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Effect of Dietary Supplementation of *Spirulina platensis* on the Growth and Haematology of the Catfish *Clarias gariepinus*

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ABSTRACT

The effect of feeding *Spirulina platensis* on the growth and haematological parameters of the Nile catfish; *Clarias gariepinus* exposed to food shortage stress were investigated. *S. platensis* was added to the basal diet at 0.0, 1.25, 2.5 and 5.0 g *Spirulina* /kg diet and fed for two months to the Nile catfish. Our results showed that the final fish weight, weight gain and specific growth rate of *C. gariepinus* fed on the experimental diets were not significantly different ($p > 0.05$). The fish fed on *Spirulina* diets exhibited higher RBC's counts, WBC's counts, haemoglobin and haematocrit levels as compared with stressed fish and control. The feeding dietary *Spirulina* resulted in significant decreases in mean cell volume, mean cell haemoglobin and neutrophils, while, the mean cell haemoglobin concentration was not affected. The platelets and monocytes were increased with *Spirulina* levels increase. A significant decrease in eosinophils and small lymphocytes were observed in the fish treated with *Spirulina*, however, the large lymphocytes were increased. The percentage of altered erythrocytes of fish treated with *Spirulina* was significantly decreased in comparison to stressed fish and control. We can conclude that *Spirulina* supplementation is promising for improving the hametological parameters in *Clarias gariepinus*, as immun-inducer and growth factor in food ingredients for fish feeding.

Keywords: *Spirulina platensis*; *Clarias gariepinus*; growth performance; haematology.

Authors' contributions

The two authors have the same contribution in this work

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1- INTRODUCTION

Marine macro- and microalgae were receiving increasing attention as a possible protein source for fish diets, particularly in tropical developing countries, because of their high protein content and production rate (Venkataraman et al., 1980). Microalgal species are of great value because of their high bioactive materials content, including polyunsaturated fatty acids, β- carotene and other pigments (antioxidants) (Bhat and Madyastha, 2000). The use of algae as a feed additive might help in effective utilization of artificial diets in cultured fish (Mustafa and Nakagawa, 1995). *Spirulina* is a freshwater blue-green filamentous alga, and has been one of the most widely used microalgal species in aquafeeds due to its high contents of proteins, vitamins, essential amino acids, minerals, essential fatty acids and antioxidant pigments such as carotenoids (Nakagawa and Montgomery, 2007). β-carotene in *Spirulina* firmly maintains the mucous membrane and thereby prevents the entry of toxic elements into the body (Henrikson, 1994). Chlorophyll in *Spirulina* acts as a cleaning and detoxifying factor against toxic substances (Henrikson, 1994). Growth studies with *Spirulina*, have confirmed that it improves carcass quality (Liao et al., 1990), encourages growth (Mustafa et al., 1994a). Therefore, it has been used as a nutrient for fish larvae (Lu and Takeuchi, 2004; Lu et al., 2002) and as an ingredient in fish diet for juveniles and adults common carp (Palmegiano et al., 2008). Tilapia fed solely on raw *Spirulina* could maintain normal reproduction from parents to progeny throughout three generations (Lu and Takeuchi, 2004). It has been verified that larval tilapia fed solely on raw *Spirulina* cultivated in photo-bioreactors can grow normally from the onset of exogenous feeding without any nutrient supplements (Lu et al., 2002). The addition of small amounts of algae to fish feed exerted pronounced effects on growth, lipid metabolism, body composition and disease resistance (Mustafa et al., 1994b). As well as, haematological studies in fish have diagnostic as well as economic significance. In recent years fish haematology has become an increasingly important tool of fisheries biologists and research ichthyologists (Mekkawy et al., 2011;Sayed et al., 2013; Sayed et al., 2007). Hence, *S. platensis* may have potential to be used as a natural feed supplement for increasing fish growth. Therefore, the main objective of the present study is to investigate the effect of diets containing *S. platensis* on the growth and some haematological parameters of *Clarias gariepinus*.

