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Evaluation of polyherbal formulation on high energy and low protein diet induced fatty liver syndrome: implications on performance, carcass characteristics, biochemistry and liver histopathology in Cobb 430 broilers

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Abstract

Aim: The study was conducted to investigate the effect of polyherbal formulation (PHF) on performance and carcass characteristics, biochemical parameters and liver histopathology in Cobb 430 broiler chickens fed with high energy low protein diet (HELP).

Methods: 720 one-day-old chicks were randomly distributed into three groups, namely, normal control (basal diet), HELP control (diet to induce fatty liver syndrome) and PHF (HELP diet plus PHF (400 g/ton)).

Results: HELP diet exhibited a negative impact on performance indices (body weight gain - BWG, average daily feed intake - ADFI and feed conversion ratio - FCR) and carcass traits and increased the level of triglycerides ($p<0.05$) and total lipids in liver samples as compared to normal control group. HELP diet also induced fatty vacuolations, ballooning degeneration of hepatocytes in the liver. However, supplementation of PHF significantly improved the performance characteristics and liver triglycerides level. Similarly, histopathology of liver tissue showed normal hepatocytes with regenerative hyperplasia (restoration of hepatic parenchyma).

Conclusion: HELP diet induced a negative effect on production performance, liver fat, and histopathology. However, supplementation of PHF reversed these undesirable changes to normal with histological amelioration of fatty liver indicating the hepatoprotective function of PHF in birds fed with HELP diet.

Keywords: Chicken, fatty liver syndrome, HELP, polyherbal formulation

1. Introduction

High energy and low protein (HELP) diet is used to shorten the rearing period and increase the profitability in the poultry industry, however, it predisposes the broiler chickens to fatty liver syndrome (FLS)^[1] and high abdominal fat pad^[2]. FLS is a metabolic disorder that generally affects the fast-growing broilers fed HELP diet, which is caused by the deficiency of methyl group donors in feed and excessive fat accumulation in the liver accompanied by hepatic damage^[3]. This was supported by XiaoQuan *et al.* (2012)^[4] and Rozenboim *et al.* (2016)^[5], who suggested that a low protein and high-energy/high-fat diet is the predisposing factor for the induction of metabolic disorder in chickens. Therefore the dietary supplementation of choline becomes an appropriate nutritional strategy for the effective utilization of HELP diets, which in-turn prevents the adverse metabolic consequences in broiler chickens. Choline, a rediscovered vitamin B4, plays a critical role in fat metabolism in the liver. It prevents abnormal accumulation of lipid (fatty livers) either by promoting its transport as lecithin or by enhancing the oxidation of fatty acids in the liver^[6]. Choline is thus referred as a lipotropic factor due to its function of acting on fat metabolism by hastening the removal or decreasing the deposition of fat in liver. However, the drawbacks of synthetic choline chloride such as high hygroscopicity, acceleration of oxidative loss of vitamins, and the formation of trimethylamine (TMA) in the gastrointestinal tract of the birds^[7] made the researchers to search for natural alternatives. Hence the polyherbal formulation (PHF) indexed as Kolin

PlusTM (M/s Natural Remedies Pvt Ltd, Bengaluru, India) is known to have choline-like activity in soy protein isolate induced choline deficiency model [8], can be used as an alternative natural choline replacer for the prevention of FLS induced by HELP diet in broiler chickens. With this viewpoint, the present study was conducted to develop FLS in broiler chickens by feeding high energy and low protein (HELP) diet using performance and liver biochemical parameters, and liver histopathological observation as measurable indicators of FLS. In addition, the effect of PHF on performance parameters, carcass characteristics, biochemical parameters and liver histopathology was studied in HELP diet model in Cobb 430 broiler chickens.

2. Material and Methods

2.1 Polyherbal formulation

Kolin PlusTM is a proprietary polyherbal formulation developed by M/s. Natural Remedies Pvt. Ltd., Bengaluru, India, containing *Acacia nilotica* and *Curcuma longa* plant parts.

