Aquaculture 482 (2018) 197–202

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

A preliminary investigation into the potential effect of *Artemisia afra* on growth and disease resistance in sub-adults of *Oreochromis mossambicus*

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ARTICLE INFO

Keywords:
Fish health
Medicinal plants
Infectious diseases
Survival rate
Aquaculture

ABSTRACT

This study investigated the potential of *Artemisia afra* on growth and disease resistance in *Oreochromis mossambicus*. Phytochemical analysis of *A. afra* was also undertaken. Five *A. afra*-based diets designated as D1, D2, D3, D4 and D5 were formulated and randomly fed to triplicate groups of 45 fish (32.5 ± 1 g). At the end of feeding, Food Conversion Ratio and Specific Growth Rate values were not significantly different ($F_{15,4} = 0.6$, $p > 0.05$; $F_{15,4} = 0.45$, $p > 0.05$, respectively) between D1 and the other diets. Weight gain and feed intake were higher in D1, D2 and D3 than in D4 and D5, but no significant difference (ANOVA, $p > 0.05$) was found between treatments. This suggests that safe dosages were used. Phagocytosis and lysozyme activity were higher in fish fed D4 and D5 than those fed D1 and the other diets. White blood cells were also higher in fish fed the *A. afra*-based diets. To test for disease resistance, fish from each dietary treatment were injected with varying concentrations (0, 1×10^6 cfu, 1.5×10^6 cfu, 3×10^6 cfu, 4×10^6 cfu mL^{-1}) of *Aeromonas hydrophila*. Relative percentage survival and the survival rate of *O. mossambicus* were higher in fish fed D4 and D5, indicating that the use of *A. afra*-based diets enhanced immunity of *O. mossambicus*. This is because *A. afra* contained a number of biologically active compounds that act as immunostimulants. High levels of total polyphenol (70.32 ± 3.5 mg 100 g^{-1}), total phenols (88.65 ± 5.32 mg 100 g^{-1}), total flavonoids (181.5 ± 4.13 mg g^{-1}) and alkaloids (29.63 ± 3.58 g kg^{-1}) were recorded in *A. afra*.

1. Introduction

In South Africa, Mozambique tilapia, *Oreochromis mossambicus* (Peters 1852) is the most widely cultured tilapia species. The successful culture of *O. mossambicus* is attributed to several characteristics such as fast growth rates, tolerance of adverse environmental conditions and its ability to feed on a variety of food items (El-Sayed, 2006; Nguyen et al., 2009). In spite of these qualities, fish mortalities caused by bacterial infections have been reported in tilapia farming, especially in intensive systems (Yılmaz and Ergün, 2014). *Aeromonas hydrophila*, a Gram-negative motile bacterium, is one of the most common causes of mortalities in cultured freshwater fish species throughout the world (Plumb and Hanson, 2011). In freshwater aquaculture, the bacteria is considered an opportunistic pathogen associated with unfavourable culture conditions (Bebak et al., 2015). *Aeromonas hydrophila* has been reported to cause heavy mortalities in *O. niloticus* (Linnaeus, 1758) in Egypt (Noor El Deen et al., 2014). To date, the efficient eradication of infectious diseases in high density production systems remains a challenge. Due to the complex nature of advanced aquaculture production systems used today, most of the recommended management practices that successfully prevented and minimised the propagation of infectious diseases in the past appear to be less effective at combating disease epidemics in aquaculture (Zhang et al., 2016). Poor growth performance and outbreaks of diseases result in the loss of substantial revenue in aquaculture enterprises, thus hindering the growth of the industry (Wu et al., 2013). Therefore, the improvement of growth performance and health has become important in aquaculture.

