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HEPATIC ANTIOXIDANT STATUS AND HEMATOLOGICAL PARAMETERS IN AFRICAN CATFISH, *CLARIAS GARIEPINUS* JUVENILE EXPOSED TO SUBLETHAL CONCENTRATION OF *PSYCHOTRIA MICROPHYLLA*

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ABSTRACT

The present study was conducted to determine the hepatic antioxidant responses and hematological parameters in juvenile African catfish *Clarias gariepinus* exposed to sublethal concentrations of *Psychotria microphylla* leaf extract. Based on the 96 hr LC_{50} value of 0.69 mg/L, two sub-lethal concentrations of 35µg/L and 69µg/L and control were selected for exposure to fish. Samples of liver and blood were taken on day 1, 5, 10, and 15 for assessment of hepatic antioxidant responses and hematological parameters, respectively. Results indicated that *P. microphylla* leaf extract enhanced significantly ($p<0.05$) levels of lipid peroxidation, superoxide dismutase, and catalase but significantly reduced ($p<0.05$) glutathione reductase and glutathione peroxidase activities in the liver in both concentrations of the piscicide. There was decrease in the mean values of red blood cell count, hemoglobin, packed cell volume, mean cellular hemoglobin concentration but a mixed trend in the values of white blood cell, mean cellular hemoglobin and mean cellular volume. While glucose levels increased throughout the duration of the experiment, significant decrease ($p < 0.05$) was observed in protein values. *Psychotria microphylla* should be handled with adequate precaution to avoid possible ecotoxicological risk associated with its use.

Keywords: *Psychotria microphylla, Clarias gariepinus,* piscicide, hematology, antioxidants, lipid peroxidation.

INTRODUCTION

The extensive and indiscriminate use of synthetic pesticides to increase crop yield and maximize productivity in modern agriculture has resulted in serious environmental problems (El-Sayeed *et al*., 2013). Pesticides have been associated with problems of environmental contamination of water system, resistance, pest resurgence and detrimental effects on non-target organisms because of their non-degradability (Kavitha *et al*., 2012). Recently, the use of botanicals for pest control has gained attention as they are easily available, biodegradable, safe for mankind and leave no residues in the environment (Ramanujam and Ratha, 2008). Pesticidal plants including *Psychotria microphylla* (PM) are extensively used in various parts of the world as folk medicines (Devadoss *et al*., 2013) and for capturing fish (Obomalu *et al*., 2007). The genus *Psychotria* belongs to the family *Rubiaceae* with an estimated 1000 to 1650 species widely distributed in many countries of the world (Nepkroeff *et al*., 1999). *Psychotria microphylla* is one of the ichthyotoxic botanicals used to stupefy fish before cropping in ponds, lakes and rivers in Africa (Orji *et al*., 2014).

In Nigeria, there is paucity of information available on the hepatic antioxidant status and hematological parameters in tropical fish species exposed to botanical pesticide, *Psychotria microphylla*. Environmental contaminants including some plant extracts are known to modulate antioxidant defense systems and to cause oxidative stress through production of reactive oxygen species (ROS) (Dar *et al*., 2014). The ROS produced may react with biological macromolecules leading to tissue damage, lipid peroxidation, DNA damage and even cell death (Singh *et al*., 2014). Aquatic organisms including fish are however equipped with antioxidant defense systems such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and glutathione peroxidase (GPx) for neutralizing ROS. Blood parameters are considered as good pathophysiological indicator for assessing the overall health conditions of vertebrates particularly fish (Nwani *et al*., 2013). *Clarias gariepinus* is one of the most commercial food fish and cheap source of animal protein in Africa (USAID Markets, 2010). The present study was thus designed to investigate whether exposure to sublethal concentrations of *P. microphylla* leaf extract can alter the hepatic antioxidant status and hematological

parameters in the juveniles of African catfish *C. gariepinus*.

