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# The Role of the Dietary Supplementation of Fenugreek Seeds in Growth and Immunity in Nile Tilapia with or without Cadmium Contamination

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**Abstract**

This study is aimed at evaluating fenugreek seeds (*Trigonella foenum-graecum*) as a feed additive for the enhancement of growth and immunity in Nile tilapia (*Oreochromis niloticus*). It is also aimed at assessing their role in fish experimentally exposed to cadmium toxicity. Fish was distributed into five groups as follows: two groups were given crude fenugreek seeds at the concentrations of 5 % and 2.5 %, two groups received 3 % and 1 % concentrations of an alcoholic fenugreek seed extract, in addition to the control group. Each group contained two subgroups; one subjected to cadmium and the other without cadmium exposure. After thirty days of feeding, the fish were bacterially challenged with *Aeromonas hydrophila*. Then the growth and hematological parameters were assessed. The interleukins IL6 & IL8 gene expressions were also estimated. The results indicate a decrease in growth parameters in the cadmium-exposed groups, whereas the growth parameters improved in the fenugreek-fed fish, especially those that received the crude fenugreek seeds at 5 % and the seed extract at 3 % concentrations. Hemoglobin, lymphocyte percentages and the total leukocyte count increased in the group treated with a 2.5 % concentration of crude fenugreek seeds. Also the crude fenugreek seeds at a 5 % concentration induced the lowest mortality (70 %) following the bacterial challenge test. Moreover, the IL6 and IL8 genes expressions were up-regulated in the groups treated with crude fenugreek seeds at 5 % and the seed extract at 3 % concentrations. It can be concluded that fenugreek seeds can be used as feed additives to improve fish growth and immunity, and to help reduce the hazardous effects of cadmium pollution.

**Keywords:** *Oreochromis niloticus*, Fenugreek, Growth performance, Cadmium, *Aeromonas hydrophila*, Interleukins.

## 1. Introduction

Aquaculture is the fastest growing sector in the world of food production accounting for almost 50 % of the world's food fish (Martins *et al.*, 2011; FAO, 2018). Aquaculture production should be increased to cover the continuous outpaced population growth (Aly, 2009). Such high rates of aquaculture production depend mainly on raising the growth rates and immunity of fish to afford the disease outbreaks which constitute the main impediment to aquaculture development. The strength of aquaculture lies in growing fish that are resistant to diseases and contamination with heavy metals, pesticides, and other stressful contaminants. Unfortunately the agricultural drainage and irrigation waters contain high concentrations of different pesticides, fertilizer runoff and heavy metals (Abumourad *et al.*, 2013).

Heavy-metal pollution is one of the greatest problems for fish consumers. Heavy metals are inorganic non-biodegradable chemicals that cannot be metabolized and broken down into harmless forms since they leave the biological cycles very slowly (Abumourad *et al*., 2013 and Wani *et al*., 2017). Elements such as cadmium, copper, lead, and zinc are considered the most dangerous in the ecotoxicological effects (Golovanova, 2008). Cadmium (Cd) is a xenobiotic which is widely used in electronics, metal plating, batteries, dye, and plastic industries. It has toxic effects on animal health even at very low concentrations (Sorensen, 1991). Filipoviæ and Raspor (2003) reported that Cd enters the aquatic ecosystems from different resources and causes many physiological alterations and DNA disorders.

Although chemical drugs are traditionally used for fish-disease treatment (Herman, 1970 and Chowdhury *et al*., 2015), they can give rise to the emergence of drug-resistant bacteria, environmental pollution and residues (Lee and Gao, 2012). Recently, more interest has been directed at the development of immunostimulants especially those of natural origins that operate on the principle of stimulating innate immunity which is the first line of defense against pathogenic invaders (Abbas and Awad, 2016; Awad and Awaad, 2017). Medicinal plants are biodegradable, cheap, easy to prepare, and effective, with fewer side effects during the treatment. They also act as immunostimulants and have anti-bacterial activities in

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fish and shellfish without causing any environmental problems (Harikrishnan *et al.*, 2011). Accordingly, they can be used in aquaculture as a natural source of food and immunostimulant in exchange for many chemical additives.

The effect of some plant extracts on several physiological functions in different fish species have been investigated by many studies; for example, Awad *et al.* (2013) studied the effect of black cumin seed oil (*Nigella sativa*) and nettle extract (Quercetin) on the enhancement of immunity in rainbow trout (*Oncorhynchus mykiss*). The effects of fenugreek (*Trigonella foenum graecum*) on gilthead seabream's (*Sparus aurata L.*) immune status and growth performance were also studied by Awad *et al.* (2015).

Studies of different growth parameters, hematological changes, and immune gene expressions create good biomarkers for aquaculture production and fish health. In this context, this work has been carried out for studying the effect of fenugreek seeds (*Trigonella foenum-graecum*) on different growth, hematological, and immune parameters of Tilapia being the most common freshwater-cultured fish all over the world. Also the study aims to evaluate the role of fenugreek seeds in reducing the toxic effects of cadmium on fish.