Broad-spectrum antimicrobials remain the most effective method of controlling fish diseases. However, reports of several bacteria being resistant to treatment have emerged in aquaculture (Verschuere et al., 2000; Batista et al., 2015). The use of antibiotics in aquaculture is also being questioned as several studies have shown that they may negatively affect both fish consumers and the environment (Caruana et al., 2012). Consequently, the European Union has regulated the use of chemical agents to treat or control disease in cultured fish (Srivastava et al., 2004; Stammati et al., 2005; Makkar et al., 2007). However, in South Africa, as in other developing countries, antimicrobials are still widely used in aquaculture (Gram et al., 2001). Thus, there is a need to

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http://dx.doi.org/10.1016/j.aquaculture.2017.09.047
Received 16 May 2017; Received in revised form 18 August 2017; Accepted 30 September 2017
Available online 02 October 2017

look for alternatives in controlling the outbreak of diseases in aquaculture. Several studies have shown that medicinal plants can be used to enhance growth performance and act as prophylactics (e.g. Gopalakannan and Arul, 2006; Sudhakaran et al., 2006; Christybapita et al., 2007; Makkar et al., 2007; Harikrishnan et al., 2011; Park and Choi, 2012; Arulvasu et al., 2013). In addition, medicinal plants have other beneficial characteristics such as anti-microbial activity, promotion and stimulation of appetite (Citarasu, 2010).

Artemisia afra Jacq. ex Willd. is among the many herbal plants that have been successfully used to treat and control several ailments in South Africa and its neighbouring states (Thring and Weitz, 2006). *Artemisia afra* (Wormwood) belongs to the Asteraceae family. Despite its success in treating various diseases (e.g., colds, influenza, asthma, allergies, rheumatic pains) in humans (Watt and Breyer-Brandwijk, 1962; Thring and Weitz, 2006), no investigation has been conducted to determine its potential to enhance growth and health in fish. Attempts to produce *A. afra* extract on an industrial scale are underway in South Africa. Some fish farmers in southern Africa are feeding *A. afra* to *O. mossambicus* in order to boost its growth and health. However, this is done without any information on appropriate dosages. It was thus deemed necessary to investigate the effect of different dosages of *A. afra* on growth and disease resistance in *O. mossambicus*.

2. Materials and methods

2.1. Feed preparation

Pure *Artemisia afra* leaf powder was purchased from Highland Essential Oils in the Free State, South Africa. Ingredients (fish meal, canola meal, sunflower meal, maze gluten, wheat middlings and maize meal) were purchased from a local supplier, situated in the Limpopo Province, South Africa. Five dosage levels were prepared by adding *A. afra* to dry ingredients at concentrations of 0%, 3%, 6%, 9% and 12%, hereafter referred to as D1, D2, D3, D4 and D5, respectively (Table 1). The dry ingredients were mixed by adding water in a mixer (Hobart, Troy, Ohio, USA) until a dough formed. The mixture was then pressed through a meat mincer to produce extruded strings. The strings of each diet was placed on a tray, clearly labelled with the name of the diet, and placed in a windstill area, outdoors in direct sunlight until air-dried. Trays were taken inside just before sunset and taken out again the following morning until dry (3 days). Once dry, the strings were broken into smaller pieces (3-4 mm), placed in labelled sealable containers and stored at −4 °C until needed. The containers were labelled with a corresponding diet name as D1, D2, D3, D4 and D5. The experimental diets were formulated to be nutritionally identical (40% protein and 15.6 MJ kg^{-1}, Table 1). Proximate composition of experimental diets was analysed in triplicate according to AOAC (Association of Official Analytical Chemists) International (2012). There were no significant differences in the moisture, ash, crude protein, or ether extract of the proximate analyses of the experimental diets (Table 1).

Table 1
Feed ingredient (g kg^{-1}) and proximate compositions of the experimental diets.