2.2 Ethical approval

The study was conducted by authorized, qualified and trained veterinarians, scientists, and technicians, in compliance with

the guidelines of the Institutional Animal Ethics Committee (IAEC) approved by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

2.3 Study design

A total of 720 one-day-old Cobb 430 broiler chicks weighing between 30 and 60 g were purchased from M/s. Venkateshwara Hatcheries Pvt. Ltd., Bangalore and healthy chicks were selected for the study. The experiment was carried out in the poultry research station, recognized by Department of Scientific and Industrial Research (DSIR), India; DSIR Reg No.: TU/IV-RD/2000/2016, located in Anniyalam, Tamil Nadu for a period of 42 days. The experiment was conducted using Completely Randomized Block Design (CRBD) with the location of poultry pens as a blocking factor. Chicks were randomly assigned to 3 groups with 6 replicates having 40 birds each, namely normal control (NC) fed basal diet, HELP control (fed HELP diet to induce fatty liver syndrome) and PHF (HELP diet plus PHF (400 g/ton)). The composition and specifications of the experimental diets are provided in Table 1.

Table 1: Composition of experimental diet (Kg/ton)

Type of Feed	Starter Feed (Day 1-21)		Finisher (Day 22-42)	
	Normal Control	HELP Control	Normal Control	HELP Control
Maize	577.5	624.5	640.5	655.5
Rice Polish	50.0	40.0	20.0	0.0
De-oiled Rice Bran	0.0	39.0	0.0	79.0
Soya Bean Meal	317.0	212.0	270.0	163.0
Calcite	8.0	9.0	8.0	8.0
Di-Calcium Phosphate	20.0	20.0	17.0	17.0
Oil	12.0	35.0	30.0	59.0
Methionine	2.4	3.0	2.3	2.8
Lysine	2.5	5.5	2.0	5.0
NSP Enzyme	0.5	0.5	0.5	0.5
Phytase 2500	0.2	0.2	0.2	0.2
Sodium Bicarbonate	2.0	2.0	1.5	1.5
Salt	3.0	3.0	2.5	2.5
Vitamin premix	0.5	0.5	0.5	0.5
Antibiotic	0.5	0.5	0.5	0.5
Anticoccidial	0.5	0.5	0.5	0.5
Toxin binder	1.0	1.0	1.0	1.0
Trace mineral Premix	1.0	1.0	1.0	1.0
Threonine	1.0	2.0	0.9	2.0
Carrier (calcite)	0.4	0.8	1.1	0.55
ME (Kcal) / CP %	3000 / 20	3150 / 16	3150 / 18	3300 / 14

2.4 Housing of birds

The chicks were housed in a semi-closed house being divided into pens with floor space of 60 square feet. The approximate size of the individual pen was 6'x10'x5' (Length x Width x Height). Each individual pen was provided with facilities for a brooder, bell drinker, chick feeder and/or jumbo feeder. Each chick was provided a floor space of 1 square feet. The size and floor space of the pen was modified as per the number of chicks housed in it with the help of PVC sheet or thermocol sheet.

2.5 Environmental conditions

The house temperature was maintained between 32 and 34 °C from days 1 to 7, and progressively reduced by 2 °C weekly until week 6. The house relative humidity (RH) was maintained between 40 and 70% throughout the study period.

The lighting was provided 24 hours daily during the first 3 days, 23 hours light and one-hour darkness between day 4 and 7, and 20 hours light and 4 hours of darkness till day 42.

2.6 Management of birds

Upon arrival, chicks were provided with 4% sugar-added water for first four hours to replenish the depleted energy and stimulate feed intake. The chlorinated potable drinking water (InnocleanTM, Natural Remedies Pvt. Ltd., 1 tablet/500 L) and poultry mash feed manufactured by Higain Feeds & Farms India Pvt. Ltd., Bangalore (starter feed from day 1 to 21, and finisher feed from day 22 to 42) were provided *ad libitum*. The chicks were vaccinated against Marek's disease immediately after hatching and then vaccinated against Newcastle disease (ND; LaSota strain) and infectious bursal disease (IBD) by eye drop on days 5 and 14, respectively.

2.7 Productive performance

The chicks in the individual pen were observed for clinical signs and mortality twice a day throughout the experimental period. The body weight of individual chicks was recorded using a weighing balance (Essae DIGI DS-450) on day 1 and thereafter weekly on days 7, 14, 21, 28, 35 and 42. Weekly feed consumption for each replicate of all groups was calculated by deducting the quantity of leftover feed from the total quantity of feed offered to the particular replicate of the group, and then average daily feed intake (ADFI) was calculated for the particular week. Thereafter weekly feed conversion ratio (FCR) was calculated as total feed consumption divided by body weight gain. European production efficiency factor (EPEF) was calculated as follows: Live weight in kg x Liveability in % x 100 / FCR x Age in days.

