

## CHAPTER IV

### RESULTS

#### 4.1 Chemical composition in experimental diets

Chemical composition (dry matter basis) in five experimental diets showed that slight differences in dietary constituents (components) occurred; however, for each, differences were not significant among diets ( $P>0.05$ ).

#### 4.2 Mycotoxin analysis of five experimental diets using HPLC and LC-MS/MS

The determination of mycotoxins in five experimental diets using LC-MS/MS is presented in Table 5. As a result of the replacement of clean wheat with contaminated wheat in five experimental diets, graded concentrations of DON/ZON in each diet were 0.07/0.01, 0.31/0.09, 0.50/0.21, 0.92/0.37 and 1.15/0.98 ppm, respectively.

##### 4.2.1 Determination of mycotoxins in five experimental diets using LC-MS/MS

A total of 50 secondary fungal metabolites namely regulated and non-regulated *Fusarium* metabolites, ergot alkaloids, plant metabolites and other non-regulated metabolites, which are produced by *Penicillium*, *Aspergillus* and *Alternaria*, were detected at trace concentrations in most of diets. However, *Fusarium* mycotoxins were detected in all diets that were accounted for 22 *Fusarium* metabolites of all detectable fungus metabolites. DON and ZON as ones of the regulated mycotoxins were found in all diets, for DON, ranged from 0.07 to 1.15 ppm and, for ZON, from 0.01 to 0.98 ppm. Concentrations of DON and ZON occurred were higher than those of other regulated *Fusarium* mycotoxins, such as OTA, DON-3-Glucoside, NIV, Zearalenon-4-Sulfat, alpha zearalenol and beta zearalenol. Notably, some non-regulated *Fusarium* metabolites, including aurofusarin, rubrofusarin, culmorin, 15-hydroxyculmorin, were detected in all diets. Besides, *Fusarium* metabolites, namely aurofusarin, culmorin and 15-hydroxyculmorin, were found in all diets with higher concentrations than those of *Fusarium* mycotoxins, in

particularly DON and ZON. The contamination of *Fusarium* and *Alternaria* metabolites in five experimental diets were detected as graded concentration of the metabolites which ranged from 0.01 to 2.46 ppm aurofusarin, 0.02 to 0.49 ppm rubrofusarin, 0.02 to 1.39 ppm culmorin, 0.06 to 1.83 ppm 15-hydroxyculmorin, 0.001 to 0.12 ppm alternariol, 0.01 to 0.14 ppm alternariolmethylether, respectively.

#### 4.2.2 Determination of mycotoxins in the five experimental diets using HPLC

The most frequently occurring mycotoxins in five experimental diets analyzed by HPLC (Table 8) were *Fusarium* mycotoxins, such as DON and ZON. However, mycotoxins (e.g. FB<sub>1</sub>, FB<sub>2</sub>, OTA, AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub> and AFG<sub>2</sub>) were not detected in all diets.

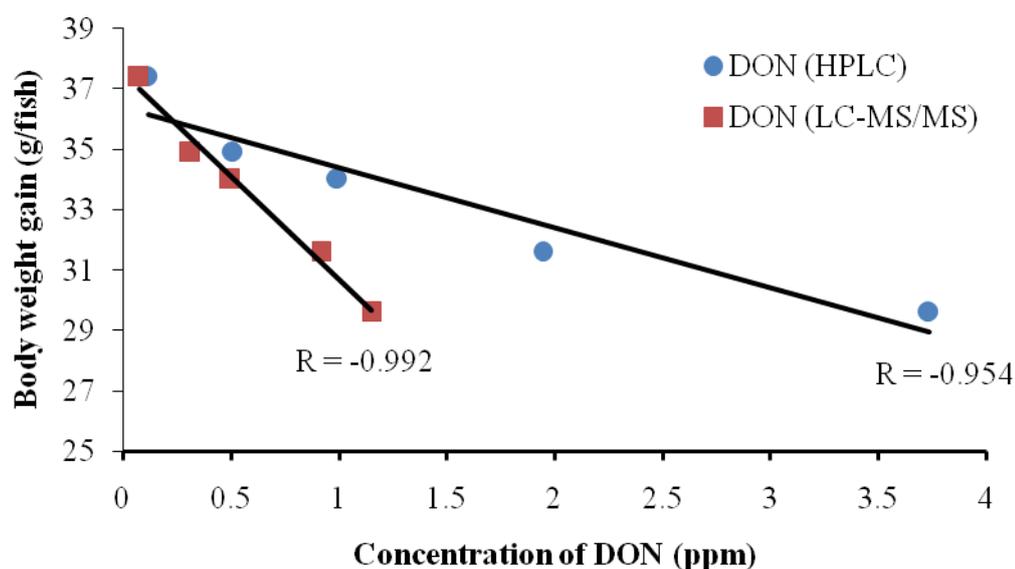
**Table 8** Mycotoxins in five experimental diets analyzed by HPLC (ppm)

Mycotoxins (ppm)	Dietary treatment				
	1	2	3	4	5
<b><i>Fusarium</i> mycotoxins</b>					
Deoxynivalenol (DON)	0.12	0.51	1.00	1.95	3.74
Zearalenone (ZON)	0.01	0.07	0.01	0.44	0.82
Fumonisin (FB <sub>1</sub> )	ND <sup>a</sup>	ND	ND	ND	ND
Fumonisin (FB <sub>2</sub> )	ND	ND	ND	ND	ND
<b><i>Aspergillus</i> mycotoxins</b>					
Ochratoxin (OTA)	ND	ND	ND	ND	ND
Aflatoxin B <sub>1</sub> (AFB <sub>1</sub> )	ND	ND	ND	ND	ND
Aflatoxin B <sub>2</sub> (AFB <sub>2</sub> )	ND	ND	ND	ND	ND
Aflatoxin G <sub>1</sub> (AFG <sub>1</sub> )	ND	ND	ND	ND	ND
Aflatoxin G <sub>2</sub> (AFG <sub>2</sub> )	ND	ND	ND	ND	ND

<sup>a</sup> ND: not detectable

#### 4.2.3 Comparison of detected concentrations of regulated mycotoxins in five experimental diets using HPLC and LC-MS/MS

The results of detected mycotoxins analyzed by differently analytic methods showed different concentrations and numbers of mycotoxins in the same experimental diets. In particular, the detected concentrations of DON (ranged from 0.12 to 3.74 ppm) using HPLC were greater than the detected concentrations of DON (ranged from 0.07 to 1.15 ppm DON) using LC-MS/MS. However, the relationship between graded concentrations of DON and response of red tilapia in terms of weight gain is shown in Figure 15. The relationship between the reduction of weight gain of red tilapia and the increase of graded concentrations of DON using LC-MS/MS ( $R = -0.992$ ) was greater than the relationship between the reduction of weight gain of red tilapia and the increase of graded concentrations of DON using HPLC ( $R = -0.954$ ).



