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Haematological and histoarchitectural alterations in *Cyprinus carpio* (Linnaeus 1758) fed with Sesbania leaf meal

G. Anand, P.P. Srivastava*, T. Varghese, N.P. Sahu, M. Xavier, V. Harikrskna, A. Prabhakaran and P. Kumari

¹ICAR-Central Institute of Fisheries Education, Mumbai-400 061, India*Corresponding Author Email : ppsrivastava@cife.edu.in

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Abstract

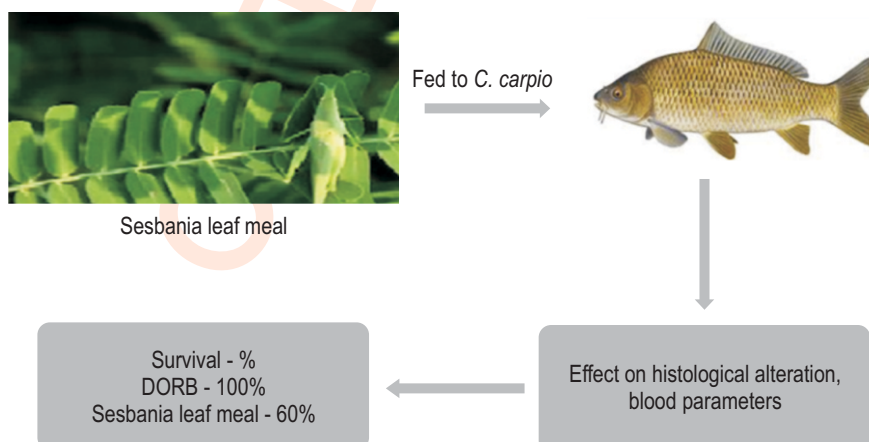
Aim: To determine the effect of feeding *Sesbania* leaf meal (SLM) in the diet of *Cyprinus carpio* (Common carp) in replacement of de-oiled rice bran (DORB)

Methodology: A 60-day-feeding trial was conducted by feeding common carp with diets containing 15% to 30% *Sesbania* leaf meal replacing DORB. Liver and intestine tissues were collected for histological examination, while blood was collected for estimation of blood count and serum metabolites (glucose, white blood cell (WBC), red blood cell (RBC), packed cell volume (PCV) and haemoglobin).

Results: Serum glucose value was significantly ($P < 0.05$) higher in the leaf meal fed groups compared to the control group. RBC and PCV value were highest in control group while WBC values were lower in the control group. Intestine and liver histology showed pathological alterations in *Sesbania* leaf meal fed groups compared to the control group.

Interpretation: The study indicated that raw *Sesbania* leaf meal had an obvious adverse effect on the health of *Cyprinus carpio* fingerlings. The mortalities observed due to leaf meal inclusion indicate their intolerance to the toxic factors present in the *Sesbania* leaf meal.