MATERIALS AND METHODS

Experimental fish: Fresh water African catfish *Clarias gariepinus* (Family: Clariidae, Order: Siluriformes) of average (±SD) weight and length 23.65 ± 1.18g and 13.44 ± 2.16 cm, respectively were procured from local outlets at Nsukka, Nigeria. They were subjected to a prophylactic treatment by bathing twice in 0.05% Potassium permanganate ($KMnO_4$) for 2 minutes to avoid possible dermal infections. They were acclimatized in the fisheries wet laboratory for 21 days under static systems in 300 L water capacity plastic tank. The fish were fed twice a day @ 3% body weight with locally pelleted diet (formulated from fishmeal, soya bean, maize meal, blood meal, premix vitamins and minerals) and containing 30% crude protein. The physicochemical characteristics of the test water following the standard method (APHA, AWWA, WPCE, 2005) were: temperature 25.65 ± 1.74°C, dissolved oxygen (DO) 7.75 ± 0.18 mg/L, pH 7.12 ± 0.40, conductivity 263 µSc/m, total alkalinity 26.80 ±3.70 mg/L and total hardness 16.74 ± 2.34 mg/L. Feeding of the fish was terminated 24 h before the commencement of the acute toxicity test. The ethical guidelines of the Institutional Animal Health Care Committee (UNN-ECAHCC, protocol no. 0620/2014) were strictly followed.

Plant material and preparation of extract: Fresh leaves of *Psychotria microphylla* were collected from Abakaliki, Ebonyi State, Nigeria. The plant was identified and authenticated by the plant taxonomist at the Department of Plant Science and Biotechnology, University of Nigeria Nsukka, Nigeria. The voucher specimen (UNN-PSB-01546/2014) was subsequently deposited in the laboratory for further reference. The leaves (50g) were shade-dried at room temperature and ground into fine powder using a mechanical grinder. The obtained powdered leaves were subjected to soxhlet extraction with 300 ml of purified water, since water extract of the leaves is normally used by the traditional fishing folk. Thereafter, the extract was filtered through Whatman filter paper (grade 1: 11µm) and concentrated into solid extract using a rotary evaporator (Stuart, Model RE-300, UK) at a temperature of 40°C. The extract obtained was stored in an air-tight container under refrigeration until used for the toxicity test.

Determination of sub-lethal concentrations and *in vivo* exposure experiment: The 96 hr LC_{50} value of *P. microphylla* extract on *C. gariepinus* in the present study was determined by probit analysis method (Finney 1971) to be 0.69 mg/L. Based on the 96 hr LC_{50} value obtained, two sub-lethal concentrations of 35µg/L (1/20th of LC_{50}) and 69µg/L (1/10th of LC_{50}) were selected for the *in vivo* experiment. The behavioral responses observed during the exposure period include rapid opercula and fin movements, skin discoloration, loss of equilibrium, frequent surfacing for air gulping, and attempt to jump out of water. A total of ninety fish from the acclimatized batch were used for the *in vivo* experiment. The test was conducted as a semi-static experiment in 40 L plastic aquaria (60 × 30 ×30cm) under photoperiod 12:12 light dark cycle. The fish were randomly divided into three groups of 30 fish without regard to sex. Fish in the first treatment group were exposed to tap water (control) while those in the second and third groups were treated with 35 and 69 µg/L of *P. microphylla* leaf extract, respectively. Each treatment group was further randomized into three replicates of 10 fish per replicate. The solution was renewed on alternate days to counter-balance decreasing pesticide concentrations. Liver and blood were sampled on day 1, 5, 10, and 15 for assessment of the hepatic antioxidant responses and hematological parameters respectively. The blood sample (0.5 ml) collected was stored in vials originally rinsed with heparin and used for estimation of hematological and biochemical parameters while the liver tissue (1.0 g) was quickly rinsed in cold 0.9% sodium chloride solution. The liver tissues from each triplicate experiment were pulled and homogenized in pre-chilled potassium phosphate buffer (1:10W/V, 0.1M, pH 7.0). One part of the homogenate was used for determination of thiobarbituric acid reactive substances (TBARS) and the other part was further centrifuged for 20 min at 10,500 g under 4°C to obtain the supernatant which was stored under 4°C for estimation of other antioxidant enzymes. A total of five determinations were made for each parameter and the average recorded as mean ± SE.