## 2. Materials and Methods

### *2.1. Medicinal Plant*

Crude crushed fenugreek (*Trigonella foenum-graecum*) seeds and an alcoholic extract of seeds were prepared to be used as fish feed additives. The alcoholic extract was obtained by washing, drying, and crushing 1 Kg of fenugreek seeds to be soaked in a double volume of absolute ethyl alcohol in a stopper container for five days. The mixture was shaken more than once each day. It was filtered, evaporated by a rotary evaporator. Finally it was left for complete dryness and was weighed (Azwanida, 2015).

### *2.2. Fish Diets*

A commercial pellet diet (35 % protein) was firstly crushed, mixed with the appropriate ratios of crude crushed fenugreek seeds or their alcoholic extract, and wet with water, then turned again into pellets. The diet was allowed to dry in the open air, and was then stored at 4$^{o}$C until use.

### *2.3. Fish and Experimental Design*

Around 450 Nile Tilapia fish (*Oreochromis niloticus*), of about 30-40 g were obtained from the private fish farm at Kafr El-Shaik, Egypt. The fish were allowed to acclimatize in an aerated free-flowing and de-chlorinated tap water for two weeks. Water quality parameters during the study period were as follows: temperature (23.3$^{o}$C); pH (7.6); dissolved oxygen (6.4 ppm); alkalinity (115 ppm $CaCO_3$) and hardness (130 ppm $CaCO_3$). After acclimatization, the fish were distributed into five groups as follows: two groups received crude fenugreek seeds of 5 % and 2.5 % concentrations, while the other two groups which fed on the alcoholic seed extract of 3 % and 1 % concentrations. The last group was the control group that fed on the basal diet. Each group was subdivided into two subgroups; a group subjected to 1/10 $LC_{50}$ of cadmium toxicity [1.5 mg of $CdCl_2$, according to Garcia-Santos *et al.*, 2006], and another group non-treated with Cd. The applied ratios of the crude and extract fenugreek seeds were chosen based on some previous references (Zaki *et al.*, 2012; Awad *et al.*, 2015). Fish were fed twice daily at 3 % of fish body weight. After thirty days of feeding, the growth performance and hematological parameters were investigated. The other subgroups of fish that were not exposed to Cd were bacterially challenged, and noticed for mortality rates for ten days, and tissue samples were collected for interleukins (6 & 8) gene expressions to assess the immune function induction by the fenugreek plant.

### *2.4. Growth Performance*

Fish sampling was done twice; at the beginning of the experiment and after thirty days of being fed on the fenugreek. Fish were firstly anaesthetized by keeping them in aquaria containing clove oil (Merck, Germany) at a concentration of 50µl $L^{-1}$ for five minutes. (Hamackova *et al.*, 2006). Total length and total weight were recorded in cm and gram respectively. After the dissection of each fish from each treated group, the liver and gonads weights were recorded to calculate growth indices. Weight gain (WG), specific growth rate (SGR), and some growth indices [hepato-somatic index (HSI), gonado-somatic index (GSI) and the condition factor (CF)] were calculated according to the following formulas (Tukmechi *et al.*, 2011).

WG = final weight – initial weight

SGR = 100* (ln final weight – ln initial weight)/days of feeding

HSI = (liver weight/total weight)*100

GSI = (Gonads weight/total weight)*100

CF = (total weight/total length$^{3}$)*100

### *2.5. Hematological Assays*

Blood samples were collected from the caudal veins in EDTA tubes. Red blood cells (RBCs) and white blood cells (WBCs) were counted using an improved Neubauer hemocytometer (Natt and Herrick, 1952). Leukocytic differential counts were also recorded using Giemsa stain. Hematocrit (Hct %) was determined using heparinized capillary tubes, centrifuged in a microhematocrit centrifuge. Hemoglobin concentration (Hb) was recorded using spectrophotometer according to the cyanmethemoglobin method (Drabkin, 1948). The red blood indices [mean corpuscular volume (MCV), mean corpuscular Hemoglobin (MCH), and mean corpuscular Hemoglobin concentrations (MCHC)] were calculated using Hct, Hb and RBC measurements.

### *2.6. Immunological Assays*

#### *2.6.1. Experimental Challenge with Bacteria*

At the end of the feeding experiment, a Cd non-treated subgroup was injected intra-peritoneally with 0.2 ml of a suspension containing $4\times10^{7}$ CFU/ml live *Aeromonas hydrohila* that were prepared according to Zhang *et al.* (2016). The challenged fish were observed for ten days for the recording of the mortality rates, clinical signs, and post mortem lesions. Re-isolation of *A. hydrophila* from dead fish was processed to confirm the specificity of pathogenicity. Tissue samples were collected and maintained in -80$^{o}$C for interleukins (6&8) gene expression determination.