Key words: *Cyprinus carpio*, Histology, *Sesbania* leaf meal, Serum parameters



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Introduction

Aquafeed industry is heavily dependent on deoiled rice bran (DORB) as major ingredient. However, other animal feed industries are equally in demand for rice bran, which may reduce the availability of DORB for aquafeed in near future. Thus, there is a need to search for other plant ingredients as alternative sources to fulfil the increasing demand for aquafeed industry as animal sources are expensive and less available for the manufacture of fish feed (El-Sayed, 1999; Francis *et al.*, 2012). Till date, several studies have been conducted on different parts of various plant species which has been used as an alternative available protein source for aquafeed without compromising the nutritional quality of the aquafeed. Leaves of many plants such as *Medicago sativa*, *Leucaena leucocephala*, *Moringa oleifera*, sweet potato, *Morus esculenta*, *Manihot esculenta* and *Hygrophila spinosa* (Ali *et al.*, 2003; Bairagi *et al.*, 2004; Diarra *et al.*, 2017; Meshram *et al.*, 2018; Mondal *et al.*, 2012; Maiti *et al.*, 2019) have been incorporated in fish diets. However, at the same time, the significant constraints in the use of alternative plant ingredients were observed, especially negative impact on growth performance, digestibility and health status. (Kumar *et al.*, 2010; Hemre *et al.*, 2009; Hardy, 2010; Hansen and Hemre, 2013; Wang *et al.* 2016). In this context, the present study aimed to evaluate *Sesbania* leaf meal in aquafeeds, which is considered as a valuable plant in tropical agriculture (Sileshi *et al.*, 2003). They belong to family fabaceae and are grown in agricultural fields because of their ability for nitrogen fixation (Kareem and Sundararaj, 1967; Wood and Larkens, 1987). They are also used as fodder and green manure due to their high protein and low fibre content (Onim *et al.*, 1987; Panda *et al.*, 1988). Six species of genus *Sesbania* is distributed throughout India. Among them, *Sesbania aculeata*, which is commonly known as dhaincha, was selected for the study. Previously, some studies are available on *Sesbania aculeata* seed meal as a dietary protein source for common carp (Hossain *et al.*, 2001) and tilapia (Hossain *et al.*, 2002) diets.

As no study has been conducted on the incorporation of *Sesbania* leaf meal as an aquafeed ingredient till date, the present study undertook a feeding trial of common carp. Scaled variety of common carp, *Cyprinus carpio var. communis* was selected for the study due to its exceptionally high growth rate, easy availability, broad distribution and commercial importance (Mohapatra and Patra, 2013). Common carp, being an omnivorous species expected to have an ability for utilisation of unconventional plant protein sources. Haematological and histopathological analysis was performed to understand the effect of feeding *Sesbania* leafmeal on their health status. Nowadays, fish haematological studies are gaining importance because of their role in monitoring the health status of fishes (Hrubec *et al.*, 2000). Haematological characteristics have been studied in most fishes with the aim of establishing a reasonable value range and in case any variation from it may be considered

as an indicator for disturbances in their physiological process (Rainza-paiva *et al.*, 2000). Similarly, the histopathological studies will give a clear picture of the extent of tissue damage due to the presence of antinutritional factors in the plant sources. Thus, the present study aimed at evaluating *Sesbania* leafmeal as a replacer of the most common ingredient, DORB owing to its nutritional composition similar to the leaf meals with high fibre, low cost, presence of antinutritional factors and protein content below 30%.

Materials and Methods

Collection and processing of *sesbania* leaves: *Sesbania* leaves were collected from Samastipur district of Bihar, India. The collected leaf was washed thoroughly in order to remove dirt particles. After washing, the leaves were sun dried for two days. Further, the leaves were oven dried at 60°C for reducing the moisture content below 10%. Dried leaf was powdered and sieved through 40 µ mesh.

Analysis of antinutritional factors from *Sesbania* leaves : Antinutrients present in *sesbania* leaves were analysed following the standard methods. Saponin content of leaf was determined by the method of Hiai *et al.* (1976). Phytic acid was determined according to the colorimetric assay described by Gao *et al.* (2007). Total tannin content was estimated using spectrophotometric method as described by Makkar *et al.* (2007).

Diet preparation : Three different isonitrogenous and isoenergetic diets were prepared with 30% crude protein and 6.0% lipid respectively (Table 1). The maximum content of *Sesbania* leaf meal inclusion was decided to be 30%. As leaf meal was used as a rice bran replacer, this was selected depending upon the inclusion of DORB used in the aquafeed, which was also reported to be 30% (Kumar *et al.*, 2018). As 30% inclusion of leafmeal instead of DORB becomes 100% replacement, 50% replacement level was also selected (15% inclusion) to make another feed. Lower levels were not selected as it was meaningless to incorporate any cheap ingredient below 15%, considering the energy spent for its collection and processing. Three diets were designated as T1 (control diet with 0% SLM), T2 (15% SLM) and T3 (30% SLM). Prior to processing, all the feed ingredients including *Sesbania* leaf meal, were ground individually, independently weighed and mixed properly by adding sufficient water. The dough, thus made was subjected to palletisation through a die of size 2.5 mm and prepared noodle-like strands were cut into small pieces and dried properly. Diets prepared were subjected to proximate analysis by following the standards of AOAC (1995).