2.8 Carcass characteristics

At the end of the finisher phase, 15 birds from each group were randomly selected and weighed, and were then slaughtered by cervical exsanguination. Carcasses were eviscerated, and liver and abdominal fat were immediately weighed. Cleaned carcasses (without head, neck, and feet) and parts (breast and leg (thighs + drumsticks)) were then weighed, and all the values were expressed as mean of the 15 birds.

2.9 Determination of liver lipid profile

The liver sample was collected from 8 birds in each group and stored at -80°C until analysis of the lipid profile. The total lipid content in the liver tissue was determined by the Folch *et al.* (1957) [9]. In brief, 1 gram of liver tissue was homogenized with 20 mL of chloroform-methanol (2:1) mixture and centrifuged. The supernatant was mixed with 0.73% sodium chloride in the ratio of 5:1 and centrifuged. The lower organic phase consisting of lipids was transferred to a round bottom flask. After solvent evaporation, total lipid content was estimated by the gravimetric method. The residue obtained was dissolved in 1 mL of isopropanol and analyzed for triglyceride levels using commercial assay kit (Arkray, Surat, India) as per manufacturer's protocol.

2.10 Histopathology of liver

2.10.1 H&E staining

The liver sample was collected from 6 birds per group (1 bird/replicate) and fixed in 10% neutral-buffered formalin

until processing. The tissue processing consisted of dehydration in ascending grades of alcohol (70%, 80%, 90%, 95% and absolute alcohol) and cleared in xylene, followed by embedding and fixation in paraffin. Then the blocks were sectioned using sliding microtome (Leica, Wetzlar, Germany) and de-paraffinized in xylol followed by hydration in descending grades of alcohol (100%, 95%, 80%, and 70%) and distilled water. The sections were then stained with standard Haematoxylin and Eosin method, mounted using DPX mountant (S.D. fine-chem Ltd., Bengaluru, Karnataka, India) and observed under a microscope (Olympus Corporation, Tokyo, Japan) connected with the camera (DP20).

2.11 Statistical analysis

Data are expressed as mean \pm SEM. Because of completely randomized block experimental design (CRBD) was employed, the data thus generated were analysed using one-way analysis of variance (ANOVA) technique with replicate as a blocking factor to see whether blocking was effective at reducing the random error. In case of any significant differences among treatment ($p<0.05$), means were subjected to Dunnett's multiple comparisons test (IBM SPSS Statistics Version.21.0; SPSS Inc., Chicago, IL, USA) to draw a comparison between control and treatment groups for each investigational parameter and $p<0.05$ was considered as statistically significant.

3. Results

3.1 Productive performance

Body weight gain, ADFI, and FCR are summarized in Table 2 & 3. The body weight gain of birds fed HELP diet remain unaffected till day 14 regardless of supplementation. There was a significant decrease ($p<0.05$) in body weight gain observed in the HELP group compared to continuation.

NC group on days 21, 28, 35 and 42. However, supplementation of PHF significantly improved the body weight gain on days 21, 28, 35 and 42 as compared to HELP control. ADFI and FCR were significantly worsened in birds fed with HELP diet as compared to birds raised on the basal diet. However, supplementation of PHF significantly improved the ADFI (day 42) and FCR (days 28, 35 and 42) of birds fed HELP diet. Similarly, EPEF of HELP group was decreased as compared to the NC group, but it was increased by PHF supplementation.