*2.6.2. Evaluation of IL6 & IL8 Gene Expressions Using Real-time PCR*

RNA extraction: After two days of the bacterial challenge test, total RNA was extracted from the liver using a Gene JET RNA Purification Kit (Fermantus, UK) according to the manufacturer's protocol.

cDNA synthesis: 2 µg RNA was reverse transcribed with Revert Aid First Strand cDNA Synthesis Kit™ (Fermentus life science) using hexa-nucleotides and was used as templates for Real-time PCR.

Primer designing: Primers for target genes and the internal control (18s ribosomal RNA) (Table 1) were designed through the NCBI web site and were purchased from Invitrogen Corporation (Van Allen Way, Carlsbad, Canada).

**Table 1.** Sequences of the 5' and 3' synthetic primers used in PCR

| Accession number | Primers (sense and antisense 5'→3') | Annealing Temp. |
|---|---|---|
| IL-6 XM_019350387.1 | Sense: 5'-TCCGATTGAAGACGGAAGTGT-3' Antisense: 5'-GGAGCAGTGCACTCGAAGG3' | 58 ℃ |
| IL-8 NM_001279704 | Sense: 5'-AGAGAACAGAGGAGACCGGG A3' Antisense: 5'-CTCCACCTTCTCGATGTGGC 3' | 58℃ |
| 18sRNA JF698683.1 | Sense: 5'-GGACACGGAAAGGATTGACAG3´ Antisense:5´-GTTCGTTATCGGAATTAACCAGAC3´ | 58℃ |

*Quantitative Real-Time PCR*

cDNA was PCR amplified using corresponding primers for IL6, IL8, and 18S rRNA was used as a reference housekeeping gene. PCR-Applied Biosystems was used with the use of SYBR green PCR Master Mix (Applied Biosystems) and gene specific primers where specimens' analysis were done in a final volume of 12 µL in MicroAmp® Optical 96-well reaction plates (Applied Biosystems, Foster City, CA) using ABI PRISM 7500 instrument, Applied Biosystems (The qPCR mixture consisted of 1 µl of cDNA (equivalent to 10 ng of RNA), 1 µl of 0.5 µM gene-specific forward primer, 1 µl of 0.5 µM gene-specific reverse primer, 6 µl of 2× SYBR Green SuperMix and 3 µl of DEPC-treated water. Q PCR was performed in triplicate for each cDNA sample. The qPCR thermal cycling parameters were 50 ºC for two minutes, 95 ºC for two minutes followed by forty cycles of 95 ºC for fifteen seconds and 60 ºC for one minute. The PCR products were also gel-excised, purified, and sequenced to confirm that they match the target genes sequences.

*2.6.3. Molecular Data Analysis*

The relative transcriptional levels of different genes were determined by subtracting the cycle threshold (Ct) of the sample by that of the 18S ribosomal RNA, the calibrator, using the formula: ΔCt =Ct (sample) - Ct (calibrator). The relative expression level of a specific gene in the immunized fish was compared to that of non-immunized fish to obtain 2ΔΔCt, where ΔΔCt = ΔCt (control) - ΔCt (Pfaffl 2001). Statistical analyses for the mRNA transcription levels were performed with the aid of the SPSS.16 statistical package (SPSS Inc., Microsoft Co., Redmond, USA).

*2.7. Statistical analysis*

Results are presented as means ± standard error (SE). Significant differences were determined by one-way ANOVA test (Duncan, 1955). All statistical analyses were performed using a computer program of SPSS Inc. (version 17.0 for Windows) at $P$ <0.05.

*2.8. Ethical Approval*

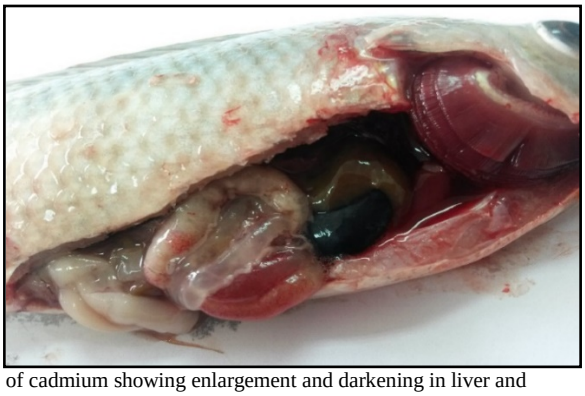
The study was ethically cleared by the ethical review board of the National Research Centre.

## 3. Results

*3.1. Clinical Investigation*

The clinical investigation of *O. niloticus* subjected to 1/10 $LC_{50}$ of Cadmium (1.5 mg/l) for thirty days showed some pictures of illness-like ascites, dark liver, distended gall bladders and enlarged intestines (Figure 1).