**Figure 15** Comparison of the relationship between response of red tilapia in terms of weight gain of red tilapia and graded concentration of dietary DON analyzed by LC-MS/MS and HPLC

### **4.3 Growth performance and mortality of red tilapia**

All feed pellets were consumed promptly indicating good feed acceptance. However, feeding behavior of fish fed the highest concentrations of dietary DON (1.15 ppm) appeared after feeding a diet for a while; for example, fish snapped pellets without swallowing and spit pellets from their mouths. They stopped responding to diet faster than other groups. (Diet 1 to 4).

Results of weight gain, TGC, feed intake, FCE and percentage of mortality of red tilapia fed diets containing with graded concentrations of DON ranging from 0.07 to 1.15 ppm for eight weeks are reported in Table 9. Weight gain, TGC, FI and FE of fish linearly decreased ( $P < 0.01$ ) throughout the experiment with increasing dietary concentrations of DON (Figures 16 (A-B), 17 (A-B), 18 (A)). Additionally, increasing concentrations of DON in diets resulted in linear and quadratic decreases in the mortality ( $P > 0.05$ ). The graph (Figure 18 (B)) shows that the percentage of mortality of red tilapia from all treatment groups slightly increased in the first three weeks and mortality of red tilapia from all treatment groups stopped decreasing at the fourth week.

### **4.4 Hematological and biochemical parameters and hepatosomatic index**

Hematocrit, plasma aspartate aminotransferase, plasma alanine aminotransferase and hepatosomatic index as the health parameters are shown in Table 10 and Figure 19 (A-B), and hematocrit and hepatosomatic index are shown in Figure 20 (A-B). Feeding diets containing graded concentrations of dietary DON for eight weeks did not significantly ( $P > 0.05$ ) affect Hct, AST, ALT and HSI of fish. However, there was slightly decreasing trends in both of the Hct and HSI with increasing dietary concentrations of DON. In contrast to ALT was likely to increase with increasing dietary concentrations of DON.

### **4.5 Chemical composition in muscle of red tilapia**

Pooled samples of red tilapia flesh (muscle) collected from each aquarium assigned representatives of each treatment were analyzed for chemical composition, namely moisture, crude protein, lipid, ash and gross energy (Table 12).

#### **4.6 Histopathological changes in organs of red tilapia (n=12 per treatment)**

Organs of red tilapia (e.g. kidney, spleen, intestines and liver) were collected for histopathological examination; unfortunately, some organs including kidney, spleen and intestines were damaged during the fixative process.

For microscopic examination, five liver specimens from tilapia in the lowest and the highest concentration group of DON were randomly assigned for histopathological examination of liver. Most of the liver specimens of fish from all treatments showed a normal structure (Figure 21-A and 21-B). Hepatocytes are spherical in shape with nuclei in the center. Bile ducts are round in cross section. The central veins and hepatopancreases are randomly distributed within the liver tissue and vary in size and shape. Some of central vein and bile ducts contained with red blood cells.

In contrast to fish from control group, only seven samples of fish fed Diet 2, 3 and 4 appeared subcapsular edema in both of the gross and histopathological examination (Figure 23-A). Focal necrosis was observed in hepatic tissues of two fish fed Diet 3, 4 and 5 (Figure 22-A). Besides, cytoplasmic vacuolation was observed in livers of fish fed Diet 2 and 3 (Figure 23-B). Though some hepatic lesions appeared in the hepatic tissue, there was no significant difference of dose-response relationship between exposure to DON and histopathological alteration, based on the scoring system as a standard method for the assessment of histological changes published by Bernet et al. (1999).

**Table 9** Effects of the dietary DON on weight gain, growth rate, feed intake, feed conversion efficiency and percentage of mortality of red tilapia (initial body weight = 4.3 g/fish; mean  $\pm$  standard error of mean) in the 8-week feeding trial

Diet (DON ppm <sup>a</sup> )	DON intake <sup>b</sup> (mg)	Weight gain (g/fish)	TGC <sup>c</sup>	Feed intake (g/fish)	FCE <sup>d</sup> (gain/feed)	Mortality (%)
1 (0.07)	3.7	37.4 $\pm$ 0.5	0.108 $\pm$ 0.001	52.5 $\pm$ 0.8	0.71 $\pm$ 0.01	12 $\pm$ 1.9
2 (0.31)	15.3	34.9 $\pm$ 2.3	0.104 $\pm$ 0.004	49.5 $\pm$ 2.4	0.70 $\pm$ 0.02	14 $\pm$ 1.2
3 (0.50)	24.7	34.0 $\pm$ 1.4	0.102 $\pm$ 0.002	49.5 $\pm$ 1.3	0.69 $\pm$ 0.01	17 $\pm$ 2.9
4 (0.92)	43.4	31.6 $\pm$ 0.7	0.099 $\pm$ 0.001	47.2 $\pm$ 0.6	0.67 $\pm$ 0.01	10 $\pm$ 2.7
5 (1.15)	52.7	29.6 $\pm$ 0.7	0.095 $\pm$ 0.001	45.8 $\pm$ 0.5	0.65 $\pm$ 0.01	6 $\pm$ 1.8
Significance <sup>e</sup>						
Linear		P<0.01	P<0.01	P<0.01	P<0.01	P<0.05
Quadratic		N.S. <sup>f</sup>	N.S.	N.S.	N.S.	P<0.05

n = 4 for each treatment

<sup>a</sup> DON = Deoxynivalenol (ppm)