Estimation of lipid peroxidation and antioxidant enzymes: Lipid peroxidation (LPO) was determined by measuring the rate of thiobarbituric acid reactive substance (TBARS) formation (Sharma and Krishna-Murti, 1968). The specific activity was expressed in nanomoles of TBARS/mg protein. Tissue SOD activity was analyzed by measuring the inhibition of autoxidation of adrenaline at 420 nm, pH 10.2 at 30°C as described by Misra and Fridovich (1972). The SOD specific activity was expressed in units/min/mg of protein. GPx activity was measured by monitoring the rate of NADPH oxidation at 340 nm by the coupled reaction with glutathione reductase. The specific activity of GPx was determined using the extinction coefficient 6.22 Mm/cm (Lawrence and Burk, 1976) and expressed in unit/min/mg protein. Activity of CAT was measured using spectrophotometry by the decrease in absorbance at 240 nm resulting from H_20_2 consumption for 1 minute (Aebi, 1984). Enzyme specific activity was expressed as µM H_20_2 decomposed /min/mg protein.

Estimation of hematological and biochemical parameters: The red blood counts (RBC) and total leukocyte counts (WBC) were determined using Neubauerhemocytometer with Toisson's fluid as the diluting fluid for RBC and Turk's solution for WBC (Rusia and Sood, 1992). Determination of the hemoglobin level was done following the cyanmethemoglobin method (Blaxhall and Daisley, 1973). Hematocrit (PCV) was determined using the microhematocrit method (Nelson and Morris, 1989). Hematological indices (MCHC, MCH and MCV) were calculated from the results of Hb, RBC count and PCV (Dacie and Lewis, 1984) according to standard formulae:

$$\text{MCHC (g/dl)} = \frac{\text{Hb (g/dl)}}{\text{PCV (\%)}} \times 100$$

$$\text{MCH (pg/cell)} = \frac{\text{Hb (g/dl)}}{\text{RBC count in millions/mm}^3} \times 10$$

$$\text{MCV (fl/cell)} = \frac{\text{PCV (\%)}}{\text{RBC counts in millions/mm}^3} \times 10$$

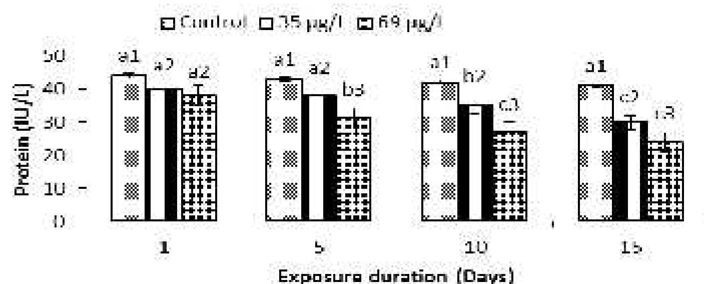
Total protein content was estimated spectrophotometrically by the Folin-Phenol reaction method as described by Lowry *et al*. (1951), using bovine serum as a standard. Glucose levels were estimated by the method described by Cooper and McDaniel (1970).

Statistical analysis: Data obtained were analyzed using the statistical package SPSS 17.0 computer program (SPSS Inc. Chicago, Illinois, USA). The data were subjected to two-way analysis of variance (ANOVA) followed by Duncan's multiple range tests to determine the significant differences at 5% probability level. Results were expressed as means ± standard error.