Table 2: Effect of PHF supplementation on body weight gain in Cobb 430 broilers fed with HELP diet

Day	NC	HELP Control	PHF (400 g/ton)
7	92.92 \pm 0.77	86.70 \pm 0.70	87.20 \pm 0.80
14	337.48 \pm 2.14	292.84 \pm 2.23	298.14 \pm 2.33
21	***708.24 \pm 4.99	620.89 \pm 5.34	**645.01 \pm 5.41
28	***1175.36 \pm 8.30	953.30 \pm 8.70	***1015.69 \pm 8.49
35	***1627.67 \pm 9.43	1267.67 \pm 13.04	***1406.97 \pm 10.74
42	***2137.70 \pm 12.61	1623.79 \pm 19.48	***1878.25 \pm 14.26

NC-Normal control; HELP-High energy low protein diet; PHF-Polyherbal formulation; Data were expressed as mean \pm SEM; n= 237-240; *** $p<0.001$ and ** $p<0.01$ as compared to HELP control based on one-way ANOVA with location as a blocking factor followed by Dunnett's Multiple Comparison test

Table 3: Effect of PHF supplementation on ADFI, FCR and EPEF in Cobb 430 broilers fed with HELP diet

Day	NC	HELP Control	PHF (400 g/ton)
ADFI, g			
7	***16.917 ± 0.09	18.137 ± 0.28	17.929 ± 0.20
14	*32.726 ± 0.23	34.027 ± 0.18	33.753 ± 0.42
21	49.413 ± 0.48	49.518 ± 0.40	49.605 ± 0.39
28	***69.211 ± 0.37	65.845 ± 0.74	66.612 ± 0.60
35	***79.786 ± 0.50	74.431 ± 0.88	76.385 ± 0.47
42	***92.920 ± 0.61	83.477 ± 0.98	***88.153 ± 0.48
FCR, g/g			
7	0.851 ± 0.01	0.942 ± 0.01	0.932 ± 0.01
14	1.190 ± 0.00	1.398 ± 0.02	1.367 ± 0.01
21	***1.375 ± 0.01	1.556 ± 0.02	1.505 ± 0.01
28	***1.585 ± 0.01	1.842 ± 0.01	**1.759 ± 0.01
35	***1.679 ± 0.01	1.980 ± 0.01	***1.848 ± 0.01
42	***1.792 ± 0.01	2.097 ± 0.01	***1.938 ± 0.01
EPEF	289.14	189.8	233.66

NC-Normal control; HELP-High energy low protein diet; PHF-Polyherbal formulation; ADFI- Average daily feed intake; FCR-Feed conversion ratio; EPEF-European production efficiency factor; Data were mean ± SEM of six replicates of forty birds within each replicate; ** $p<0.001$ and ** $p<0.01$ as compared to HELP control based on one-way ANOVA with location as a blocking factor followed by Dunnett's Multiple Comparison Test

3.2 Carcass characteristics

HELP diet caused a significant ($p<0.05$) worsening of the carcass traits percentage (eviscerated carcass, abdominal fat, breast, and liver) and non-significant decrease ($p>0.05$) in leg and carcass yield percentage as compared NC group.

However, supplementation of PHF numerically improved the percentages of carcass traits (eviscerated carcass, breast, leg, liver, abdominal fat and carcass yield) as compared to the HELP group (Table 4).

Table 4: Effect of PHF supplementation on carcass traits in Cobb 430 broilers fed with HELP diet

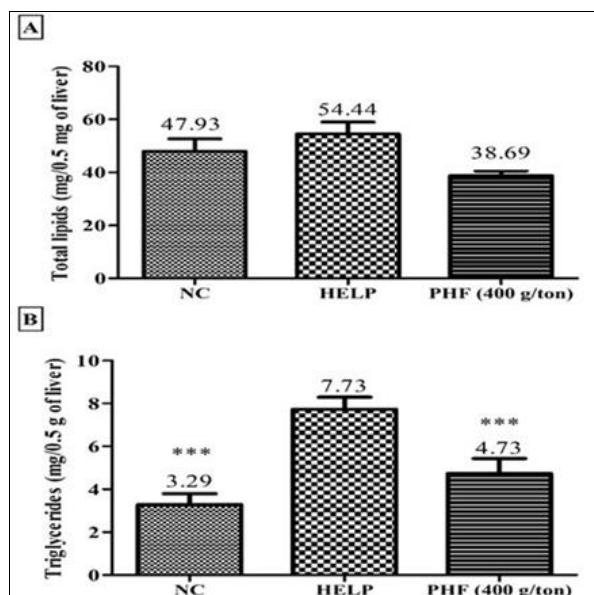
Carcass traits	NC	HELP Control	PHF (400 g/ton)
Eviscerated carcass (%)	**70.72 ± 1.00	67.26 ± 0.71	67.78 ± 0.50
Liver (%)	***1.70 ± 0.06	2.12 ± 0.06	2.02 ± 0.04
Abdominal fat (%)	***1.31 ± 0.10	1.97 ± 0.13	1.86 ± 0.10
Leg (%)	22.03 ± 0.51	20.33 ± 0.45	20.86 ± 0.39
Breast (%)	*26.45 ± 0.62	24.70 ± 0.32	24.92 ± 0.40
Carcass yield	73.73 ± 0.94	71.35 ± 0.71	71.66 ± 0.56