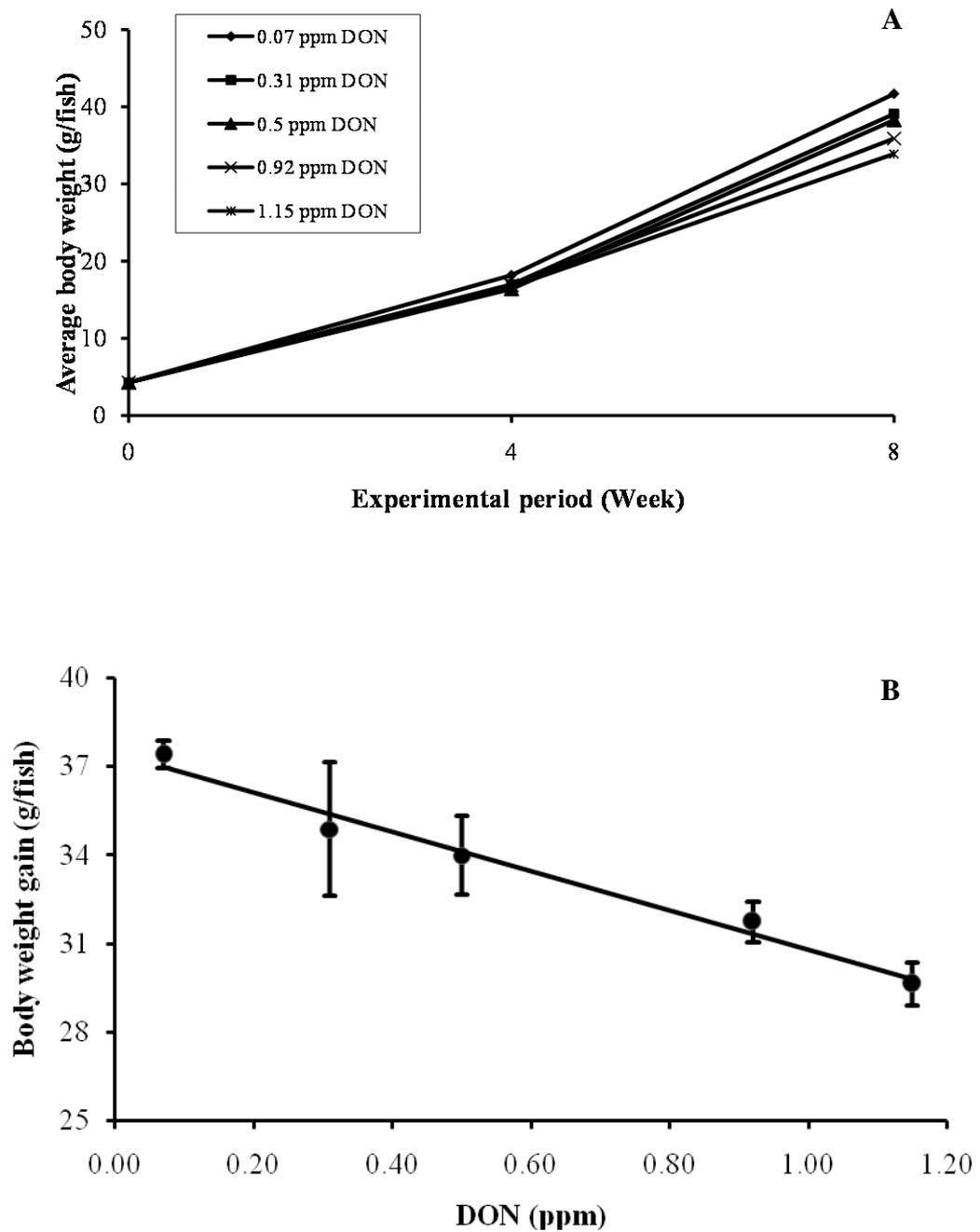
<sup>b</sup> DON intake = total amount of DON which red tilapia were fed for 8 weeks

<sup>c</sup> TGC = Thermal-unit Growth Coefficient expressed as growth rate

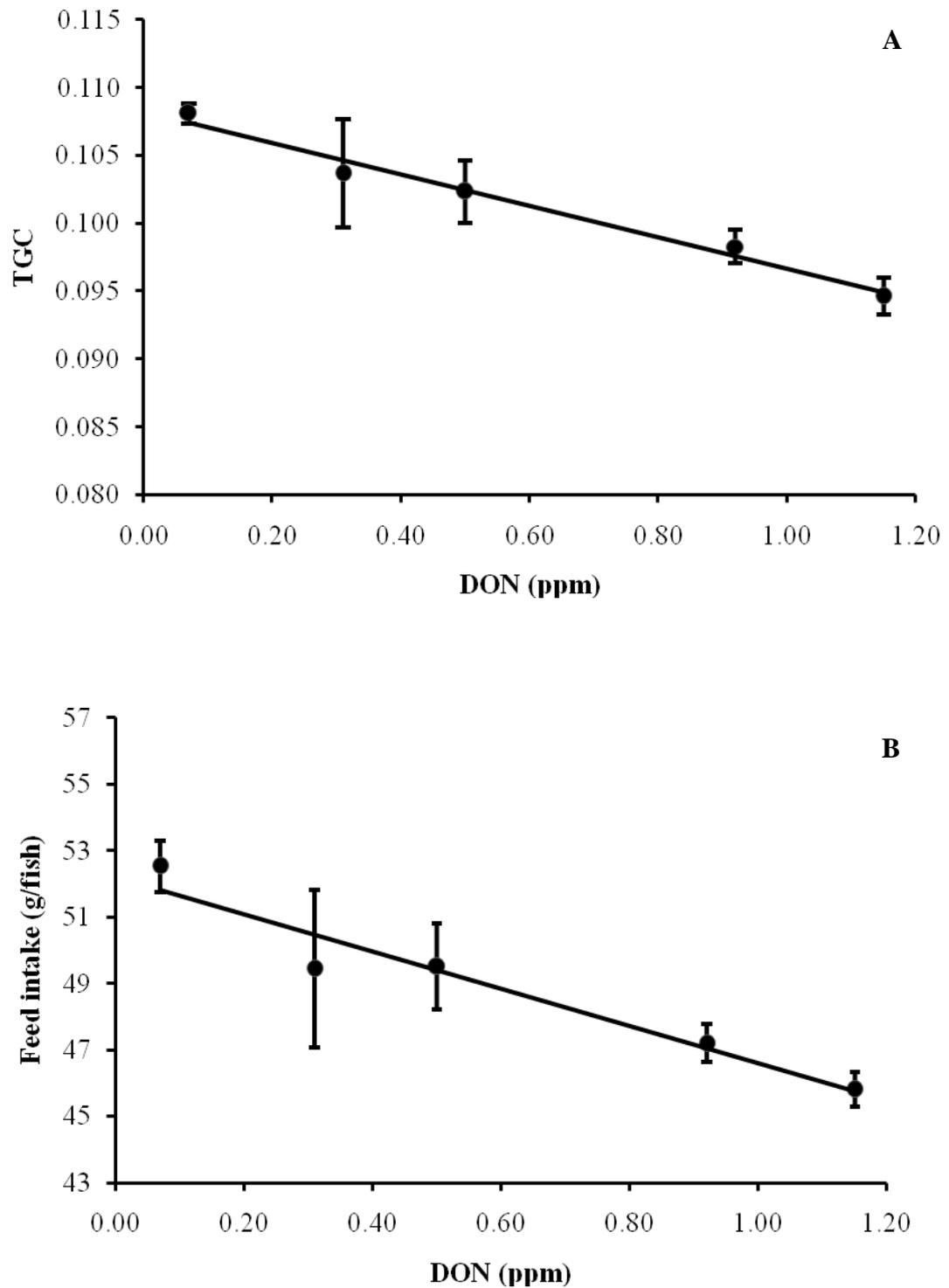
<sup>d</sup> FCE = Feed conversion efficiency