RESULTS

Effects on lipid peroxidation and antioxidant enzymes: The effects of different sublethal concentrations of *P. microphylla* leaf extract on lipid peroxidation in the form of TBARS formation and the responses of other antioxidant enzymes in the liver of *C. gariepinus* are presented in Table 1. Both sublethal concentrations of the piscicide increased the activities of LPO, SOD and CAT although the effects were somewhat different for each. In *C. gariepinus* exposed to 35 and 69µg/L, LPO in form of TBARS formation increased in the liver by a factor of 2.36 and 2.80 on day 1 and 2.72 and 4.99 on day 15 respectively. SOD enzyme activity responded in time and dose dependent manner with 46.41-88.01% increase at different exposure levels. Exposure of the fish to 69 µg/L of *P. microphylla* increased the activity of CAT by 37.69% on day 1 which further increased by 74.35% on day 15 compared to the control. There was however both concentration- and time-dependent decrease in GR and GPx in the fish exposed to both sublethal concentrations of *P. microphylla* throughout the duration of the experiment. On day 10 of exposure, GR activity reduced by 29.77% and 38.06% in fish exposed to 35 and 69 µg/L of *P. microphylla* and further reduced by 40.95% and 42.48% on day 15 at the same concentrations respectively. At 69 µg/L concentration, GPx activity reduced by 29.50% on day 1 and further declined by 41.25% on day 15 compared to the control.

Hematological and biochemical parameters: Changes in hematological parameters such as PCV, Hb, WBC, RBC, MCH, MCV and MCHC in *C. gariepinus* exposed to sublethal concentrations of *P. microphylla* leaf extract are presented in Table 2. There were appreciable reduction in the values of RBC counts, Hb, PCV, MCH and MCHC in fish exposed to both sublethal concentration of *P. microphylla* compared to the control. The WBC counts decreased in both *P. microphylla* concentrations on day 1 and 5 but significantly ($p < 0.05$) increased on day 10 and 15 of the exposure. For MCV, there was significant decrease in fish exposed to 35 and 69µg/L *P. microphylla* on days 15 and 10 respectively. Generally, there were no significant differences ($p > 0.05$) in the Hb, RBC, MCH and MCHC values between both sublethal concentrations of *P. microphylla* in the exposed fish throughout the duration of the experiment. The effects of *P. microphylla* on the plasma glucose and total protein are presented in Fig1a and b. There was decline in the level of protein in the exposed fish compared with the control throughout the duration of the experiment. There was however concentration and duration dependent significant ($p < 0.05$) increase in the level of glucose in *C. gariepinus* exposed to both sublethal concentrations of *P. microphylla* leaf extracts throughout the exposure period.



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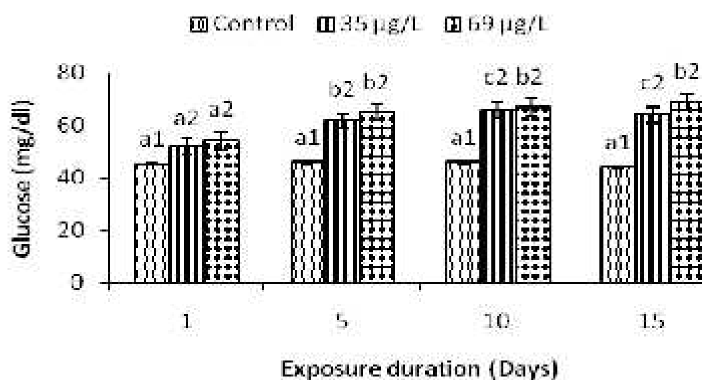

1b

Fig 1a and b. Changes in (a) plasma protein and (b) plasma glucose in *C. gariepinus* exposed to control and different concentrations of *P. microphylla* (35 and 69 µg/L) for 15 days. Different letters indicate significant difference ($p < 0.5$) in mean values among durations of exposure; different numerals indicate significant difference ($p < 0.05$) in mean values among concentrations