2- MATERIALS AND METHODS

2.1. Algal Culture

The culture of *Spirulina platensis* (Gomont) was isolated from agriculture faculty farm in Assiut University (Egypt) and identified according to (Prescott, 1978). S. platensis was grown in Zarrouk's medium (Zarrouk, 1966) at pH 9 and incubated at 30 ^{o}C under continuous illumination fluorescent light of 48.4 µmole.m-2.s-1. The alga was cultivated in 50 L photobioreactors using a unialgal semi-continuous culture (Morist et al., 2001). To determine the algal biomass, a 100 mL aliquot of the algae suspension was filtered through Whatman (GF/A) glass fiber (0.45 µm), and the obtained pellet was then oven-dried at 85^{o}C for 4h and weighed. Chlorophyll-a and carotenoids were extracted in methanol (80%) and determined according to (Marker, 1972). Phycobiliproteins contents of *S. platensis* were determined according to the method described by (Bennet and Bogorad, 1973). Total proteins in S. *platensis* were determined according to (Lowry et al., 1951). For the determination of total carbohydrates, the anthrone sulfuric acid method was used (Badour, 1959). Total lipids in S. *platensis* were determined by the sulfophosphovanilin method (SPV) (Drevon and Schmitt, 1964) (Table 2).

2.2. Fish sample collection and pre-experimental adaptation

Nightly six healthy fishes of the Nile catfish; *Clarias gariepinus* (490 ± 17.8 g) in weight were caught from the fish farm of Faculty of Agriculture, Assiut University, Egypt in April 2013. Fishes immediately transported to the Fish Biology laboratory in the Department of Zoology, Faculty of Science, Assiut University. The experimental fishes were reared in aerated glass tanks (100 L capacity) and acclimatized for two weeks before being used in the experimental study. The experimental fish fed commercial pellets at a rate of 5 % of wet weight twice daily to help the fish adapt to their new environment before the experiment. Feces and residual food were aspirated regularly. The water temperature, pH, dissolved oxygen (DO) concentrations and electrical conductivity (EC) were measured daily (25.2 ± 0.08 ºC, pH: 6.8 ±11, DO: 6.5± 0.89 mg L-1 and EC: 260 ± 0.2 µmho.cm-1).

2.3. Experimental setup

The adapted fishes were exposed to food shortage stress (feed at 2% of body wet weight daily) for two months (May and June 2013). This experiment was done under the same conditions of the adaption period. After that, fishes were weighed and classified randomly into 4 groups according to the concentrations of *Spirulina*. Each group was contained three replicates (8 fish/ 100 L tank). Then, fishes were exposed to

different concentrations of *Spirulina* within food (0.0 (control), 1.25, 2.5 or 5.0 g *Spirulina* /kg diet). The exposure was for two months and experimental fish were fed on commercial pellets (5 % of wet weight twice daily) with changing all the tap water every day.

2.4. Diet preparation

Isolated *Spirulina* culture was added to the basal diet which represented in Table (1) to represent various concentrations as 0.0 (control), 1.25, 2.5 or 5.0 g *Spirulina*/kg diet. The nutritional composition of *Spirulina platensis* was shown in Table (2). Each concentration of *Spirulina* was suspended in 100 ml distilled water and added to the ingredients of the experimental diet, and blended for 40 min at least to make a paste of each diet. The pastes were separately passed through a grinder, and pelleted (1 mm diameter) in a paste extruder. The diets were air-dried and stored in plastic bags in a refrigerator (-2 ^{o}C) for further use. Water quality such as temperature, dissolved oxygen pH and electrical conductivity were measured every 20 day during the experiment.