<sup>e</sup> Significance = significance of the orthogonal linear and quadratic contrasts

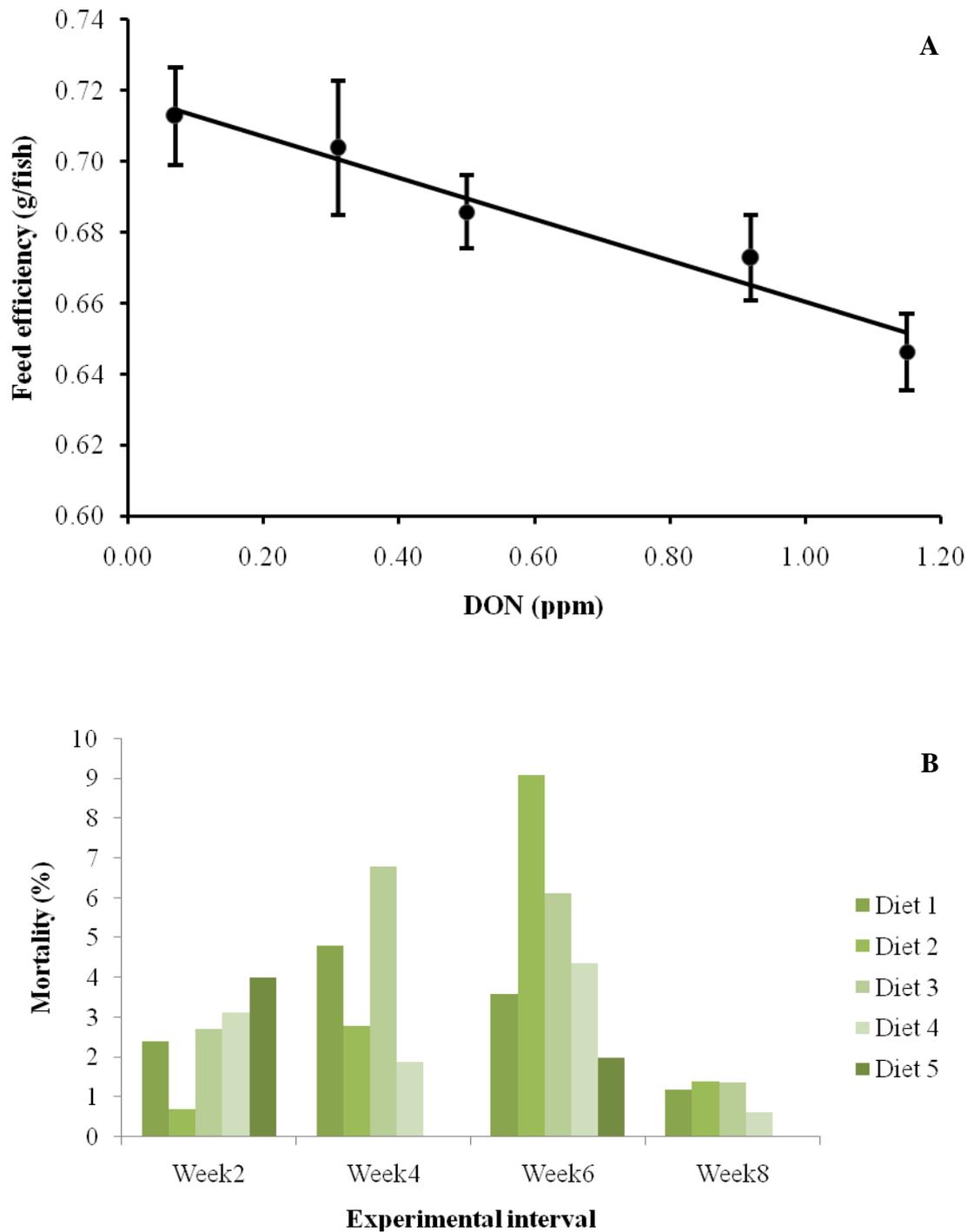
<sup>f</sup> N.S. = not statistically significant ( $P \geq 0.05$ )