Table 1. Activity of lipid peroxidation (TBARS, nmol TBARS mg protein^{-1}), catalase (CAT, µmol min^{-1} mg protein^{-1}), superoxide dismutase (SOD, U mg protein^{-1}), glutathione reductase (GR, nmol mg protein^{-1}) and glutathione peroxidase (GPx, nmol min^{-1} mg protein^{-1}) in the liver tissue of *C. gariepinus* exposed to sublethal concentrations (35 and 69 µg/L) of *P. microphylla.*

		Duration (Days)			
Parameter	**Concentration (µg/L)**	**1**	**5**	**10**	**15**
LPO	Control	5.49 ± 0.32^{a1}	3.82 ± 0.18^{a1}	4.27 ± 0.41^{a1}	4.93 ± 0.22^{a1}
	35	12.93 ± 0.61^{a2}	12.83 ± 0.54^{a2}	10.68 ± 0.71^{a2}	13.42 ± 0.63^{a2}
	69	15.26 ± 0.55^{a3}	15.13 ± 0.43^{a3}	16.93 ± 0.91^{a3}	24.58 ± 0.83^{a3}
CAT	Control	10.40 ± 0.44^{a1}	11.06 ± 0.77^{a1}	10.10 ± 0.03^{a1}	11.07 ± 0.01^{a1}
	35	11.80 ± 0.51^{a1}	15.41 ± 0.33^{b2}	16.39 ± 0.04^{b2}	16.54 ± 0.02^{b2}
	69	14.32 ± 0.06^{a2}	17.34 ± 0.04^{b2}	18.33 ± 0.06^{b2}	19.30 ± 0.01^{b2}
SOD	Control	19.80 ± 0.69^{a1}	19.58 ± 0.68^{a1}	22.21 ± 0.76^{a1}	22.27 ± 0.81^{a1}
	35	25.24 ± 0.97^{a2}	22.23 ± 0.83^{b2}	32.21 ± 0.76^{c3}	38.27 ± 0.81^{c2}
	69	28.99 ± 0.78^{a2}	36.32 ± 1.11^{a3}	40.99 ± 1.14^{c3}	41.87 ± 0.98^{c2}
GR	Control	10.3 ± 0.45^{a1}	10.20 ± 0.67^{a1}	10.01 ± 0.89^{a1}	10.52 ± 0.93^{a1}
	35	9.41 ± 0.55^{a1}	8.04 ± 0.49^{a1}	7.03 ± 0.72^{a1}	6.20 ± 0.88^{b2}
	69	8.74 ± 0.71^{a1}	7.23 ± 0.43^{a1}	6.20 ± 0.91^{a2}	6.04 ± 0.48^{a2}
GPx	Control	24.07 ± 0.44^{a1}	25.03 ± 0.33^{a1}	25.09 ± 0.64^{a1}	22.01 ± 0.56^{a1}
	35	21.87 ± 0.67^{a1}	20.26 ± 0.63^{a2}	18.58 ± 0.94^{a2}	16.90 ± 1.10^{b2}
	69	16.97 ± 0.83^{a2}	15.91 ± 0.71^{a3}	13.86 ± 0.51^{a3}	12.93 ± 1.22^{b3}

Values with different alphabetic superscript (lower case) differ significantly ($p < 0.05$) between different exposure durations within the same concentration. Values with different numeric superscripts differ significantly ($p < 0.05$) between concentrations within the same duration.