Experimental setup : The present study was conducted at the Wet Laboratory of Central Institute of Fisheries Education, Rohtak, India. Two hundred and twenty-five juveniles of common carp with the average weight of 15.9 ± 0.05 g were purchased from sampla village of Haryana. Fishes were acclimatised for

Table 1 : Composition of different experimental diets (% dry matter)

Ingredients	C (0% SLM)	SLM 15%	SLM 30%
DSBM	40	40	40
GNOC	21	15.6	10
Wheat flour	1.70	7.20	13
DORB	30	15	0
SLM	0	15	30
Vit - min mix	1.20	1.2	1.2
Choline chloride	0.20	0.20	0.20
CMC	1.50	1.5	1.5
BHT	0.1	0.1	0.1
Oil*	4.3	4.2	4.0
Proximate composition of feed (%)			
Moisture	8.92	9.01	9.05
Crude protein	32.83	33.02	33.29
Ether extract	5.92	6.04	5.80
Total ash	7.02	7.20	7.10
Crude fibre	7.54	7.14	6.81
NFE	46.69	46.60	47.00
Dry matter	91.08	90.99	90.95

*Oil-sunflower :fish-1:1. Note. BHT, butylated hydroxytoluene; CMC, carboxymethyl cellulose; DORB, de-oiled rice bran; DSBM, defatted soybean meal; GNOC, groundnut oil cake; NFE, nitrogen-free extract. (NFE = 100-(CP + CL + CF + ash); SLM, *Sesbania* leaf meal; vitamin mineral mix (PRE-EMIX PLUS) (quantity/kg) –Vitamin A- 5 500,000 IU; Vitamin D₃- 1 100 000 IU; Vitamin B₂-2000 mg; Vitamin E- 750 mg; Vitamin K-1000 mg; Vitamin B₆- 1000 mg; Vitamin B₁₂- 6 mcg; Calcium Pantothenate- 2,500 mg; Nicotinamide- 10 g; Choline Chloride- 150 g; Mn- 27 000 mg; I-1000 mg; Fe- 7500 mg; Zn- 5000 mg; Cu- 2000 mg; Co- 450 mg; L- lysine- 10 g; DL- Methionine- 10 g; Selenium- 50ppm

twenty days and were randomly distributed in circular FRP tanks of 500 l capacity at a stocking density of 15 fishes in each tank in triplicates. Fishes were fed twice a day at 8:00 and 18:00 hrs. During the acclimation period, fishes were fed with control diet (0% SLM). All the physico-chemical parameters of water were maintained throughout the experimental period as per the requirement of common carp.

Collection of blood and haematological analysis : After 60 days of feeding trial sampling was performed by following all the ethical guidelines of the animal cares for ICAR-Central Institute of Fisheries Education, Mumbai India. Fishes were anaesthetised before blood collection using clove oil (50 µl l⁻¹). Medical syringe (No 23) was used for puncturing the caudal vein which was previously rinsed with 2.7% EDTA for blood collection. The collected blood sample was used for haematological analysis (RBC, Hemoglobin, WBC, PCV). The haemoglobin level of blood was analysed following the Cyanmethemoglobin method using Drabkins Fluid (Qualigens, Mumbai, India). Blood (20 µl) was mixed with 5 ml of Drabkin's working solution. The absorbance was read on a spectrophotometer at 540 nm. The final concentration was calculated by comparing with the standard cyanmethemoglobin (Qualigens Diagnostics). Haematocrit value (PCV) was determined by drawing non-dotted blood by capillary action into microhaematocrit tubes. One end of the tubes was sealed with synthetic sealant. The sealed tube was centrifuged in a microhaematocrit centrifuge for 5 min at 10,500 rpm and the

values were recorded in microhaematocrit as percentage. WBC and RBC count was performed by using leucocytes and erythrocytes diluting fluids respectively (Qualigens). Blood (20 µl) was mixed with 3980 µl of RBC diluting fluid in a clean test tube. The mixture was shaken well to suspend the cells uniformly in the solution. A small drop of this mixture was charged to Neubauer's counting chamber of haemocytometer. The following formula was used to calculate the leucocytes and erythrocytes per ml of the blood samples: No. of cells/ml= (No. of cells counted × dilution)/ (Area counted × depth of fluid).