**Figure 16** (A) Growth curves and (B) body weight gain of red tilapia (initial average weight = 4.3 g/fish) fed experimental diets containing 0.07 (control), 0.31, 0.50, 0.92 and 1.15 ppm DON for eight weeks (Standard error bars; n = 4 for each treatment)



**Figure 17** (A) Thermal-unit co efficiency (TGC) and (B) Feed intake of red tilapia (initial average weight = 4.3 g/fish) fed experimental diets containing 0.07 (control), 0.31, 0.50, 0.92 and 1.15 ppm DON (standard error bar; n = 4 for each treatment)



**Figure 18** (A) Feed efficiency of red tilapia (initial average weight = 4.3 g/fish) fed experimental diets containing 0.07 (control), 0.31, 0.50, 0.92 and 1.15 ppm DON (standard error bar; n = 4 for each treatment), and (B) percentage of mortality of red tilapia in each week

**Table 10** Effects of the dietary DON on blood chemistry, hematocrit and hepatosomatic index of red tilapia (initial body weight = 4.3 g/fish) in the 8-week feeding trial

Diet	DON <sup>a</sup> (ppm)	AST <sup>b</sup> IU/L	ALT <sup>c</sup> IU/L	Hct <sup>d</sup> (%)	HSI <sup>e</sup> (%)
1 (control)	0.07	592±164 <sup>h</sup>	96±24	34±1.5	2.40±0.17
2	0.31	477±120	69±15	32±1.1	2.18±0.13
3	0.50	574±159	103±24	33±1.1	1.94±0.08
4	0.92	350±63	90±42	32±1.0	1.87±0.16
5	1.15	527±118	146±49	31±1.3	1.97±0.12
<b>Significant<sup>f</sup></b>					
Linear		N.S. <sup>g</sup>	N.S.	N.S.	N.S.
Quadratic		N.S.	N.S.	N.S.	N.S.

n = 12, (12 individual fish per treatment were measured)