Table 2. Effects of exposure to various sublethal concentrations of *P. microphylla* (35 and 69 µg/L) on RBC parameters in *Clarias gariepinus*

Parameter	Concentration (µg/L)	Duration (Days) 1	5	10	15
PCV (%)	Control	23.01 ± 0.86^{a1}	20.01 ± 0.94^{a1}	21.50 ± 0.70^{a1}	20.02 ± 0.63^{a1}
	35	21.50 ± 0.56^{a1}	20.01 ± 0.67^{a2}	20.01 ± 0.69^{a1}	18.02 ± 0.83^{a2}
	69	20.01 ± 0.35^{a1}	18.02 ± 0.23^{a2}	16.53 ± 0.33^{b2}	16.05 ± 0.44^{b2}
Hb (g/dl)	Control	7.15 ± 0.85^{a1}	6.83 ± 0.93^{a1}	7.25 ± 0.88^{a1}	7.80 ± 0.67^{a1}
	35	6.80 ± 0.93^{a1}	6.05 ± 0.31^{a1}	6.00 ± 0.22^{a1}	5.20 ± 0.23^{a1}
	69	6.01 ± 0.13^{a1}	6.00 ± 0.19^{a1}	5.01 ± 0.14^{a1}	5.05 ± 0.13^{a1}
WBC ($\times 10^4$ cell/mm^3)	Control	7175 ± 4.11^{a1}	6750 ± 2.14^{a1}	6500 ± 3.09^{a1}	6450 ± 6.23^{a1}
	35	6400 ± 4.11^{a2}	6550 ± 4.45^{a2}	6750 ± 3.31^{b2}	6625 ± 3.41^{b2}
	69	6675 ± 3.86^{a3}	6475 ± 4.45^{b3}	7600 ± 2.33^{c3}	6750 ± 3.62^{c3}
RBC ($\times 10^6$ cell/mm^3)	Control	5.61 ± 0.13^{a1}	4.51 ± 0.63^{a1}	4.19 ± 0.45^{a1}	4.12 ± 0.33^{a1}
	35	5.21 ± 0.23^{a1}	5.09 ± 0.40^{a1}	4.61 ± 0.36^{a1}	3.31 ± 0.33^{a1}
	69	4.57 ± 0.35^{a1}	4.37 ± 0.45^{a1}	4.07 ± 0.55^{a1}	3.17 ± 0.66^{a1}
MCH	Control	13.46 ± 0.28^{a1}	13.30 ± 0.36^{a1}	12.92 ± 0.07^{a1}	12.03 ± 0.87^{a1}
	35	10.95 ± 0.45^{a1}	10.01 ± 0.68^{a1}	9.30 ±0.93^{a1}	8.60 ± 0.87^{a1}
	69	10.66 ± 0.88^{a1}	9.76 ± 0.43^{a1}	8.53 ± 0.63^{a1}	8.03 ± 0.54^{a1}
MCV	Control	43.76 ± 0.23^{a1}	39.91 ± 0.18^{a1}	41.77 ± 0.14^{a1}	39.42 ± 0.18^{c2}
	35	37.84 ± 0.38^{a1}	41.45 ± 0.24^{a1}	39.22 ± 0.83^{a1}	37.18 ± 0.46^{c2}
	69	41.00 ± 0.71^{a1}	42.83 ± 0.53^{a1}	37.89 ± 0.44^{a2}	41.74 ± 0.45^{a1}
MCHC	Control	30.75 ± 0.71^{a1}	33.33 ± 0.82^{a1}	30.71 ± 0.93^{a1}	30.53 ± 0.37^{a1}
	35	28.94 ± 0.46^{a1}	27.29 ± 0.77^{a1}	30.36 ± 0.81^{a1}	30.00 ± 0.71^{a1}
	69	28.87 ± 0.31^{a1}	27.78 ± 0.21^{a1}	26.43 ± 0.54^{a2}	26.03 ± 0.67^{a1}

Values with different alphabetic superscript (lower case) differ significantly ($p < 0.05$) between different exposure durations within the same concentration. Values with different numeric superscripts differ significantly ($p < 0.05$) between concentrations within the same duration.