Serum samples were collected without using EDTA and these samples were allowed to clot for 2 hr then centrifuged (5000 g for 5 min at 4°C). Serum glucose was analysed by commercial kit (Erba®Diagnostic Mannheim, Transasia Biomedicals Ltd, Solan, HP, India) following the manufacturers' instructions, based on the glucose oxidase-peroxidase method.

Histological studies of liver and intestine : After 60 days of feeding trial, liver and intestine sample of each experimental groups (3 each) were dissected and were instantly fixed in neutrally buffered formalin (NBF) and embedded using paraffin wax. Tissue blocks, thus prepared were sectioned at 5 mm thickness with the help of microtome, and they were collected on glass slides. The sections were exposed hydration and dehydration cycles using varying concentrations of alcohol solution. The slides were then dewaxed using xylene and stained

with haematoxylin and eosin following the method of Roberts (1989). Tissue sections were examined under light microscopy and mounted with a coverslip. The photos were taken at different magnification (20X and 40X) and selected the appropriate ones under light microscope.

Results and Discussion

In the present study, the highest survival was recorded in T1 (control) group, and the lowest was observed in T3 (R30%) followed by T2 (R15%) SLM fed groups. The survival percentage was reduced drastically with the inclusion of *Sesbania* leaf meal. The toxicity of leaf meal could explain this mortality. Our study is supported by the report of Brown *et al.* (1987) and Shequeir *et al.* (1989) in which high mortality, low feed intake and growth depression were found when chicks were fed with *S. sesban*. This may be due to the presence of toxic compounds like saponin, tannin, amines and alkaloid sesbanine (Bell, 1978; Kinghorn and Smolenski, 1978). The analysis of antinutritional factors in the present study revealed that the saponin, phytic acid and tannin contents in *Sesbania* leaf meal was 18.12 ± 0.81 , 16.20 ± 1.20 and $14.10 \pm 1.01 \text{ g kg}^{-1}$, respectively.

In the present study, haematological and histopathological changes were observed after feeding *Cyprinus carpio* fingerlings with the feed containing *Sesbania* leaf meal in comparison to the control group fed without any leaf meal (Table 2). Similarly, Brown *et al.* (1987) and Shqueir *et al.* (1989) observed decrease in growth performance, low feed intake as well as mortality in growing broiler when they were fed with *Sesbania sesban* leaf meal. Haematological parameters are generally considered as important indicators for the evaluation of fish health (Gabriel *et al.*, 2004). The previous study conducted by Osuigwe *et al.* (2005) revealed that haematological parameters in fishes vary due several factors such as size, age, physiological status, environmental conditions as well as dietary regime. Blood profiles can be used to evaluate the physiological responses of fish. Stress responses in animals can be observed from the changes of cortisol, blood glucose, haemoglobin, and hematocrit levels. In the present study, RBC and PCV values decreased significantly ($P < 0.05$) with increasing the quantity of *Sesbania* leaf meal in the diet and the highest value was observed in the control group. The PCV value in the blood decreased due to the presence of toxic factors which showed an adverse effect on blood formation

(Oyawoye and Ogunkunle, 1998). However, a decrease in RBC value with the inclusion of *Sesbania* leaf meal was due to the presence of antinutritional factors such as tannin, saponin and alkaloids (Bell, 1978; Kinghorn and Smolenski, 1978).