Ingredients	Diets				
	D1	D2	D3	D4	D5
Fish meal	10	10	10	10	10
Soybean meal	7	7	7	7	7
Canola meal	16	16	16	16	16
Sunflower meal	14	14	14	14	14
Cellulose	12	9	6	3	0
Maize gluten	11	11	11	11	11
Wheat middlings	2	2	2	2	2
Maize meal	24	24	24	24	24
Canola oil	1	1	1	1	1
Vitamin/mineral premix	1	1	1	1	1
Binder	2	2	2	2	2
Artemisia afra (g kg^{-1})	0	3	6	9	12
Moisture	8.05	8.07	8.02	8.05	8.04
Crude protein	40.11	39.10	41.12	40.07	41.10
Crude lipid	8.06	7.90	8.03	7.89	8.07
Ash	6.90	6.40	6.39	6.06	6.42
Gross energy (MJ kg^{-1})	15.8	15.8	15.9	15.1	15.4

Vitamin/mineral premix: Vitamin A, 4500, I. U. Vitamin D, 11,252.U; vitamin E. 71.U; vitamin K3, 2 mg; Vitamin B12 0.015 mg, panthothenic acid; 5 mg, nicotinic acid, 14 mg; folic acid, 0.4 mg; biotin, 0.04 mg; choline, 150 mg, cobalt 0.2 mg; copper 4.5 mg; iron 21 mg; manganese 20 mg; iodine, 0.6 mg; selenium 2.2 mg; zinc 20 mg, antioxidant, 2 mg.

Methanol was used to extract compounds from *A. afra* by adding 5 g plant leaves to 50 mL of methanol in a 250 mL Erlenmeyer flasks which was then mixed by shaking for 5 min. The tubes were left for 48 h at room temperature. The extracts were then filtered using a Pasteur pipettes and whatman No.1 filter paper and evaporated to dryness under a stream of air over a 48 h period. The extracts were subsequently used to screen for the presence of bioactive compounds using various assays. Total soluble phenolic content of the extract was determined with Folin-Ciocalteu reagent using pyrocatechol as standard. Total flavonoid was determined by the aluminium chloride colorimetric method. Alkaloids were determined using the method described by Harborne (1998).

2.2. Experimental design

A completely randomised design was used in this feeding trial. The Mozambique tilapia (*Oreochromis mossambicus*) used in this study were bred and reared at the Aquaculture Research Unit, University of Limpopo. Six hundred and seventy-five (675) healthy sub-adults of *O. mossambicus* were randomly chosen from grow-out ponds and acclimatised to experimental conditions two weeks prior to the commencement of the experiment. During the two-week acclimatisation period, fish were fed a commercial tilapia diet (Aqua-Plus, Avi-Products (Pty) Ltd) at 09:00 h and 15:00 h. The fish were fed to satiation. The experiment was run in a recirculating system equipped with 21 rectangular concrete tanks (1200 L) and thermo-regulated. The tanks were filled to a 900 L mark. The recirculating system is one of four independent systems housed in a solar heated tunnel. The systems are supplied with aged municipal water that is matured for at least two weeks prior to being released into the systems. Each system is independently controlled and backwashed once a week. After the two-week acclimatisation period, fish were anaesthetised with 2-phenoxyethanol (1 mL L^{-1}), weighed (32.5 ± 1.0 g) and stocked in 15 tanks in groups of 45 per tank. Each diet was randomly assigned to the triplicate groups of 45 fish. The fish were fed the experimental diets to satiation (one pellet remain after one-two mins), at 09:00 h and 15:00 h for 45 days. The following water quality parameters were monitored: water temperature (27 ± 0.1 °C), pH 7.6 ± 0.2, dissolved oxygen 7.14 ± 0.4 mg L^{-1}, and conductivity 610 ± 10 µs.

2.3. Growth performance indices

At the end of the feeding trial, growth performance indices for each diet were determined by calculating the following growth indices. Specific Growth Rate was calculated according Winberg (1956) as: $SGR = \frac{\ln W_f - \ln W_0}{t} \times 100$; where: Wf = final body weight (g), W0 = initial body weight (g), ln = natural Logarithm (log) $^{-10}$, t = feeding period (days). Feed utilisation was determined using feed conversion ratio (FCR) = $\frac{\text{food consumed (g)}}{\text{weight gained (g)}}$, Feed Intake (FI, g $fish^{-1}$ 45 $days^{-1}$) = dry feed intake (g)/number fish/days.