DISCUSSION

Several physiological and biochemical alterations have been reported in fish exposed to various botanical extracts (Devadoss *et al*., 2013; Audu *et al*. 2015). Results of the present study indicated that exposure to sublethal concentrations of *P. microphylla* leaf extract significantly enhanced levels of LPO in form of MDA in the liver of *C. gariepinus* thus reflecting oxidative stress and lipoperoxidation. The elevated values of LPO obtained are in agreement with previous reports in fish exposed to different ichthyotoxic botanicals (Piner and Uner, 2013; Thadani and Salunke, 2014). In order to deal with oxidative stress and associated tissue damage organisms are equipped with multiple systems of antioxidants such as CAT, SOD, GPx and GR to ensure optimum protection in the environment. The CAT-SOD enzyme system work cooperatively and provide the first line of defense in eliminating ROS. Under exposure to leaf extracts of *P. microphylla*, levels of CAT and SOD were significantly ($p < 0.05$) higher compared to the control throughout the duration of the experiment. The elevation in CAT and SOD as obtained in the present study could be adaptive mechanism against stress and thus suggest the ability of *P. microphylla* leaf extract to induce oxidative stress in *C. gariepinus*. Although CAT and SOD activities in the fish were elevated, they were probably not sufficient to remove ROS and neutralize oxidative stress as significant ($p < 0.05$) increase in LPO was observed throughout the experiment. GPx is a protective antioxidant that acts in cooperation with CAT to counteract the negative effects of ROS by scavenging out hydrogen peroxide and other peroxides (Dabas *et al*., 2011). Under exposure to *P. microphylla* leaf extract, GPx and GR activity in the liver of *C. gariepinus* were significantly reduced compared with the control throughout the duration of the experiment. Deb and Dutta (2013) also reported similar reduction in GPx and GR in rats administered with aqueous extracts of *Prunus persica* and *Clerodendrum colebrookianum*. Exposure of *C. gariepinus* to the two sublethal concentrations of *P. microphylla* leaf extract in the present study substantially decreased the values of RBC counts, Hb, PCV and MCHC. The reduction in these parameters may be attributed to the disorders in hemopoietic processes, defective Hb biosynthesis and accelerated disintegration or shrinkage of RBC cell membranes induced by *P. microphylla* leaf extracts on the erythropoetic tissue of the fish. The significant increase in the level of WBC count in the blood of *C. gariepinus* from day 10 of

exposure may indicate immune and protective defense mechanisms in response to *P. microphylla* intoxication. Similar increase in leukocyte counts have been reported in fish exposed to different plant extracts (Adewoye, 2010; Musa *et al*., 2013).

The decreased protein content as observed in *C. gariepinus* exposed to the leaf extract of *P. microphylla* may be due to their utilization for metabolic purposes or impairment in protein synthesis pathway by the presence of the alkaloid bioactive molecule emetine (Moller *et al*., 2007). Similar results were obtained in *Euphorbia royleana* and *Jatropha gossypifolia* lattices (Singh and Singh, 2002) and in *Cyprinus carpio* exposed to *Moringa oleifera* seed extract (Kavitha *et al*., 2012). The increased hyperglycemia noted in the *P. micropylla* exposed fish may be a typical stress response to the toxic plant extract (Orji *et al*., 2014). Ansari and Kumar (1998) attributed the hyperglycemia to the blocking of glucose receptors of pancreatic -cells by cholinesterase inhibitors. The leaves of some *Psychotria* species contain alkaloids such as psychotridine, brachycerine, N-dimethyl-tryptamine (DMT), beta-carbolines with opium-like analgesic effects and have been reported to be toxic and cause physiological impairment in fish (Fafioye, 2005).

In conclusion, although the aqueous leaf extract of *P. microphylla* may be valuable to stupefy freshwater fish before cropping, the present study demonstrated its effect on physiological, hematological and biochemical parameters in *C. gariepinus*. The discharge of *P. microphylla* and its extracts into nearby streams and ponds should be highly regulated to guide against ecotoxicological hazards that may arise. Further research on isolation, characterization and degradation kinetics of the bioactive ingredients in *P. microphylla* for effective use in medicine and aquaculture is thus recommended.

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