Similarly, Bello *et al.* (2013) found a reduction in RBC count when fishes were fed with *M. oleifera* leaf meal-based diet. Douglass and Janes (2010) stated that the amount of WBC in blood have an implication in immune responses and the ability of animal to fight against the existing infection. In the present study, WBC count increased with inclusion of leaf meal as compared to the control group. This may be due to the feed toxicity caused by ANFs in *Sesbania* leaf meal. According to Oyawoye and Ogunkunle (1998), elevated WBC count is usually related to microbial infection or the presence of a foreign body or antigen in the circulating system. In the present study, blood glucose showed an increasing trend with increasing inclusion level of *Sesbania* leaf meal in the diet of *Cyprinus carpio*. According to Varghese *et al.* (2019), elevated blood glucose level is an indicator of stress due to metabolic alterations in carp exposed to hypoxia. However, *Sesbania* seed meal did not cause any metabolic stress in tilapia (Hossain *et al.*, 2002) which may be due to lower antinutrient content in the seeds as compared to leaves.

Intestine histology in *C. carpio* showed pathological alterations in *Sesbania* leaf meal fed groups compared to control groups (Fig. 1). Shorter villi and reduced length of intestinal fold were observed when fed with 15% and 30% SLM containing feed compared to the control group (DORB). The longer villi found in the intestine of control group indicates higher efficiency in the absorptive process resulting in better growth and production (Da Silva *et al.*, 2012; Caballero *et al.*, 2003). Furthermore, the enterocytes of these fish intestine showed an increase in the number of goblet cells and microvilli degeneration. The widening of central stroma, with high infiltration of inflammatory cells in the lamina propria and epithelium, were more evident in the group fed with 30 % SLM compared to 15 % SLM fed groups. The reduction in villi height results in reduced surface area for nutrient absorption (Da Silva *et al.*, 2012).

Furthermore, the intestinal histology revealed an increase in goblet cell number and microvilli degeneration with increase in the inclusion of SLM in the diet. The increase in the number of goblet cells may be an indication of increased irritation of brush border lining leading to more mucus production. This mucus serves as a lubricant providing protection against chemical and

Table 2 : Haematological paramters of *Cyprinus carpio* fed with *Sesbania* leaf meal based diet after 60 days of experiment

Treatments	PCV (%)	RBC ($10^6 \text{ cells mm}^{-3}$)	Hb (g dl ⁻¹)	WBC ($10^3 \text{ cells mm}^{-3}$)	Glucose (mg dl ⁻¹)
T1 (Control)	21.18 ^b ± 0.83	2.03 ^b ± 0.08	8.07 ± 0.33	124.13 ^a ± 5.37	36.26 ^a ± 2.25
T1 (15%R)	19.38 ^{ab} ± 0.52	1.76 ^a ± 0.04	7.83 ± 0.22	181.32 ^b ± 9.05	41.32 ^{ab} ± 1.88
T2 (30% R)	18.27 ^a ± 0.48	1.57 ^a ± 0.07	7.5 ± 0.23	221.36 ^c ± 8.85	44.47 ^b ± 1.68
p-value	0.045	0.009	0.372	0.001	0.064

Values are mean of six replicates ± SE. Values in the same column with different superscripts are significantly ($P < 0.05$) different, n=6. PCV, packed cell volume; RBC, red blood cell; Hb, haemoglobin; WBC, white blood cell

mechanical damage. The increase in goblet cell number may also be an immune response against anti-nutrients as reported by Marchetti *et al.* (2006). Further, saponin was present in the SLM which may have surface-active constituents that may damage biological membranes resulting in increased permeability of mucosal cells (Bureau *et al.*, 1998). Higher saponin concentrations in 30 % SLM may explain the negative effect of this

leaf meal in the digestive tract. Bureau *et al.* (1998) reported a reduction in weight and significant intestinal damage in Chinook salmon and rainbow trout fed with saponin containing diets. Similarly, they also reported infiltration of inflammatory leucocytes cells in the lamina propria and epithelium (Krogdahl *et al.*, 2000; Refstie *et al.*, 2000). Healthy hepatopancreatic histoarchitecture was seen in *C. carpio* fed with control diets (Fig. 2). Centrally