2.5. Growth parameters

Fishes were weighed at the beginning and end of the experiment. Growth was calculated as the difference between the wet weights at the beginning of the experiment and on the day of calculation. Percentage weight gain PWG (%) was calculated as (Final mean body weight/ Initial mean body weight) x 100 (Tacon, 1990). Specific growth rate (SGR) was calculated as $(Wt_1 – Wt_0)/t1 \times 100$, where Wt_0 and Wt1 are the weights of the fish at the beginning and end of each sampling period and t1 is the period between samplings in days (Hevrory et al., 2005). The survival rate was calculated as (No of fishes remaining at the end of the experiment/ No of fishes at the beginning of the experiment) x 100 (Ai et al., 2006).

2.6. Hematological parameters

Blood samples were taken from the caudal vein into heparinzed tubes. The concentration of Hb and blood cells count was immediately estimated. Other samples of blood were centrifuged at 5000 rpm for 10 min and serum samples were stored in polyethylene Eppendorf test tubes at -20 °C until serum analysis. The RBC's, WBC's, blood Platelets, Haematocrit (HCT), Hemoglobin (Hb) were determined by using automated technical analyzer (Mindray Bc-2800). Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated using the formulae mentioned by (Dacie and Lewis, 1991). MCHC (g/dl) = Hb/ HCT x 100, MCH (pg) = Hb/ RBC's x 10, MCV (μm^3) = HCT/ RBC's x 10. Differential WBC's were counted using blood smears stained with Giemsa satin according to (Tavares-Dias and Moraes, 2003)

2.7. Erythrocytes alterations

Blood smears were obtained by the caudal incision on clean grease free microscopic slides after exposure. The smears were fixed in absolute methanol for 10 min after drying at room temperature. Slides were stained with haematoxylin and eosin. It was followed by dehydration in ascending grades of alcohol (30, 50, 70, and 90%, absolute). Finally the slides were cleared in xylene and permanently mounted by DPX (Pascoe and Gatehouse, 1986). Many slides were selected on the basis of staining quality, then coded, randomized and scored blindly. In each group 10,000 cells (a minimum of 1000 per slide) were examined (Al-Sabti and Metcalfe, 1995) at 40× objective and 10× eyepiece for morphologically altered erythrocytes in separate studies. The morphologically altered erythrocytes were described according to (Mekkawy et al., 2011).

2.8. Statistical analysis

Statistical analyses were performed on the data obtained from completely randomized design with three replications after two months of the experimental period. One-way ANOVA was used to test the effect of the dietary treatment. Duncan Multiple Range test was also applied to compare the means when a significant difference ($p < 0.05$) was detected by ANOVA. All the statistical analyses were done using SPSS program version 10 (SPSS, Richmond, VA, USA).

2.9. Ethical statement

All experiments were carried out in accordance with the Egyptian laws and University guidelines for the care of experimental animals. All procedures of the current experiment have been approved by the Committee of the Faculty of Science of Assiut University, Egypt.

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3- RESULTS

3.1. The physico-chemical characteristics of the water

The water quality was measured every 20 day during the experiment (Table 3). Water temperature ranged between 28.6 and 29.1^{o}C at twenty day and first day of the experiment, respectively. With respect to the dissolved oxygen and electrical conductivity, the maximum value was recorded at the forty day. The pH value was tented to weakly alkaline.

3.2. Nutritional composition of *Spirulina platensis* and Growth performance

Nutritional composition of *Spirulina platensis* was shown in Table (2). *Spirulina platensis* were contained high percentage of total proteins (43.4 %), total carbohydrates (28.1 %), total lipids (5.02 %), chlorophyll-a (0.68 %), carotenoids (0.65 %) and phycobiliproteins (18.43 %).

The effects of *Spirulina* diets on the growth parameters for *Clarias gariepinus* throughout the experimental periods are given in Table (4). The final fish weight ranged from 471 g to 535 g for the fishes exposed to food shortage stress (after stress) and 2.5 g *Spirulina*/kg diet, respectively; and was not significantly lower in fish fed after stress than all *Spirulina* diets ($p > 0.05$). Also, the weight gain and specific growth rate of *Clarias gariepinus* fed on the experimental diets was not significantly different ($p > 0.05$). Furthermore, fish survival was not statistically different among dietary treatments, and its range was 91.7-100% (Table 4).