2.4. Haematological and biochemical analyses

After 45 days, 5 fish per replicate were randomly selected and anaesthetised with 2-phenoxyethanol (1 mL L^{-1}) in order to draw blood

Table 2
Results of the quantitative phytochemical analysis of *Artemisia afra* (n = 3).

Phytochemical	*Artemisia afra*
Total polyphenols	70.32 ± 3.5 mg/100 g
Total phenols	88.65 ± 5.32 mg/100 g
Total tannins	7.79 ± 0.65 g/kg
Total saponins	14.65 ± 5.32 g/kg
Total flavonoids	181.5 ± 4.13 mg/g
Phytate	–
Total alkaloids	29.63 ± 3.58 g/kg
Carotenoids	18.42 ± 4.96 mg/100 g
Vitamin C	0.0013 ± 0.00014 mg/100 g
Vitamin E	–

Key: - = absent. Values are presented as means ± standard deviation.

for white blood cell count (WBC), nitro-blue tetrazolium (NBT) (which quantifies the activities of neutrophils) and lysozyme assays. Fish that were used to draw blood were not returned to the holding tanks. Approximately, 1 mL of blood was collected from the caudal vein using a 1 mL sterile syringe. Some of the blood was used to determine white blood cell count (WBC × 10^6/L) using a Systemex, XT-1800i blood analyser. The NBT was determined following the protocol outlined by Secombes (1990). Briefly, 0.1 mL of blood was added into wells of a microtitre plate containing 0.1 mL NBT followed by incubation at 37 °C for 60 min. In order to halt the reaction and fix the cells, 100% methanol was added to the mixture. Seventy percent methanol was then used to wash the cells three times and allow them to dry. One hundred microliters of potassium hydroxide (KOH) and 120 μL of dimethyl sulphoxide (DMSO) was added into each well to solubilize the reduced formazan in cells. The resultant mixture was subsequently incubated on a shaker for 30 min. After incubation, the absorbance of the reaction was measured at 655 nm wavelength. Potassium hydroxide and dimethyl sulphoxide served as blanks.

The lysozyme assay was carried out as described by Ellis (1990). Briefly, *Micrococcus lysodeikticus* was grown on a nutrient agar plate. After a 12 h-period, three clearly isolated colonies were dissolved in 100 mL of sodium phosphate buffer (0.06 M, pH 6.3). One hundred microliters (100 μL) of blood was added to 3 mL of the suspension of the culture and the absorbance was read at 550 nm wavelength.

2.5. Challenge trial

The purpose of the challenge trial was to assess whether the inclusion of *A. afra* in the diets of *O. mossambicus* will enhance disease resistance. For that purpose, *Aeromonas hydrophila* was obtained on tryptone soya agar (TSA) from the IDEXX laboratories in Johannesburg, South Africa. The isolates were maintained at 4 °C until used. In preparation for the challenge trial, subcultures were grown on TSA and incubated at 37 °C for 24 h. After 24 h, 5 mL of tryptone soya broth (TSB) was inoculated with *A. hydrophila* and briefly vortexed and then incubated for 24 h at 37 °C. The suspension was then adjusted to five concentrations, namely, 0 (broth without bacteria to serve as control), 1 × 10^6 cfu, 1.5 × 10^6 cfu, 3 × 10^6 cfu, 4 × 10^6 cfu mL^{-1}, hereafter referred to as C1 (Control), C2, C3, C4 and C5, respectively. To determine viability of cells, serial dilutions (1:10) were performed and the spread plate technique was used to plate 0.1 mL of the suspensions on TSA. The plates were then grown at 37 °C for 24 h.

