Zhang, Lau, Yao, Vilches, Merino, Tomas & Howard 2005). However, specific single-variable tests would need to be done to identify which of the major components is primarily responsible for the antibacterial activities. Enhancement of the nonspecific immune parameters by the dietary *C. limon* fruit peels extract could have possibly been an influencing factor in increasing survival by protecting the fish against *A. hydrophila* infection. Previous studies in this direction have revealed that dietary supplementation of EO from leaves, flowers and fruits of various plants extracts reduced the mortality and increased the survival against bacteria, virus, fungi and parasites *in vivo* and vitro. The present study suggest that the EO extracted from citrus lemon fruits strengthen immune system of *L. victorianus* when supplemented in the diet at 2% and 5% and can help in management against *A. hydrophila* outbreaks.

Presently, the EO extract from lemon fruit peels is an effective agent to inactivate *A. hydrophila* and are useful not only as remedies but as growth promoters, stress resistance boosters, immune promoters and preventatives of infections in fish. This knowledge will provide many aquaculturists

important information on management of outbreak of bacteria in the fish culture units, which can encourage the mass utilization of the lemon fruit peels that conventionally would have been a waste product from the lemon fruit users.

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