3.3. Hematological parameters

Fishes were fed on *Spirulina* diets exhibited significant increasing in RBCs counts and their ranges were 2.98-3.8 × 106/µL for the fishes fed after stress and 5 g *Spirulina*/kg diet, respectively ($p < 0.05$; Table 5). The high counts of WBC's were obtained at 5g/kg diet (12.03 × 105/µL). Dietary *Spirulina* significantly affected the haemoglobin of fishes and varied from 7.51 to 9.83 mg/dl for the fishes fed after stress and 1.25 g *Spirulina*/kg diet, respectively. Haematocrit tended to increase with increasing dietary *Spirulina* levels than the control fish ($p < 0.05$). Mean cell volume ranged between 100.1 and 116.2 µm³ for the fishes fed 5 g *Spirulina*/kg diet and the control fish, respectively. The highest level of mean cell haemoglobin was found in control diet; however, there were no differences in mean cell haemoglobin concentration of the fishes fed *Spirulina* diets. Platelets were significantly increased than the control fish with increasing *Spirulina* levels in fish diet ($p < 0.05$) and the high value was recorded at 5 g *Spirulina*/kg diet. The number of neutrophils was decreased with the *Spirulina* levels while monocytes increased in comparison with control fish (Table 5). The highest number of neutrophils and monocytes were recorded in fish fed after stress. The fish were fed 1.25 g *Spirulina*/kg diet displayed high number of basophils. Generally eosinophils were significantly decreased with *Spirulina* levels ($p < 0.05$).

The control diet produced the highest percentage of small lymphocytes, which decreased with the increase of *Spirulina* levels in fish diet ($p < 0.05$). Fish fed 5 g *Spirulina*/kg diet had a significantly ($p < 0.05$) higher number of large lymphocytes compared to the control fish (Table 4).

3.4. Altered erythrocytes

As shown in Fig.1 the percentage of altered erythrocytes of control fish was 3.14 ± 0.26% and this percentage increased significantly in group exposed to feeding shortage stress. With increasing the concentration of *Spirulina* levels the percentage of altered erythrocytes significantly decreased as 3.00 ± 0.44 %, 2.29 ± 0.52 % and 1.00 ± 0.31 % for 1.25, 2.5 and 5.0 g/kg fish diet, respectively. Fig. 2 shows the important variations in erythrocytes such as acanthocytes, tear drop like cells, sickle cells, and enucleated erythrocytes (arrows).

4- DISCUSSION

The current investigation was performed to test the feasibility of including dietary *Spirulina* meal in practical diets for *Clarias gariepinus*. The water quality in the present study was within an acceptable range for catfish culture (Bhujel, 2000). The data in this study showed that, the growth performance of *Clarias gariepinus* fed on *Spirulina* diets was not significantly different. These results are in accordance with (Ungsethaphand et al., 2010) who recorded that the final weight gain and specific growth rate of hybrid red tilapia were not affected by S. *platensis* supplementation. Also, (Teimouri et al., 2013) found that, S. *platensis* supplemented diets did not change growth related parameters in rainbow trout. (Olvera-Novoa et al., 1998) and (Dernekbasi et al., 2010) observed that replacing fishmeal with S. *platensis* up to 40% did not change growth rate in tilapia and guppy. On the contrary, (James et al., 2006) reported that dietary inclusion of 8 % *Spirulina* significantly elevated growth performance of the ornamental red swordtail *Xiphophorus helleri*. Because *Spirulina* is rich in proteins, vitamins, minerals, essential amino acids and fatty acids (Nakagawa and Montgomery, 2007), it has

been identified as a potential feed ingredient for cichlids and ornamental fish and appears to be a promising dietary ingredient.