**Figure 1.** *Oreochromis niloticus* subjected to 1/10 $LC_{50}$ (1.5 mg/l)



of cadmium showing enlargement and darkening in liver and gallbladder, enlargement of intestines with ascites

*3.2. Weight Gain (WG) and Specific Growth Rate (SGR)*

The results of the current study showed that the highest fenugreek concentrations; of crude fenugreek seeds at a 5 % concentration and the seed extract at a concentration of 3 % showed the highest significant increase ($P$>0.05) of WG (10.14 and 9.88 g, respectively) compared to the control (3.3 g) and SGR (0.76 and 0.81, respectively) compared to control (0.32) (Figures 2 and 3). Regarding the cadmium treated groups, there was a decrease in WG and SGR compared to their corresponding non-cadmium treated groups. Whereas the fish that fed on fenugreek seeds and subjected to Cd revealed insignificant increase in WG and SGR, especially at higher fenugreek concentrations; (crude fenugreek seeds at 5 % and the seed extract at 3 %) (Figures 2 and 3).

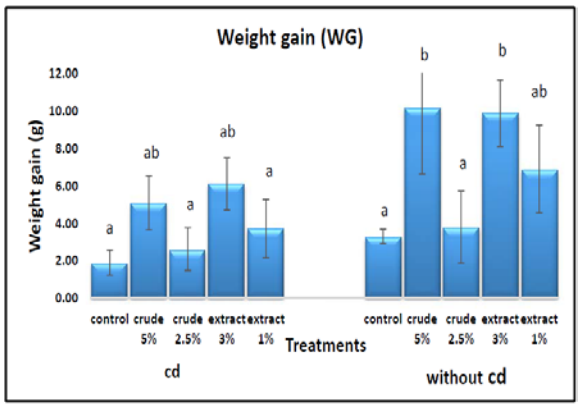



**Figure 2**. Weight gain (WG) of *Oreochromis niloticus* after experimental feeding on fenugreek seeds for 30 days with and without being subjected to 1/10 $LC_{50}$ of cadmium (1.5 mg/l). The same letters at the columns are not significantly different at $P > 0.05$

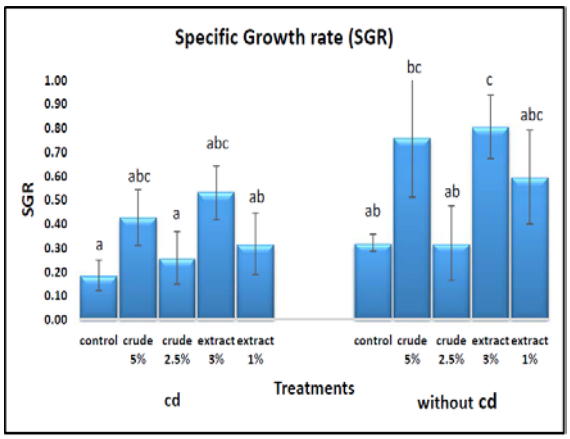



**Figure 3.** Specific growth rate (SGR) of *Oreochromis niloticus* after experimental feeding on fenugreek seeds for 30 days with and without being subjected to 1/10 $LC_{50}$ of cadmium (1.5 mg/l). The same letters at the columns are not significantly different at $P > 0.05$.

### *3.3. Hepatosomatic Index, Gonadosomatic Index and Condition Factors*

Generally, the fish group that was exposed to cadmium toxicity showed a decrease in the hepatosomatic index (HSI) and gonadosomatic index (GSI) (1.35 and 1.39, respectively) compared to the control group (1.46 and 2.60, respectively) (Table 2). There was a significant increase in HSI values ($P$>0.05) in the Cd-exposed groups at lower fenugreek concentrations; that is crude fenugreek seeds at 2.5 % and the seed extract at 1 % (2.03 and 2.2, respectively). Regarding GSI values, the group treated with crude fenugreek seeds exhibited a significant increase in the Cd-exposed groups ($P$>0.05); they were (4.09 and 3.41) in the groups that fed on the crude fenugreek seeds at 5 % and at 2.5 % concentrations, respectively compared to their comparable control groups. CF values in all groups showed insignificant changes (Table 2).

**Table 2.** Hepatosomatic index (HSI), Gonadosomatic index (GSI) and Condition factor (CF) values of *Oreochromis niloticus* that fed on fenugreek seeds for 30 days with and without being subjected to 1/10 $LC_{50}$ of cadmium (1.5 mg/l).