<sup>a</sup> DON = Deoxynivalenol

<sup>b</sup> AST = Plasma aspartate aminotransferase

<sup>c</sup> ALT = Plasma alanine aminotransferase

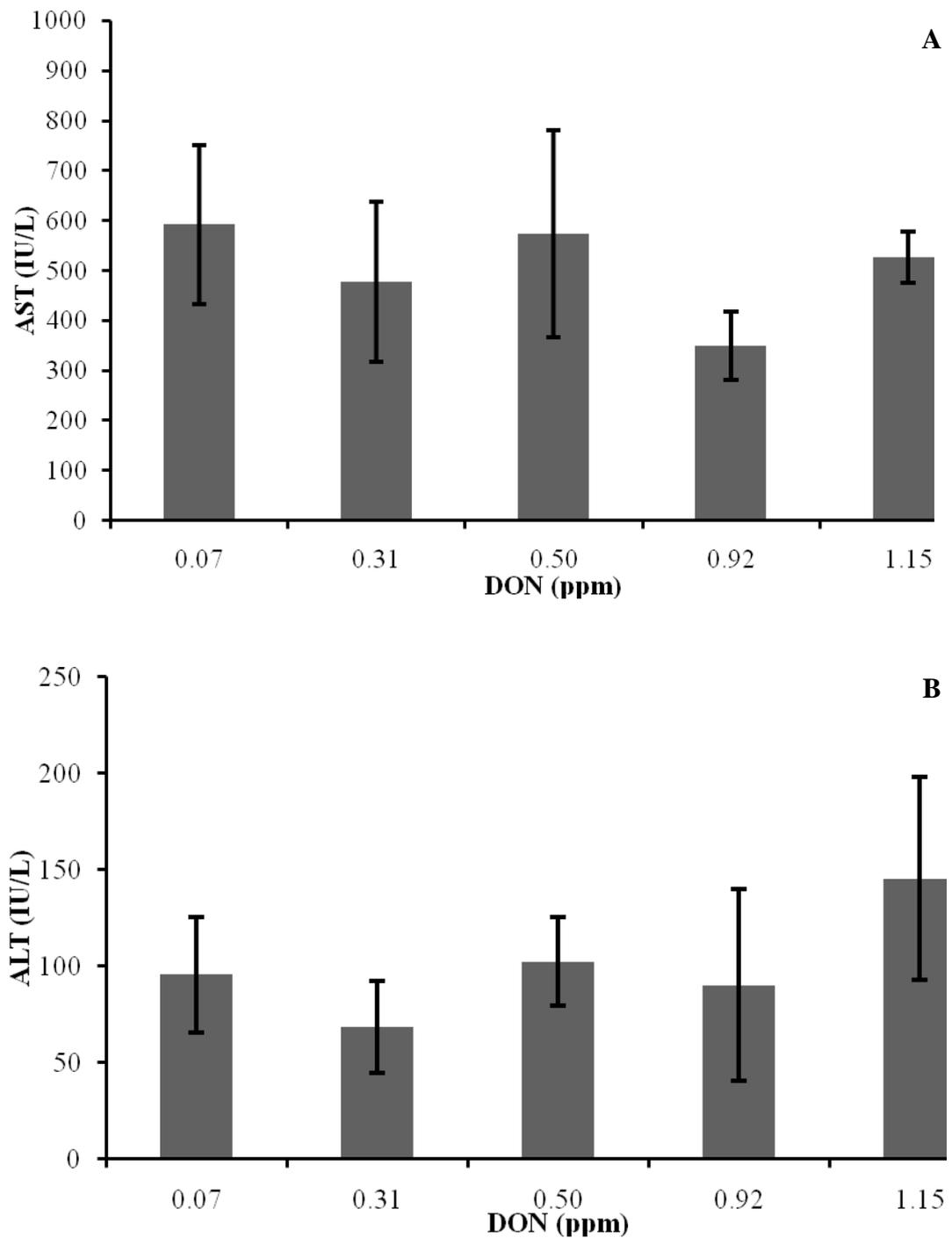
<sup>d</sup> Hct = Hematocrit

<sup>e</sup> HSI = Hepatosomatic Index

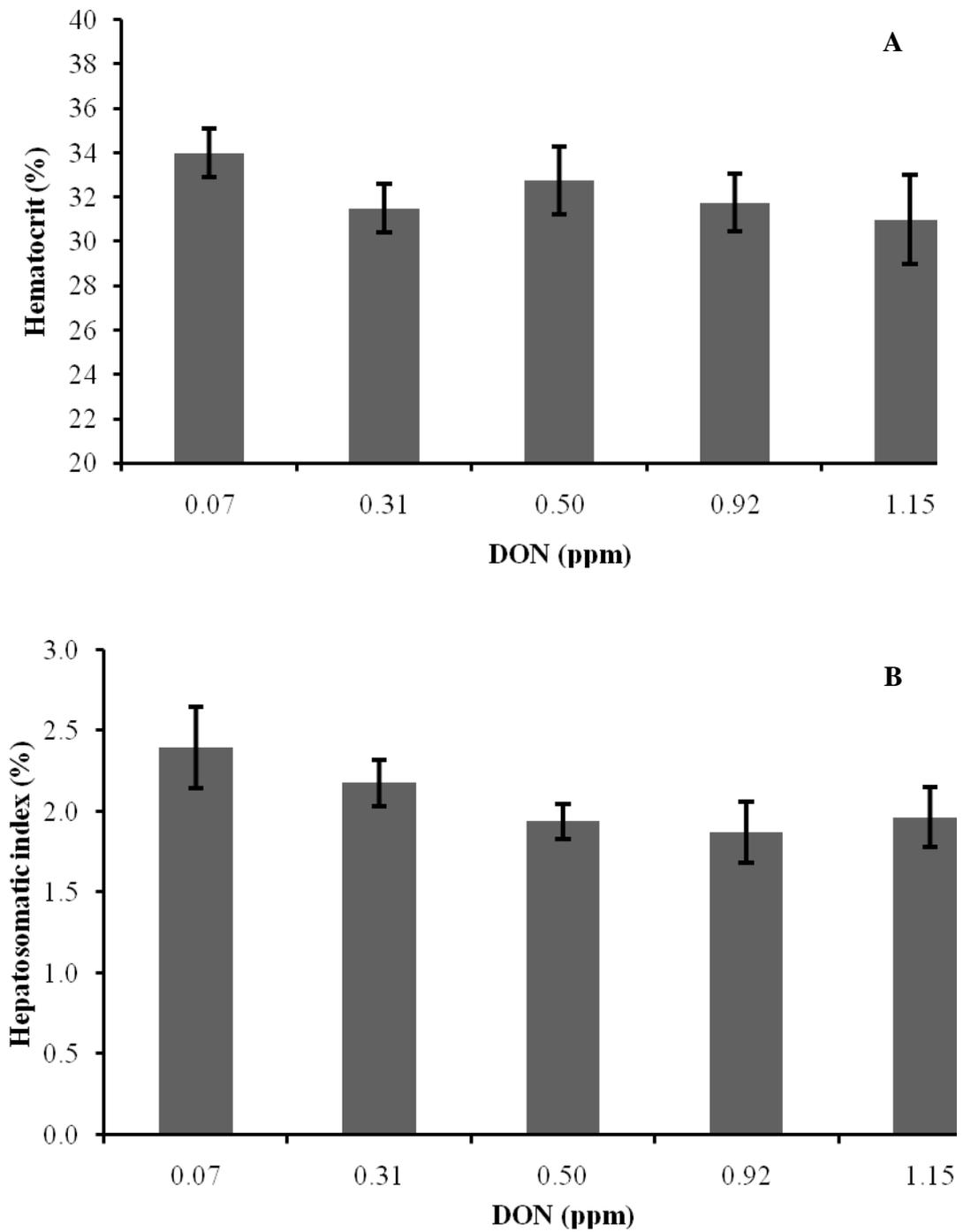
<sup>f</sup> Significance = significance of the orthogonal linear and quadratic contrasts

<sup>g</sup> N.S. = not statistically significant ( $P \geq 0.05$ )

<sup>h</sup> S.E.M. = Standard Error of the Mean



**Figure 19** (A) Plasma aspartate aminotransferase (AST) and (B) Plasma alanine aminotransferase (ALT) of red tilapia (initial average weight = 4.3 g/fish) fed with experimental diets containing 0.07 (control), 0.31, 0.50, 0.92 and 1.15 ppm DON for eight weeks (Standard error bars; n = 12 for each treatment)



**Figure 20** (A) Hematocrit (Hct) and (B) Hepatosomatic Index (HSI) of red tilapia (initial average weight = 4.3 g/fish) fed with experimental diets containing 0.07 (control), 0.31, 0.50, 0.92 and 1.15 ppm DON for eight weeks. (Standard error bars; n = 12 for each treatment)

**Table 11** Chemical composition in muscle (expressed as percentage of dry matter) of red tilapia (initial average weight = 4.3 g/fish) fed diets containing graded concentrations of DON for eight weeks