Fish from each dietary treatment (i.e., D1, D2, D3, D4 and D5) were transferred into two replicate tanks in groups of 10 and assigned to each of the five bacterial concentrations. The fish were continuously fed their allocated diets. The tanks (500 L rectangular fiberglass) were filled with 400 L aged municipal water and allowed to stand for two days before fish were transferred. Each tank was supplied with a submersible air stone to diffuse oxygen into the water. In addition, portable submersible heaters were used in each tank to keep the water temperature at 27 °C. The fish were injected with *A. hydrophila* in the third day of being transferred. One hundred microliters of each bacterial concentration (C1, C2, C3, C4 and C5) was thus allocated to fish in two replicate tanks per dietary treatment. The control (C1) only contained an equal volume of TSB without *A. hydrophila*. The tanks were labelled with the name of the dietary treatment and the bacterial concentration (i.e., C1D1, C2D1, C3D1, C4D1 and C5D1; C1D2, C2D2, C3D2, C4D2 and C5D2; C1D3, C2D3, C3D3, C4D3 and C5D3; C1D4, C2D4, C3D4, C4D4 and C5D4; C1D5, C2D5, C3D5, C4D5 and C5D5). Fish were continued on their respective diets after the injection. After injection, fish behaviour was monitored for 10 days. Mortalities were counted daily and dead fish removed from the tanks. A 30% partial water change was done 48 h after injection. There was no replacement of the dead fish. The number of dead fish was used to calculate the Relative percent survival (RPS) according to the method described by Amend (1981).

2.6. Statistics

One-way analysis of variance (ANOVA) was used to test for significant differences between means of growth parameters, NBT and lysozyme activity of fish fed the different dietary treatments. The Tukey test was used to separate means that differed significantly from others. All statistical analyses were performed at the probability level of 0.05 using the SPSS 17.0 software package (SPSS, Chicago, IL, USA).

3. Results

3.1. Phytochemical analysis

Artemisia afra contained several biologically active compounds. The dominant biologically active compounds were flavonoids, total polyphenols, total phenols and total alkaloids (Table 2).

3.2. Growth parameters

No significant changes were observed on growth parameters

Table 3
Growth performance indices calculated for *Oreochromis mossambicus* fed on *Artemisia afra*-based diets.

Growth parameters	Diets				
	D1	D2	D3	D4	D5
IBW (g)	32.34 ± 0.36	32.52 ± 0.17	32.62 ± 0.14	32.56 ± 0.11	32.65 ± 0.25
FBW (g)	67.05 ± 3.08[a]	66.02 ± 3.67[a]	67.12 ± 2.64[a]	65.56 ± 2.89[a]	65.39 ± 3.49[a]
WG (g)	35.02 ± 3.53[a]	33.67 ± 2.48[a]	34.63 ± 3.32[a]	32.89 ± 2.77[a]	32.51 ± 3.91[a]
FCR	2.56 ± 0.13[a]	2.67 ± 0.21[a]	2.59 ± 0.18[a]	2.74 ± 0.31[a]	2.77 ± 0.25[a]
SGR (%)	1.64 ± 0.31[a]	1.59 ± 0.23[a]	1.61 ± 0.26[a]	1.54 ± 0.29[a]	1.52 ± 0.19[a]
FI (g/fish/day)	1.91 ± 0.25[a]	1.86 ± 0.32[a]	1.84 ± 0.21[a]	1.77 ± 0.34[a]	1.75 ± 0.36[a]

IBW = Initial body weight, FBW = Final body weight, WG = Weight gain, FCR = Food conversion ratio, SGR = Specific growth rate, FI = Feed Intake. In each row, different letters indicate significant differences between diets ($p < 0.05$). Values are presented as means ± standard deviation.