In the current study, fish fed on *Spirulina* diets exhibited higher RBC's and WBC's counts as well as, the haemoglobin value as compared with fish fed after stress and the control diet. This increase could be due to the presence of C-phycocyanin in the *Spirulina* alga, which can help build the immunity capacity (Vonshak, 1997). These results proved the improvement of fish health when fed *Spirulina*-supplemented diets because *Spirulina* contains carotenoids, which increase the ability to fight off infections through the reduction of stress levels (Nakono et al., 2003). The major functions of WBC's are to fight infection; defend the body against foreign organisms and in immune response. Decreased in WBC's counts in control fish after food shortage stress in this study was similar to the observation of (Sunmonu and Oloyede, 2008) in catfish exposed to increased crude oil concentration. Feeding dietary *Spirulina* had a significant ($P < 0.05$) increase in the levels of haematocrit. (Moe, 2011) recorded that haematocrit value in treated fish was lower than that of control fish. On the other hand, the obtained results revealed that feeding dietary *Spirulina* resulted in significant decreases in mean cell volume as well as mean cell haemoglobin but mean cell haemoglobin concentration were not affected by S. *platensis* supplementation. These results are disagreeing with (Moe, 2011) who reported that the values of MCH were significantly higher ($p < 0.05$) than that of control fish. The platelets and monocytes in this study were increased while neutrophils were decreased with *Spirulina* levels. (Abd El-Ghany and Abd Alla, 2008) observed increasing in plasma levels of cortisol, eosinophils and monocytes in the fish treated with Fucus. The results of the present investigation exhibit that, the highest number of basophils was recorded in the fish fed 1.25g *Spirulina*/kg diet. Basophils play an important role in body immune responses (Miller, 1993). Generally, the fish treated with *Spirulina* exhibited a significant decrease in eosinophils and small lymphocytes, however, the large lymphocytes were increased. (Abd El-Ghany and Abd Alla, 2008) revealed that, the fish treated with *Fucus* exhibited a significant increase in the total leucocytic count and lymphocytes. Our study indicated that, the percentage of altered erythrocytes of fish treated with *Spirulina* was significantly decreased in comparison to fish fed after stress and the control diet. The improvement of the erythrocytes alterations was recorded after treatment with quince leaf extract in the same species after UV radiation (Sayed et al., 2013). In the present study the increase in altered cell frequencies appeared more clearly in fishes exposed to food shortage stress. The alterations in fish erythrocytes were observed in hypoxic condition (Sawhney and Johal, 2000), factors that induce apoptosis of blood cells like radiations , 4-nonylphenol (Mekkawy et al., 2011), and UVA (Sayed et al., 2013). In conclusion, the present study showed hematology profile for the *Clarias gariepinus* species after two months of food shortage stress in comparison with control and *Spirulina* diets. Also, the *Spirulina* feeding improved the hametological parameters in all exposed groups compared with stressed and control groups. Finally we recommend with using *Spirulina* as immun-inducer and growth factor in food ingredients for fish feeding in fish culture.

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Table 1. Nutrient composition of experimental diets (%)

Ingredient	Percentage
Soybean meal (48%) [a]	30.1
Cotton meal (41%)	10.0
Menhaden meal (61%)	4.0
Corn grain	33.6
Wheat middlings	20.0
Dicalcium phosphate	0.6
Catfish vitamin and mineral mix [b]	0.2
Fat/oil	1.5

[a] Values in the parentheses represent percentage protein.

[b] Commercial mix that meets all requirements for catfish.

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Table 2: Typical nutritional composition of *Spirulina platensis* (%)

Ingredient	Percentage
Total proteins	43.4±3.13
Total carbohydrates	28.1±2.2
Total lipids	5.02±1.7
Chlorophyll- *a*	0.68±0.02
Carotenoids	0.65±0.00
Phycobiliproteins	18.43±0.24

Table 3:- Physico-chemical parameters of the water during the experiment.