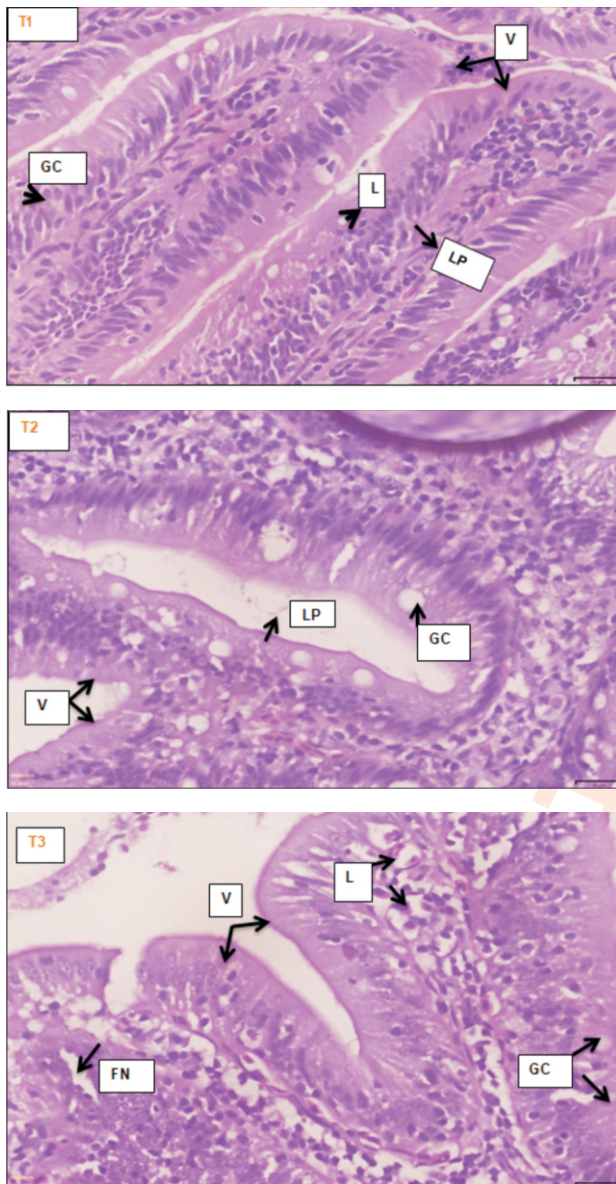


Fig. 1 : Intestine of experimental fish (*C. carpio*) fed feed. **T1**- Fed with 0% SLM showing normal architecture; **T2**- Fed with 15% SLM shows increased number of goblet cells, with slight leucocytes infiltration in the epithelium and in lamina propria and **T3**- Fed with 30% SLM showed significantly reduced length of villi and intestinal fold length as well with more leucocytes infiltration in the epithelium and lamina propria with focal necrotizing villi. Scale bar = 64 μ m (HE x 40). Here, LP- lamina propria, GC- goblet cells, V- villi, L- leucocytes, FN- focal necrosis.