| Treatment | | Hepatosomatic Index (HSI) | Gonadosomatic index (GSI) | Condition factor (CF) |
|---|---|---|---|---|
| | | Mean ± SE | Mean ± SE | Mean ± SE |
| Cd | Control | $1.35\pm0.08^{a}$ | $1.39\pm0.12^{a}$ | $1.70 \pm 0.07^{a}$ |
| | Crude 5% | $1.23\pm0.33^{a}$ | $4.09\pm0.88^{c}$ | $1.54\pm 0.03^{a}$ |
| | Crude 2.5% | $2.03\pm0.40^{bc}$ | $3.41\pm0.90^{bc}$ | $1.47\pm 0.12^{a}$ |
| | Extract 3% | $1.50\pm0.19^{ab}$ | $2.13\pm0.64^{ab}$ | $1.51\pm 0.05^{a}$ |
| | Extract 1% | $2.20\pm0.22^{c}$ | $1.61\pm0.58^{ab}$ | $1.50\pm 0.09^{a}$ |
| Without Cd | Control | $1.46\pm0.08^{ab}$ | $2.60\pm0.76^{abc}$ | $1.63\pm 0.02^{a}$ |
| | Crude 5% | $2.07\pm0.10^{bc}$ | $1.64\pm0.31^{ab}$ | $1.57\pm 0.06^{a}$ |
| | Crude 2.5% | $1.31\pm0.09^{a}$ | $1.19\pm0.20^{a}$ | $1.51\pm 0.08^{a}$ |
| | Extract 3% | $2.02\pm0.17^{bc}$ | $1.86\pm0.31^{ab}$ | $1.60\pm 0.10^{a}$ |
| | Extract 1% | $1.62\pm0.03^{abc}$ | $1.29\pm0.35^{a}$ | $1.79\pm 0.28^{a}$ |
| | *F*-Value | 3.216 | 2.869 | 0.794 |
| | Sig. | 0.004** | 0.008** | 0.623† |

Means with the same letter within the same column are not significantly different ($P$>0.05) SE= standard error $F$-value = ANOVA $F$-test. Sig. = significance level. **ANOVA (Highly significant difference, $P$<0.01). †ANOVA (Insignificant difference, $P$>0.05).

### *3.4. Hematological Results*

While there was a general decrease in the red blood cells count (RBCs), hemoglobin (Hb), packed cell volume (Hct%) values in all cadmium-treated groups compared to the control groups, only the group treated with a 2.5 % crude fenugreek seeds showed a significant increase in RBCs, Hb, Hct % and hematological indices (Table 3). Cadmium toxicity decreased the total WBCs count, while the group treated with 2.5 % crude fenugreek seeds showed a significant increase in WBCs count compared to the control cadmium group (Figure 4). Also, the Cd-exposed group showed a significant decrease in lymphocyte %, and fenugreek treatments revealed an increase in their percentages (Figure 5).

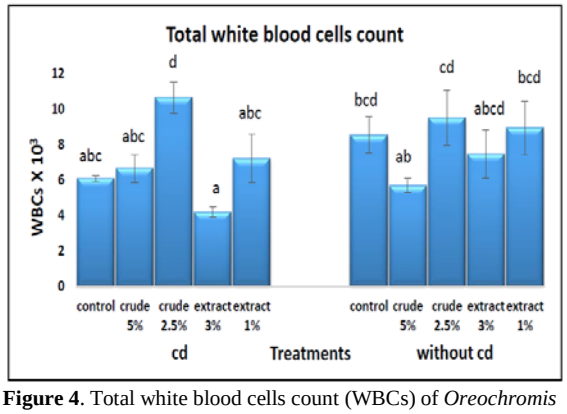



**Figure 4**. Total white blood cells count (WBCs) of *Oreochromis niloticus* after experimental feeding on fenugreek seeds for 30 days with and without subjection to 1/10 $LC_{50}$ of cadmium(1.5 mg/l) (1.5 mg/l). The same letters at the columns are not significantly different at $P > 0.05$.

**Table 3.** $RBC_S$ count, Hb, Hct and hematological indices of *Oreochromis niloticus* fish feeding on fenugreek seeds for 30 days with and without being subjected to 1/10 $LC_{50}$ of cadmium (1.5 mg/l).

| Treatments | | $RBC_S$ X $10^6$/µl | Hb (g/dl) | Hct (%) | MCV | MCH | MHCH |
|---|---|---|---|---|---|---|---|
| | | Mean ± SE | Mean±SE | Mean ± SE | (fl) | (pg) | (g/dl) |
| Cd | Control | 1.41±0.02[a] | 4.60±0.06[a] | 21.50±0.29[a] | 152.48 | 32.62 | 21.40 |
| | Crude 5% | 1.36±0.23[a] | 4.83±0.55[a] | 20.75±3.59[a] | 153.14 | 35.61 | 23.25 |
| | Crude 2.5% | 1.91±0.26[b] | 7.13±0.87[b] | 30.75±4.15[b] | 160.99 | 37.30 | 23.17 |
| | Extract 3% | 1.26±0.10[a] | 4.18±0.29[a] | 19.25±1.44[a] | 152.78 | 33.13 | 21.69 |
| | Extract 1% | 1.45±0.01[a] | 4.60±0.06[a] | 21.50±0.29[a] | 148.28 | 31.72 | 21.40 |
| Without Cd | Control | 1.40±0.03[a] | 4.80±0.12[a] | 21.75±0.48[a] | 155.64 | 34.35 | 22.07 |
| | Crude 5% | 1.58±0.12[ab] | 5.08±0.48[a] | 24.00±1.91[a] | 151.90 | 32.12 | 21.15 |
| | Crude 2.5% | 1.50±0.06[ab] | 5.13±0.22[a] | 23.25±1.03[a] | 154.74 | 34.11 | 22.04 |
| | Extract 3% | 1.52±0.11[ab] | 4.83±0.14[a] | 22.25±0.75[a] | 146.38 | 31.74 | 21.69 |
| | Extract 1% | 1.53±0.12[ab] | 5.05±0.20[a] | 23.50±0.87[a] | 153.59 | 33.01 | 21.49 |
| | *F*-Value | 1.776 | 4.163 | 2.500 | | | |
| | Sig. | 0.115† | 0.001** | 0.029* | | | |