Experiments Days / Parameters	1st	20th	40th	60th
Water temperature (°C)	29.1	28.6	29.2	29.4
Dissolved oxygen (mg L^{-1})	6.9	6.6	7.2	6.8
pH	7.5	7.4	7.6	7.5
Electrical conductivity (µmho.cm^{-1})	266	252	284	275

Table 4. Growth performance and feed utilization of *Clarias gariepinus* fed experimental diets containing different levels of *Spirulina platensis* for 8 weeks.

Items	After stress	Control (0.0)	*Spirulina* levels (g/kg diet)		
			1.25	2.5	5.0
Initial weight (g)	461.7±15.9[a]	438.3±37.7[a]	470.0±20.2[a]	465.0±22.5[a]	470.7±13.1[a]
Final weight (g)	471.0±17.1[a]	501.7±8.3[a]	521.7±18.8[a]	535±37.9[a]	510.3±4.1[a]
Weight gain (%)	102.01±0.26[a]	115.9±8.7[a]	111.8±2.0[a]	114.9±4.5[a]	108.6±2.9[a]
SGR (%/day)*	0.14±0.02[a]	1.02±0.52[a]	0.75±0.13[a]	0.98±0.28[a]	0.58±0.19[a]
Survival rate (%)	100±0.00[a]	91.7±4.82[a]	94.5±2.77[a]	97.2±2.77[a]	100±0.00[a]

* SGR= Specific growth rate

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Table 5. Hematological Parameters of *Clarias gariepinus* fed experimental diets containing different levels of *Spirulina platensis* for 8 weeks.

Items	After stress	Control (0.0)	*Spirulina* levels (g/kg diet)		
			1.25	2.5	5.0
RBC's (million/µl)*	2.98±0.07^{a}	3.00±0.1^{a}	3.5±0.06^{b}	3.7±0.06^{bc}	3.8±0.06^{c}
WBC's (thousands/µl)	10.64±0.76^{a}	10.70±0.15^{a}	10.48±0.22^{a}	11.57±0.18^{ab}	12.03±0.18^{b}
Hb (Mg/dl)	7.51±0.35^{a}	8.64±0.20^{b}	8.74±0.29^{b}	9.60±0.1^{c}	9.83±0.03^{c}
HCT (%)	33.55±0.87^{a}	34.82±0.89^{a}	36.88±0.39^{b}	37.55±0.30^{b}	38.00±0.34^{b}
MCV (µm³)	112.9±4.25^{bc}	116.2±1.50^{c}	105.4±1.15^{ab}	101.5±1.19^{a}	100.1±2.40^{a}
MCH (Pg)	25.19±0.55^{a}	28.87±1.14^{b}	24.96±0.45^{a}	25.95±0.22^{a}	25.89±0.4^{a}
MCHC (%)	22.41±1.22^{a}	24.84±0.67^{b}	23.69±0.57^{ab}	25.57±0.09^{b}	25.88±0.23^{b}
Platelets (thousands/µl)	203.7±4.18^{a}	207.7±3.38^{ab}	214.7±1.33^{bc}	222.0±1.15^{cd}	223.3±0.67^{d}
Neutrophils (%)	15.67±0.33^{c}	13.00±0.58^{b}	13.67±0.33^{b}	11.33±0.33^{a}	10.67±0.33^{a}
Monocytes (%)	4.00±0.00^{b}	2.33±0.33^{a}	3.67±0.33^{b}	2.67±0.33^{a}	2.67±0.33^{a}
Basophils (%)	0.67±0.33^{a}	0.67±0.33^{a}	1.00±0.00^{a}	0.33±0.33^{a}	0.33±0.33^{a}
Eosinophils (%)	4.67±0.33^{c}	2.67±0.33a^{b}	3.00±0.00^{b}	2.00±0.00^{a}	2.00±0.00^{a}
Small lymphocytes (%)	21.33±0.88^{c}	23.67±0.33^{d}	18.33±0.88^{b}	13.33±0.33^{a}	11.67±0.33^{a}
Large lymphocytes (%)	53.7±0.88^{a}	57.7±0.33^{b}	60.3±1.2^{c}	71.0±0.58^{d}	72.7±0.33^{d}

* RBC's= red blood cells, WBC's= white blood cell's, Hb= Haemoglobin, HCT= Haematocrit, MCV= Mean Cell Volume, MCH= Mean Cell Haemoglobin and MCHC= Mean Cell Haemoglobin Concentration.