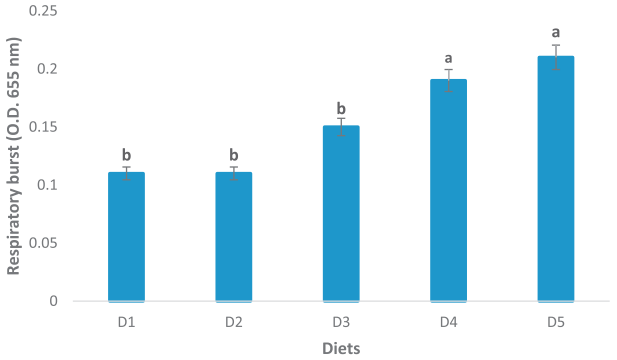

Fig. 1. NBT activity of *Oreochromis mossambicus* fed diets containing different levels of *Artemisia afra* for 45 days. Bars represent standard error. Different letters mean significant differences between diet treatments ($p < 0.05$).

between diets (Table 3). FCR and SGR were not significantly affected by the dietary treatments ($F_{15,4} = 0.6, p > 0.05$; $F_{15,4} = 0.45, p > 0.05$, respectively). Weight gain and feed intake was higher in fish fed with D1, D2 and D3 inclusion levels than in fish fed with D4 and D5, but the differences were not statistically significant (ANOVA, $p > 0.05$) (Table 3).

3.3. Biochemical parameters

There were significant variations in NBT between dietary treatments ($F_{15,4} = 6.6$, $p < 0.05$). A significantly higher NBT activity was recorded in fish fed the highest *A. afra* concentrations (D4 and D5) while D2 and D3 inclusion levels and D1 recorded the lowest NBT (Fig. 1).

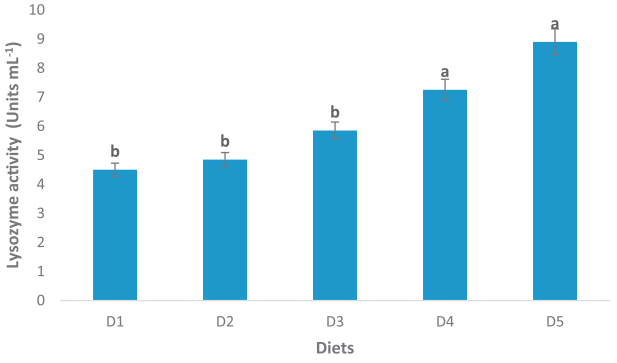

Fig. 2. Lysozyme activity of *Oreochromis mossambicus* fed diets containing different levels of *Artemisia afra* for 45 days. Bars represent standard error. Different letters mean significant differences between diet treatments (p < 0.05).

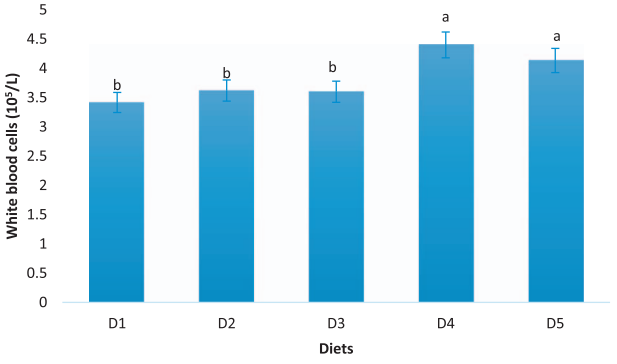

Fig. 3. White blood cell count of *Oreochromis mossambicus* fed diets containing different levels of *Artemisia afra* for 45 days. Bars represent standard error. Different letters mean significant differences between diet treatments (p < 0.05).

However, no significant difference (ANOVA, $p > 0.05$) was found between the D2-D3 and D1. The highest NBT activity was recorded in D4 and D5 (Fig. 1). There were significant variations in lysozyme activity between dietary treatments ($F_{15,4} = 7.2$, $p < 0.05$). Lysozyme activity followed a similar trend to the NBT. The highest lysozyme activity was detected in fish fed with D4 and D5 inclusion levels (Fig. 2). WBCs were significantly affected ($F_{15,4} = 3.8$, $p < 0.05$) by the addition of *A. afra*. White blood cells were higher in fish fed with D4 and D5 (Fig. 3).