Means with the same letter within the same column are not significantly different *at* $P>0.05$. SE= standard error. *F*-value = ANOVA *F*-test. Sig. = significance level.†ANOVA (Insignificant difference, $P>0.05$). *ANOVA (Significant difference, $P<0.05$). **ANOVA (Highly significant difference, $P<0.01$).

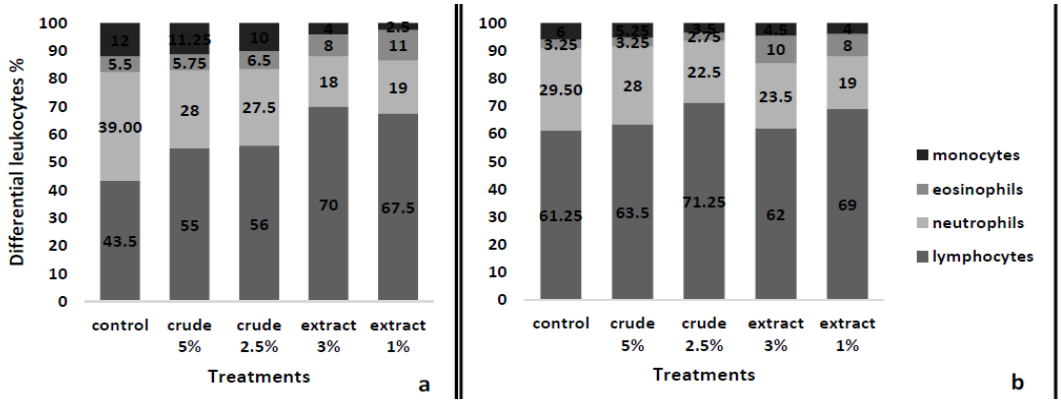



**Figure 5**. : Differential white blood cells of *Oreochromis niloticus* after experimental feeding on fenugreek seeds for 30 days with subjection to 1/10 $LC_{50}$ of cadmium (**a**) and without cadmium subjection (**b**).

### *3.5. Immunological Results*

#### *3.5.1. Bacterial Challenge*

After thirty days of fenugreek feeding, the *A. hydrophila* injection revealed the lowest mortality rate in the group that fed on the 5 % crude fenugreek seed concentration followed by the groups that fed on the seed extract at 3 % and 1 % concentrations, and finally followed by the group that fed on the crude fenugreek seeds at a 2.5 % concentration compared to the control group (Figure 6). Some clinical observations were noticed in the injected fish such as the external hemorrhage in the abdominal region (Figure 7).

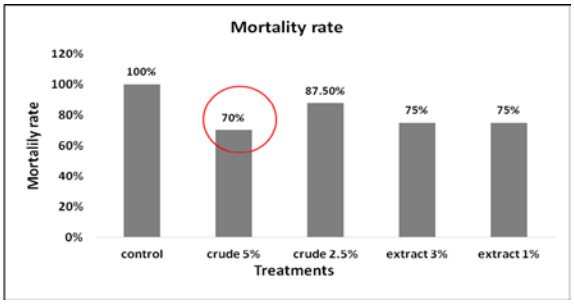



**Figure 6**. Mortality rate of *Oreochromis niloticus* injected with *Aeromonas hydrophila* after 30 days feeding on fenugreek seeds.