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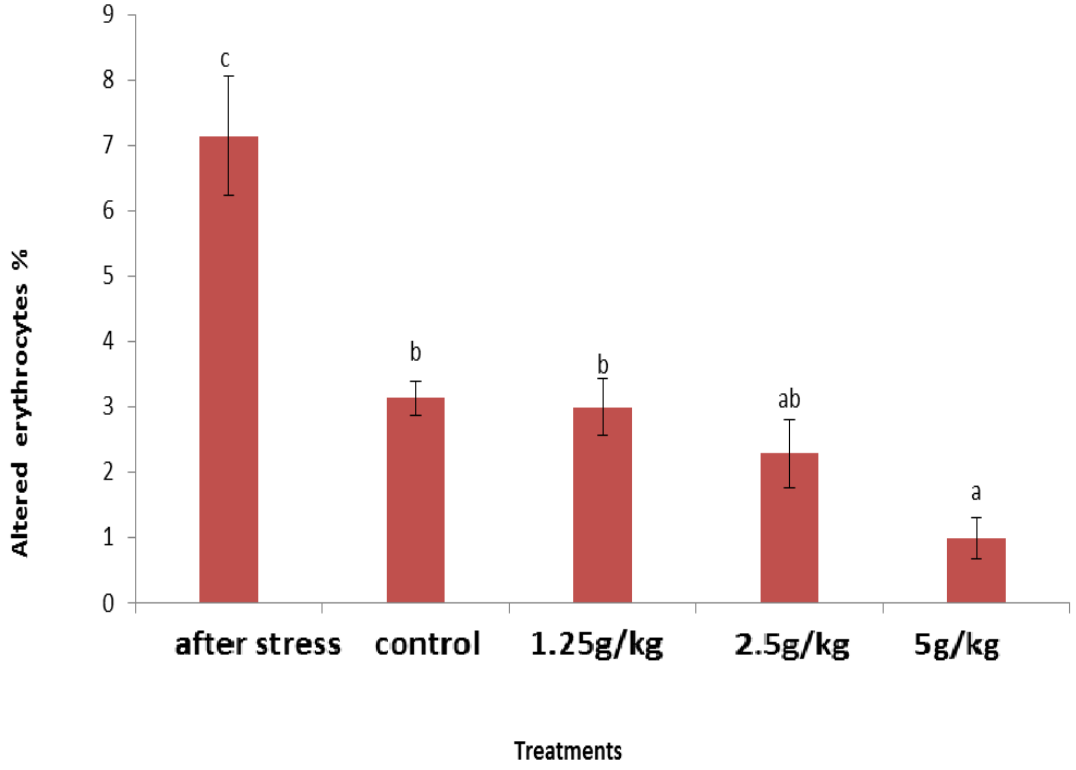

Figure1. Altered erythrocytes in the African catfish *Clarias gariepinus* fed experimental diets containing different levels of *Spirulina platensis* for 2 months.