3.4. Challenge trial

Eighty percent (80%) of all mortalities in challenged fish were occurred after 24 h of post-challenge and fish continued to die until day 5. Mortalities were monitored daily and no mortalities were recorded after the fifth day of injection. Survival was 100% in C1D1, C1D2, C1D3, C1D4 and C1D5 (Table 3). Survival was between 70 and 80% in C2D4, C2D5, C3D4, C3D5, C4D4, C4D5, C5D4 and C5D5 (Table 4). Fish fed with D4 and D5 showed low mortality and high RPS regardless of the bacterial concentrations.

4. Discussion

The incorporation of medicinal plants in fish diets is increasingly becoming popular due to their positive effect on growth and the immune system. In the present study, no significant differences on growth parameters were detected between dietary treatments. The lack of a negative effect on feed utilisation was evident in the feed intake which was not significantly affected between the treatments. The addition of several plants to fish diets has been found to have both negative and positive effect on growth. Kareem et al. (2016) reported an improvement in feed conversion ratio by *O. niloticus* when fed on diets containing extracts of *Cinnamomum camphora* (L.), *Euphorbia hirta* L. and *Carica papaya* L. They attributed their observations to better lipid metabolism. However, the mechanism through which this is possible has not been clearly demonstrated yet. Feed enriched with *Azadirachta*

Table 4
Relative Percentage Survival (RPS) (%) of challenged *Oreochromis mossambicus* fed with *Artemisia afra* supplemented diets. Values represent the average of two percentages.

Bacterial concentration	Diets	Survival (%)	RPS (%)
C1 (Control)	D1 (Control)	100	–
	D2	100	–
	D3	100	–
	D4	100	–
	D5	100	–
C2	D1 (Control)	60	–
	D2	70	25
	D3	70	25
	D4	80	50
	D5	80	50
C3	D1 (Control)	50	–
	D2	60	20
	D3	60	20
	D4	80	60
	D5	70	40
C4	D1 (Control)	30	–
	D2	40	14
	D3	70	57
	D4	70	57
	D5	90	85
C5	D1 (Control)	30	–
	D2	40	14
	D3	40	14
	D4	70	57
	D5	80	71

Key: C1 = Broth without bacteria serving as control; C2 = 1×10^6 cfu mL^{-1}; C3 = 1.5×10^6 cfu mL^{-1}; C4 = 3×10^6 cfu mL^{-1}; C5 = 4×10^6 cfu mL^{-1}.

indica A.Juss., 1830 extracts (1 g kg^{-1}) significantly enhanced growth performance in *O. niloticus*, but concentrations of 2–8 g kg^{-1} led to significant reductions (Obaroh and Achionye-Nzeh, 2011). Kareem et al. (2016) showed no changes in the growth of *O. niloticus* fed on diets supplemented with *A. indica* diets (2 g kg^{-1}).

The results from previous studies and the current findings seem to suggest that dosage plays a crucial role in growth performance. It appears that safe dosages were used in the present study and this probably explains why there were no negative effects on the growth of *O. mossambicus*. Many medicinal plants contain saponins and tannins and higher concentrations of these compounds could be harmful to fish and have been associated with poor growth performance and feed utilisation (Sakai, 1999). Higher levels of saponins and tannins have a bitter taste which reduces palatability (Sakai, 1999; Dongmeza et al., 2006), thus leading to poor feed utilisation. Tannins are reported to affect fish growth by reacting with digestive enzymes, thus minimising the availability of nutrients (Muhammad et al., 2011; Hlophe and Moyo, 2014). On the other hand, saponins are said to damage cell membranes (Bureau et al., 1998; Hlophe and Moyo, 2014). Furthermore, the literature suggests that the effect of plants on growth and health may be species-specific. For instance, Talpur and Ikhwanuddin (2013) recorded better growth performance in Asian seabass (*Lates calcarifer*) fed with 1.5 g kg^{-1} of *A. indica* whereas approximately similar concentrations resulted in the decline in the growth of *O. niloticus* (Obaroh and Achionye-Nzeh, 2011). This points to the need to always test the same plant and dosages on different fish species and compare responses in order to ascertain the most effective inclusion levels before large scale application of a particular plant can be considered.