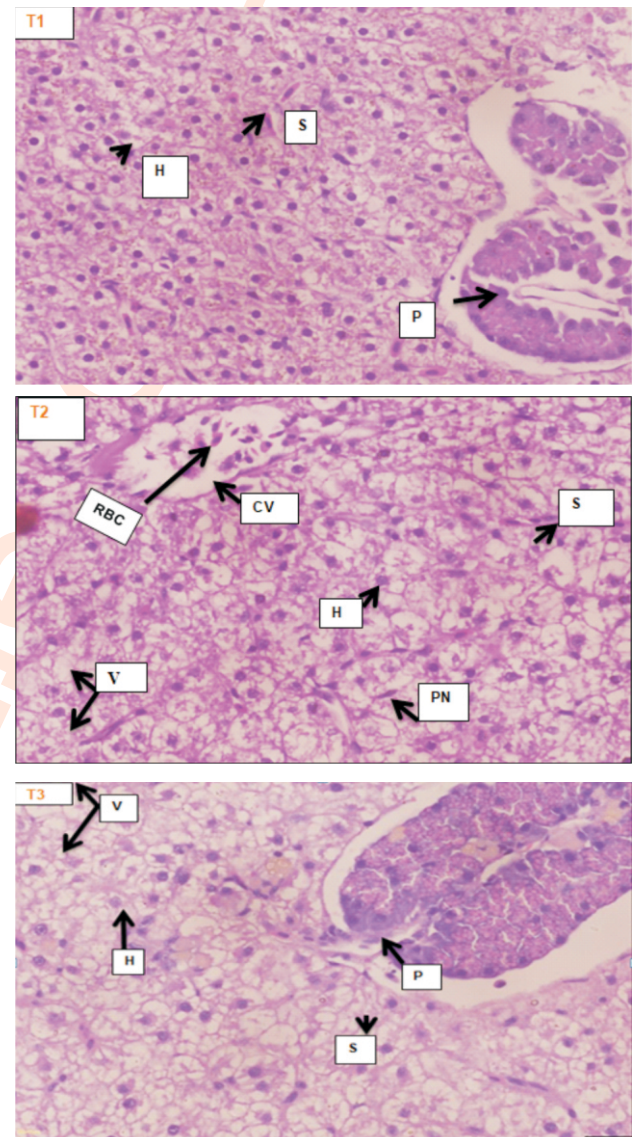


Fig. 2 : Histological section of liver of experimental fish (*C. carpio*) fed feed. **T1**- Fed with 0% SLM control group showing normal architecture of without any pathological alteration. **T2**- Fed with 15% SLM shows increased number of pyknotic nuclei with slight RBC infiltration in the CV and stroma of hepatocytes, vacuolization of hepatic cells and **T3**- Fed with 30% SLM feed with 30 % inclusion of SLM showed significantly increased vacuolization of hepatic cells, deposition of feed debris in the hepatocytes with hepatic cells degeneration. Scale bar = 64 μ m (HE x40). Here, S- sinusoids, H- hepatocyte, PN- pyknotic nuclei, CV- central vein, V- vacuolization, P- pancreas, D- debris deposits.

located nuclei and homogenous cytoplasm are the characteristics of hepatocytes. However, fish fed with SLM (15% and 30 %) showed atrophied nuclei with irregular staining of the cytoplasm. Hepatocyte degradation increased in fish fed with 30% SLM. Hepatocyte degradation may be an indication of compromised health because of nutritional imbalances (Mosconi-Bac, 1990).

Further, the increased degeneration and vacuolisation of hepatocytes of higher-level SLM fed group (30 % SLM inclusion) in the present study is concordance with the study of Tan *et al.* (2018) in *Epinephelus* species fed with high lipid diets. Vacuolisation of hepatic cells may represent a degenerative change when there is fluid distension associated with toxin handling by hepatocytes (Wolf and Wheeler, 2018). The inability of *C. carpio* to adequately digest the feed incorporated with 30% SLM inclusion compared to 15 % SLM included feed, and the control group may have caused higher levels of lipid deposition in hepatocytes, vacuolation and hepatocyte degradation in this fish is in agreement with the study of Hlophe (2014). The aberrant lipid accumulation in hepatocytes may be due to damage in the detoxification system of liver.

The results of this study indicate that the inclusion of *Sesbania* leafmeal had an apparent adverse effect on the health of *Cyprinus carpio* fingerlings as revealed by low survival rate in the *Sesbania* meal incorporated treatments. The deleterious effects are further confirmed by abnormal haematological indices and aberrated liver and intestinal histoarchitecture. This adverse effects are due to their intolerance to toxic factors present in the *Sesbania* leaf meal. Thus, the use of raw *Sesbania* leaf meal at 15% or more is not recommended in the diet of *Cyprinus carpio*, as a rice bran replacer. A further study is recommended by selecting lower percentage inclusion of 5 or 10% in order to just use as a minor ingredient. However, considering the potential of *Sesbania* leaf meal, further studies are suggested to evaluate the use of leafmeal in *Cyprinus carpio* diet after detoxification using different methods.

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