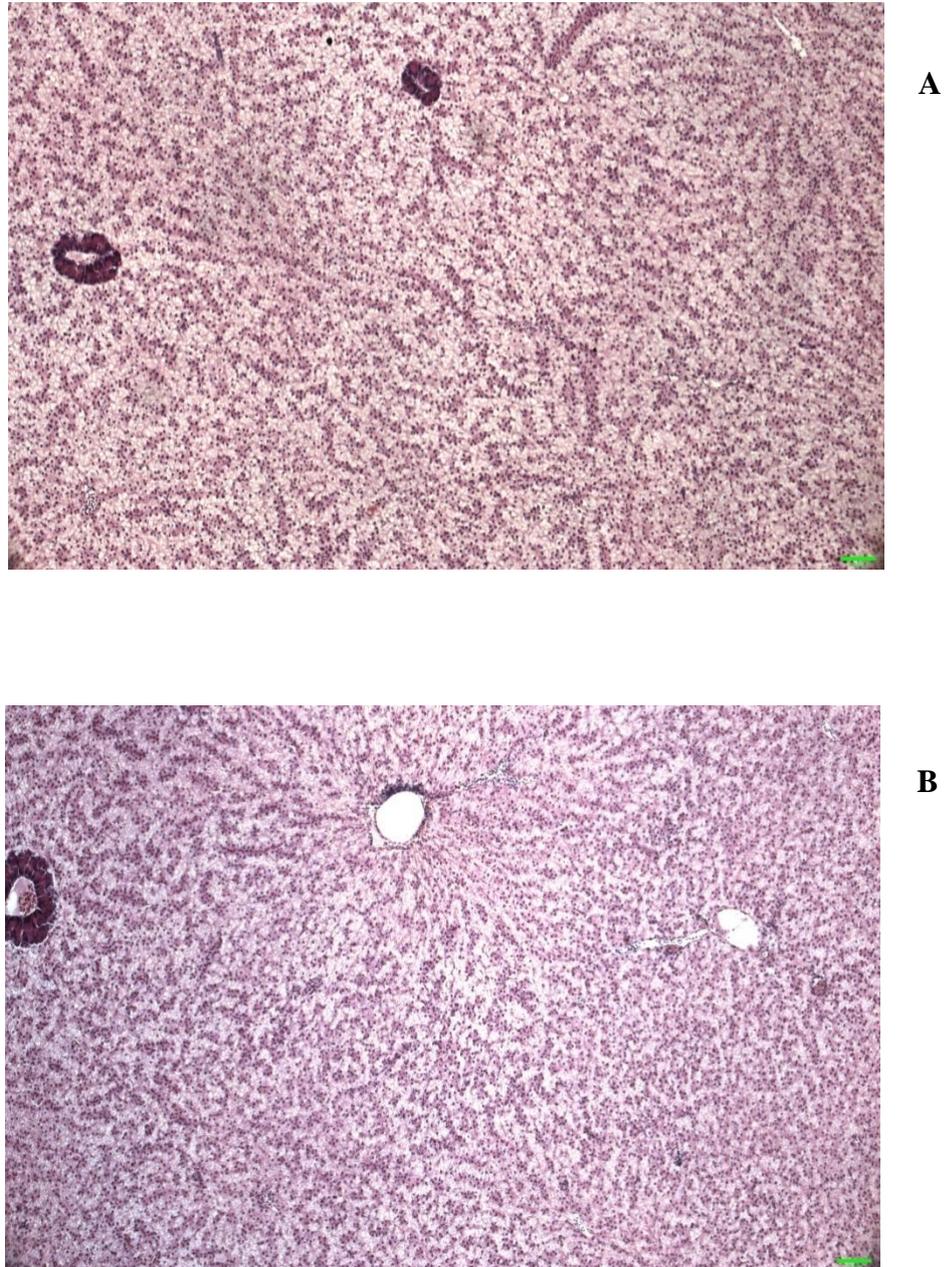
<b>Diet</b>	<b>DON<sup>a</sup> (ppm)</b>	<b>Moistur (%)</b>	<b>CP<sup>b</sup> (%)</b>	<b>Ash (%)</b>	<b>Lipid (%)</b>	<b>GE<sup>c</sup> (KJ/g)</b>
1 (control)	0.07	73.7	22.4	2.0	1.5	6.0
2	0.31	73.4	22.4	1.9	1.6	6.0
3	0.50	73.7	22.6	2.1	1.5	5.9
4	0.92	74.4	21.5	2.0	1.5	5.8
5	1.15	79.6	17.1	1.6	1.2	4.6

n = 12 (12 pooled fish per treatment were used for proximate analysis)