The current study showed an increase in WBCs in fish fed with the highest extracts of *A. afra* than those offered low *A. afra* inclusion levels. An increase in WBCs is important because these cells are key components of the first line of defence against pathogens in fish (Reda et al., 2016). The current results are similar to the results of Gültepe et al. (2014) who recorded higher amounts of WBCs in *O. mossambicus* after feeding with diets containing extracts of thyme, rosemary and fenugreek. The increase in WBCs recorded in this study was confirmed by the activities of phagocytes (quantified using the NBT technique), which were higher in fish fed with the highest concentrations (D4 and D5) of *A. afra* than those fed with lower (D1-D3) concentrations of *A. afra*. This may suggest that *A. afra* can improve the defence system in *O. mossambicus* to resist bacterial infections. The mechanism involved in the enhancement of the innate immune response by medicinal plants is not fully understood and needs further experimentation. However, it has been suggested that medicinal plants play a protective role against cellular oxidative stress in fish. It is known that a variety of medicinal plants possess anti-oxidant properties which are important in the normal functioning of cells in living organisms (Kumar et al., 2014; Elmada et al., 2016). The plant used in this study, *A. afra*, is high in secondary metabolites such as phenolics and flavonoids, which have antioxidant activity. The high phenolic and flavonoid content in *A. afra* is responsible for the bioactivity of the *A. afra* leaf extract. Both phenolics and flavonoids are effective scavengers of most oxidising molecules and other free radicals implicated in several diseases.

Increased non-specific immune response was also evident in the increased lysozyme activities in fish fed higher *A. afra* concentrations in their diets. Fish fed with D4 and with D5 had significantly higher lysozyme activity than those fed with D1, D2 and D3. This is an indication of the ability of *A. afra* to increase the activities of lysozyme in *O. mossambicus*. Lysozyme activity is also considered an essential component of innate immune response in fish (Park and Choi, 2012). Yin et al. (2009) stated that an increase in lysozyme levels indicates enhanced phagocytic activity. This is confirmed by the results from this study as both phagocytic activity and lysozyme activity were enhanced with *A. afra* inclusion.

The enhancement of the defence system was also shown in the challenge trial with *A. hydrophila* which showed an improvement in disease resistance in those fish fed on diets with high *A. afra* inclusion levels. The findings indicated a reduction in disease resistance (lower survival rate) in fish fed with D1, D2 and D3 than those fed with D4 and D5.

The present study showed that the inclusion of up to 12% *A. afra* in the diets of *O. mossambicus* did not affect growth performance, but had a positive influence on disease resistance. This suggests that it can be used as a dietary additive to improve disease resistance. It is therefore recommended that the 9 and 12% *A. afra* concentrations are safe to use in enhancing disease resistance without compromising the growth of fish.

Acknowledgements

The authors extend gratitude to the IDEXX laboratories in Johannesburg, South Africa, for providing the *A. hydrophila* isolates. We acknowledge Mrs. Eva Nephale and Mr. Gavin Geldenhuys for their technical help. Mr. DBM Moganedi is thanked for providing space for microbial work.

Compliance with ethical standards

A humane protocol was followed when handling the fish. This was done in accordance with the University of Limpopo's ethical committee regulations. An appropriate amount of 2-phenoxyethanol (1 mL L^{-1}) was used as an anaesthetic to minimise stress and harm during handling and transportation of fish.

Funding

This study was funded by the National Department of Agriculture, Forestry and Fisheries (DAFF) and the Aquaculture Research Unit (ARU), University of Limpopo.

Conflict of interest

The authors declare that they have no conflict of interest.

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