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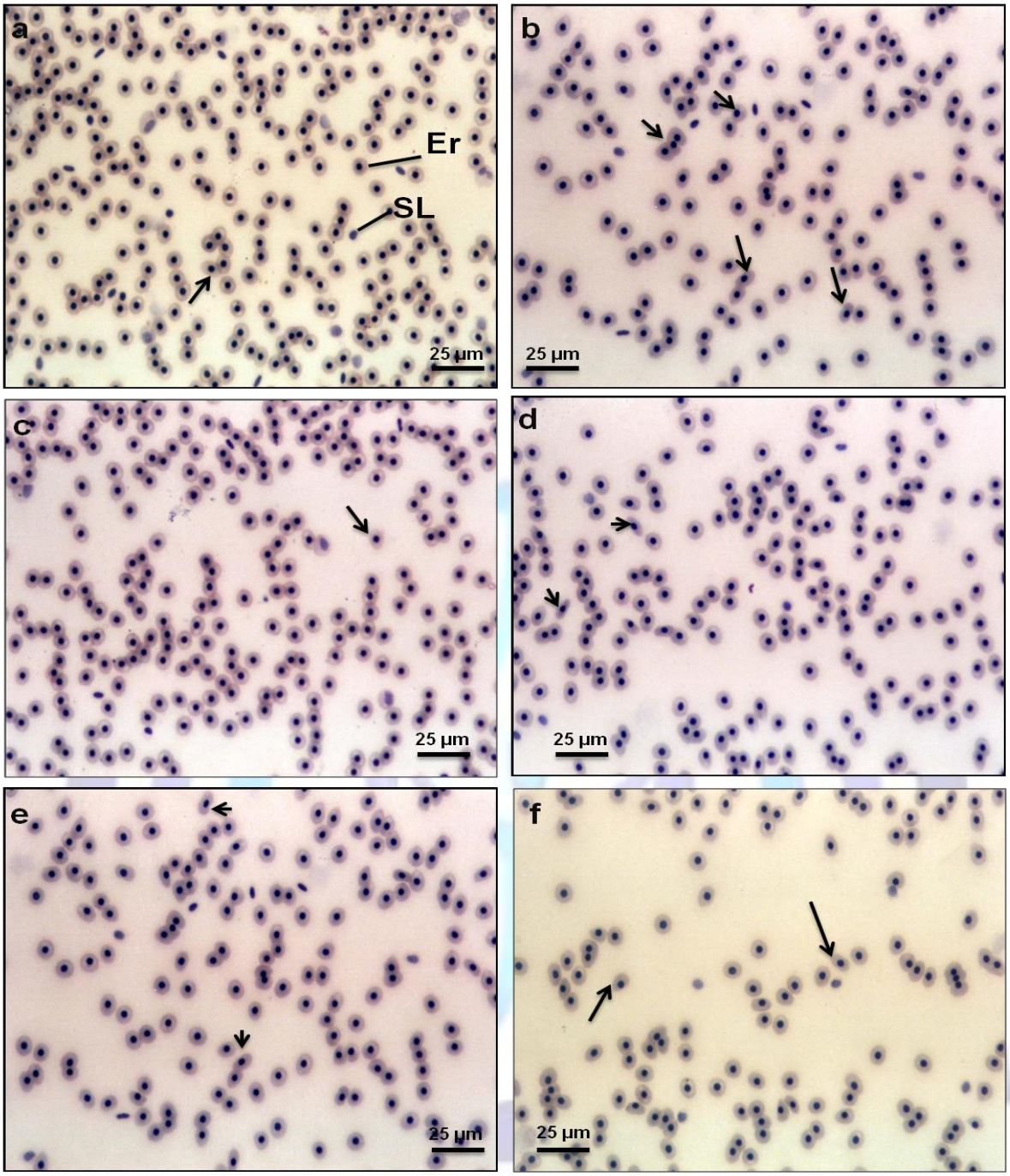

Figure 2. Blood smears of catfish *Clarias gariepinus*. (a & b) fish group after stress (fed 2% of wet body weight daily) for two months. (c) Control Fish group, (d) group exposed to 1.25 g *Spirulina*/kg diet, (e) group exposed to 2.5 g *Spirulina*/kg diet, (f) Fish group exposed to 5.0 g *Spirulina*/kg diet. Er; erythrocytes, SL; Small lymphocytes. Erythrocytes alterations were indicated by (arrows) (400×).