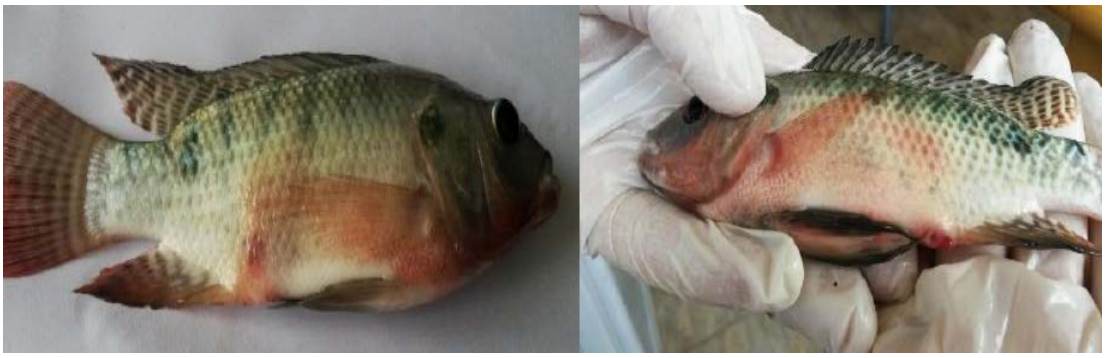


**Figure 7.** *Oreochromis niloticus* injected with *Aeromonas hydrophila* showed external hemorrhage in the abdomen and anal prolapse.

*3.5.2. Molecular Studies (Interleukins 6 & 8 (IL 6 &IL 8) Immune Genes Expression)*

The changes in the IL6 & IL8 gene expression fold with the different concentrations of the fenugreek crude seeds and seed extract show that the expressions of these genes were increased significantly with high fenugreek doses (P>0.05). They were up-regulated better in the groups treated with crude fenugreek seeds at 5 %, or with the seed extract at 3 % following the bacterial challenge. However, it was clear that the fold change of IL6 expression (8.24-8.46) was higher than the fold change of IL 8 expression (1.34-3.16) (Figure 8).

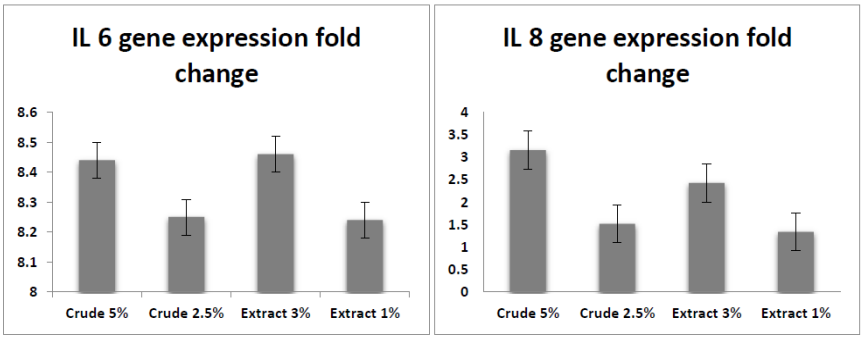



**Figure 8**. IL6 and IL8 gene expression fold changes after *Aeromonas hydrophila* bacterial challenge in fish that fed on Fenugreek crude and extracts with different concentrations.

## 4. Discussion

Aquaculture is an important industrial sector which needs combined efforts to boost its production as it constitutes the last frontier to solve the world's protein-deficiency problem. To grow fish that are bigger and healthy, alternatives from natural products must been found as feed additives and immunostimulants instead of drugs and chemicals. Many plants that possess several biological activities are currently used in traditional medicine, including fenugreek seeds. This study reveals that *O. niloticus* that were given crude fenugreek seeds at 5 % and fenugreek seeds' extract at 3 % concentrations showed a significant increase in growth performance. Also it has been found that fenugreek can modulate the growth retardation in the cadmium-exposed groups, especially at higher fenugreek concentrations; crude fenugreek seeds at 5 % and fenugreek seed extract at 3 %. The growth-promotion effect of fenugreek may be attributed to its high nutritive value, as it is rich in protein, carbohydrates and many minerals. Fenugreek also improves the digestion of protein and absorption of fats (Mansour and El-Adawy, 1994). Some previous studies reported the effect of fenugreek as food additive in rabbits (Zeweil *et al.*, 2015) and ensured its growth-promotion effect in fish (Zaki *et al.* 2012; Awad *et al.*, 2015). Similarly, the obtained results suggest that using a concentration of 5 % of crude fenugreek seeds and 3 % of an alcoholic fenugreek seed extract as a feed additive enhances fish growth and equilibrates the cadmium inhibitory effect on the growth of the Nile tilapia fish.

Environmental pollution causes a lot of physiological and metabolic changes to fish that can have bad effects on growth and reproduction (Heath, 1995). Hepatosomatic index (HSI) represents one of the main indicators of metabolic activity and status of energy reserve in animals. Thus, HSI value provides information about the health status of fish and the quality of water surrounding it. In this study, the exposure of *O. niloticus* to 1/10 $LC_{50}$ of Cd for thirty days decreased the HSI value. This may be attributed to the excessive usage of energy reserve in the liver of the cadmium-exposed group (Çiftçi *et al.*, 2015). Such result was corroborated by previous studies which reported that HSI values decreased in catfish and tilapia fish when exposed to water pollution, especially heavy-metal pollution (Bekmezci, 2010; Çiftçi *et al.*, 2015).