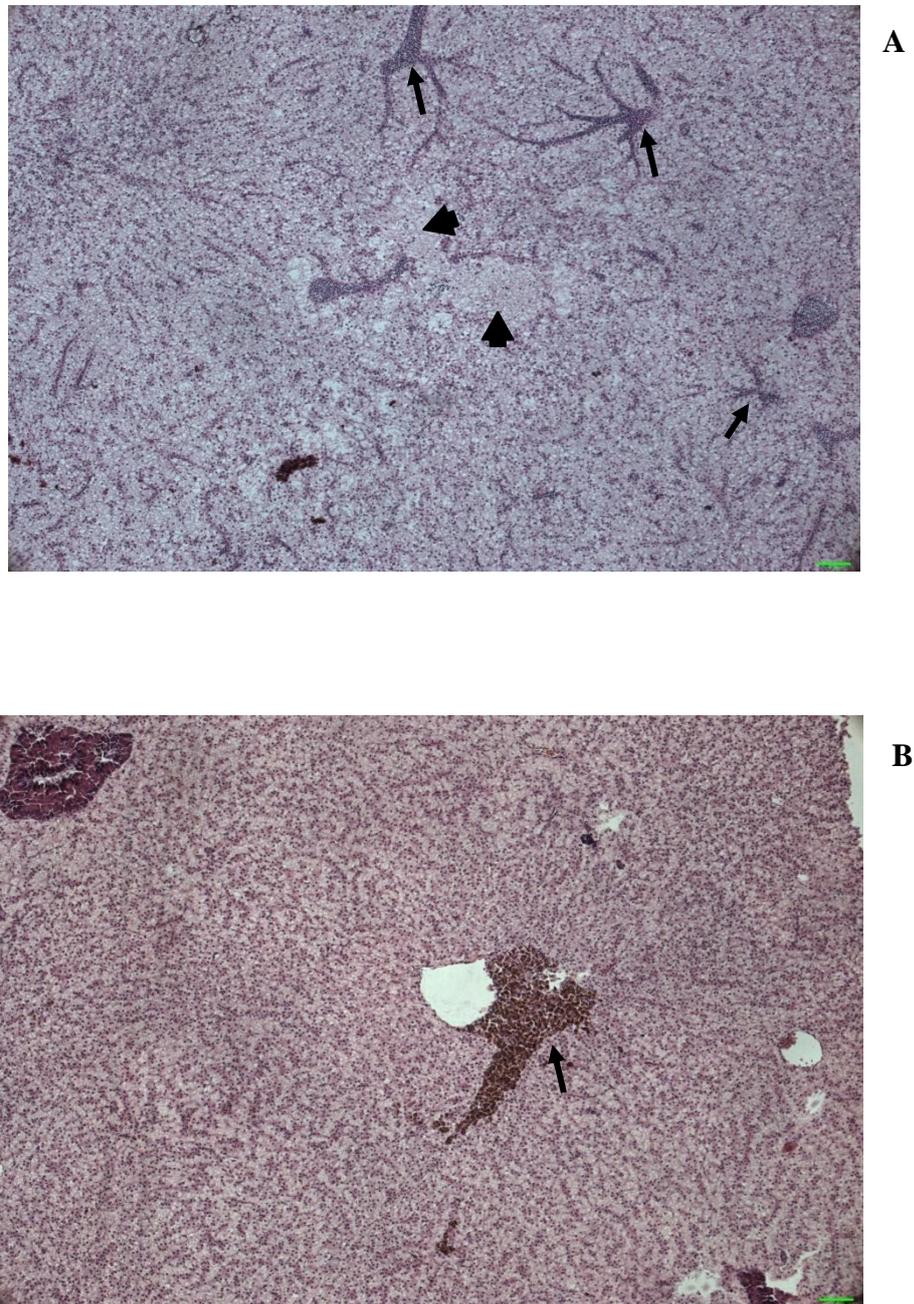
<sup>a</sup> DON = Deoxynivalenol

<sup>b</sup> CP = Crude protein

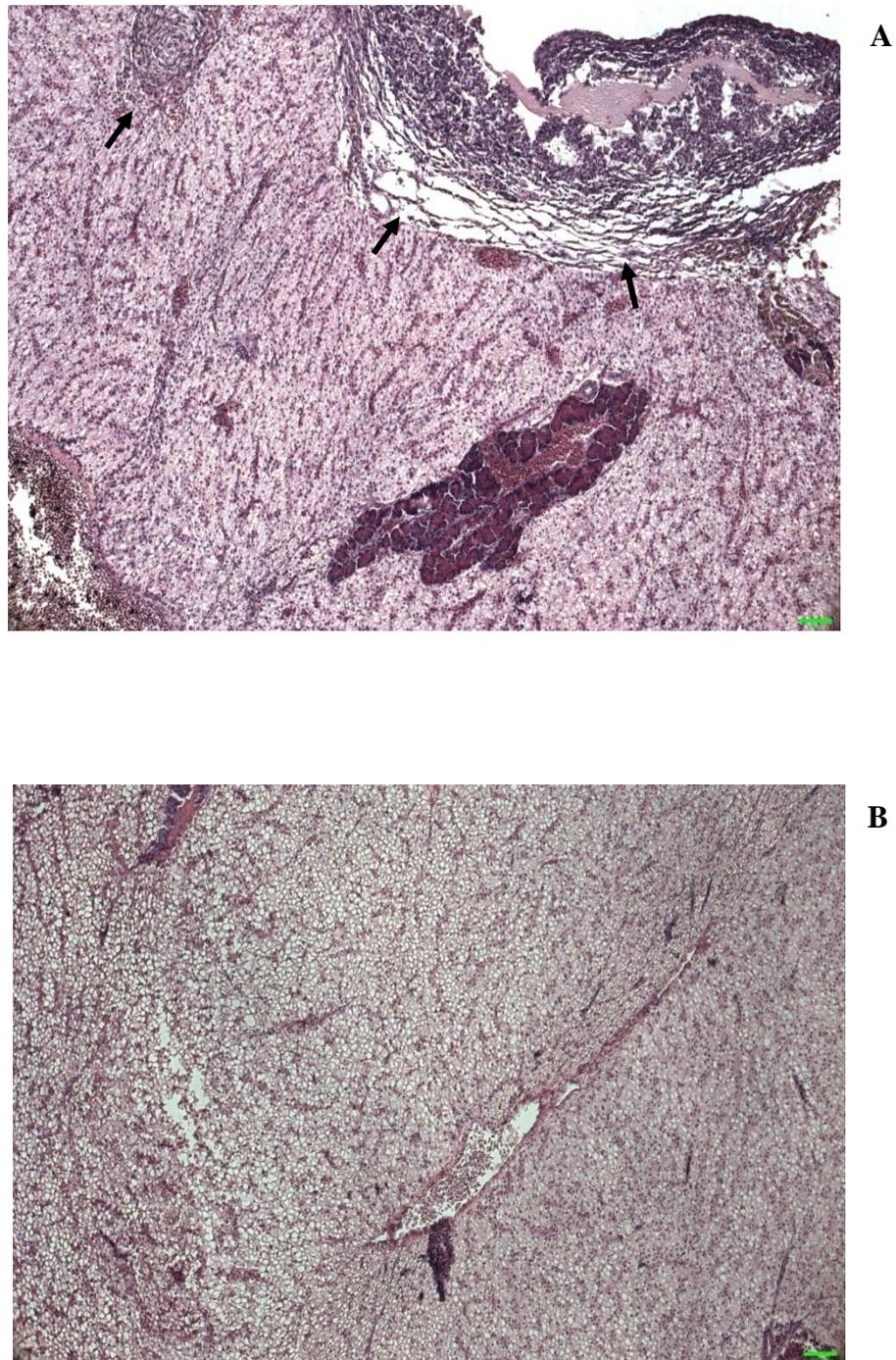
<sup>c</sup> GE = Gross energy



**Figure 21** Histological changes of liver of tilapia fed experimental diets containing 0.07 ppm DON (control diet) for eight weeks (H&E stain, scale bar = 179.48  $\mu\text{m}$ ). (A) and (B) illustrate normal hepatic architecture consists of portal vein, hepatopancreas and sinusoids



**Figure 22** Histological changes of liver of tilapia fed experimental diets containing 0.50 ppm DON (Diet 3) for eight weeks (H&E stain, scale bar = 179.48  $\mu$ m). (A) displays blood congest in central portal vein and sinusoids (arrows), and distribution of necrosis (arrowheads) (B) exhibits melanomacrophage aggregate at bile (arrow)



**Figure 23** Histological changes of liver of tilapia fed experimental diets for eight weeks (H&E stain, scale bar = 179.48  $\mu$ m). (A) Fish fed with 0.31 ppm DON (Diet 2) displays the increase in connective tissue at the portal vein and subcapsular edema (arrows). (B) Fish fed diet containing with 0.31 ppm DON (Diet 2) exhibited cytoplasmic vacuolation