Also, gonadosomatic index (GSI) is another parameter that reflects the health status of fish. It was decreased in this study in the cadmium-exposed group; such result was similar to that of Çiftçi *et al.* (2015) who reported a decrease in GSI in tilapia fish after exposure to cadmium and zinc heavy metals. They attributed such decrease in the GSI value to the inhibition of some enzymes and reproductive hormones due to chemical pollution. Fortunately, feeding on fenugreek for thirty days can neutralize the cadmium toxic effects on liver and gonads. This was obvious in some fenugreek groups that showed a significant increase in HSI and GSI values in the

cadmium-exposed groups, which may be explained by the catalyst-promoting effects of fenugreek and its usage as a growth stimulus which can also increase reproductive hormones.

Moreover, condition factor (CF) indicates the state of general health of fish that is affected by many environmental factors. It has been used to compare growth conditions of fish, since high CF values can indicate good environmental quality and vice versa. In the current study, the insignificant changes in CF in mostly all of the the treated groups may be attributed to the short duration of feeding (thirty days). Similarly, the CF values were not affected in *Clarias gariepinus* under the effect of lead acetate toxicity (Balawi *et al*., 2011).

Regarding the hematological changes, only the group that fed on a concentration of 2.5 % of crude fenugreek seeds showed a significant increase in RBCs, Hb and Hct % in cadmium- treated groups, while other groups were insignificantly affected. This may be attributed to the inability of the short duration of time to affect the hematological parameters. Also cadmium decreased the fish immunity which is represented in the total leukocyte count and lymphocyte %; however, feeding on fenugreek modulated such toxic effect of cadmium and increased the WBCs count in the group which fed on the crude fenugreek seeds at 2.5 % and the lymphocyte percentage in all of the cadmium-exposed groups, which explains the increase in the immunity response of fish, especially after the bacterial challenge (Awad and Awaad, 2017).

The immune system plays an essential role in protecting fish against stressful environmental conditions. Immunological effects of natural plants depend on the dose and time of administration as well as on the fish species (Chakrabarti, 2005; Awad and Awaad, 2017). An important and a large category of proteins that are crucial to many immunological processes are the pro-inflammatory cytokines. Cytokine genes are glycoprotein mediators produced by immune cells and contribute to cell growth and differentiation and defense mechanisms of the host against bacterial invasions (Biswas *et al*., 2012). Liver is the central organ regulating the acute-phase response by releasing specific cytokines including interleukin 1 beta (IL-1beta), interleukin 6 (IL-6) and interleukin 8 (IL-8) (Panigrahi *et al*., 2007). Some investigations have identified cytokines and immune genes in the salmonid immune systems (Secombes *et al*., 2001) and their response to invading bacteria in rainbow trout (Komatsu *et al*., 2009). The present study evaluated IL6 & IL8 cytokines gene expressions after a bacterial challenge with *A. hydrophila*, the causative agent for hemorrhagic septicemia and some frequent diseases among fish and other aquatic animals (Longyant *et al*., 2010). IL-8 is an important cytokine, which can attract neutrophils, T-lymphocytes, and basophils to inflammatory sites (Mukaida *et al*., 1998). This protein was detected in the head kidney, and spleen of catfish, and its expression was up-regulated three to five fold in channel catfish and blue catfish after infection with the pathogenic bacterium *Edwardsiella ictaluri* (Chen *et al*., 2005). IL-6 is another cytokine that plays important roles in regulating immune gene response, hematopoiesis, and inflammation in many cell types such as lymphocytes and macrophages (Naka *et al*., 2002; Zante *et al*., 2015). The present results show that fenugreek up-regulated the expression of IL6 and IL8 after the *A. hydrophila* infection more than the control group. Also it can be seen that there is a dose- immune genes expression positive relationship, which means that a high dose of fenugreek can enhance interleukins better. This may be attributed to the fact that fenugreek seeds are rich in active principles of flavonoid and alkaloids, and their high quantity led to improve the immune response. Similarly, the feeding of rainbow trout on lupin and stinging nettle stimulated the expression of IL-1b and IL-8 after being challenged with *A. hydrophila* (Awad *et al*., 2013). Also, the up-regulation of IL6 and IL8 expressions confirmed the inflammatory response in zebra fish after being challenged (Pressley & Gaskins, 2006). PCR analysis of Japanese puffer fish showed a constitutive expression of IL-6 in kidney following a bacterial infection (Bird *et al*., 2005).

## 5. Conclusion

In conclusion, this study highlights the promising role of fenugreek seeds in the *O. niloticus* production, since it can improve the growth and immunity parameters in tilapia fish. Also, fenugreek was shown to have an antibacterial effect and to decrease the mortality rate in fish challenged with *A. hydrophila*. Moreover, fenugreek plays a significant role in reducing the toxic effects of cadmium. However, further studies are still needed to assure its efficiency.

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