NC-Normal control; HELP-High energy low protein diet; PHF-Polyherbal formulation; Data were expressed as mean ± SEM; n=15; *** $p<0.001$, ** $p<0.01$ and * $p<0.05$ as compared to HELP control based on one-way ANOVA with location as a blocking factor followed by Dunnett's Multiple Comparison Test

3.3 Biochemical parameters

Triglycerides ($p<0.05$) and total lipids level in the liver were found to be increased in birds fed with HELP diet, however,

supplementation of PHF reduced the triglycerides ($p<0.05$) and total lipid levels in the liver as compared to HELP control (Figure 1).

**Fig 1:** Effect of PHF supplementation on liver lipid profile in Cobb 430 broilers fed with HELP diet. (A) Total lipids (B) Triglycerides; NC-Normal control; HELP-High energy low protein diet; PHF-Polyherbal formulation; All values are expressed as mean ± SEM; n=8; *** $p<0.001$ as compared to HELP control based on one-way ANOVA followed by Dunnett's Multiple Comparison Test

3.4 Histopathology of liver

The HELP control group showed extensive fatty vacuolations and ballooning degeneration of hepatocytes as compared to NC group which displayed a mild to moderate vacuolar degeneration and congestion with mild ballooning of hepatocytes. In contrast, mild fatty vacuolations with restoration of the hepatic parenchyma, and normal hepatic lobules were observed in birds supplemented with PHF (400 g/ton) (Figure 2).

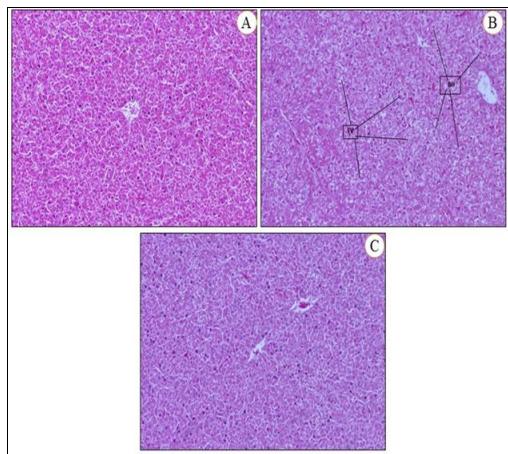


Fig 2: Histopathological staining (H&E) of liver tissues. Normal control (A): Liver showing mild to moderate vacuolar degeneration and congestion with mild ballooning of hepatocytes; HELP control (B): Liver showing ballooning of hepatocytes and fatty vacuolations (FV); PHF (C): HELP + PHF 400 g/ton feed: Liver showing mild fatty vacuolations with restoration of hepatic parenchyma, and normal hepatic Lobules

4. Discussion

FLS is a multifactorial disorder caused by the disturbances in the nutritional and metabolic factors, but the major predisposing factor is strongly associated with nutritional disturbances. This was supported by Trott *et al.* (2013)^[10] who reported that nutritionally over-fed laying hens (high energy) were at high risk for developing FLS, particularly in the spring and summer months. In addition, excess fat is removed from the liver by the lipoprotein, but its synthesis is limited by the availability of the protein moiety and the phospholipids components in birds^[11]. Thus, the deficiency of protein content in the diet can also cause FLS in birds. This statement was supported by several authors^[12-17] who used high energy and low protein diet to induce fatty liver syndrome in broilers and egg-laying or overweight backyard chickens. Hence in the present study, HELP diet was used to induce FLS in broiler chickens. It caused a significant negative impact on body weight gain (24%), ADFI, FCR (305g more feed per unit of body weight gain) and EPEF. Moreover, HELP diet increased the total lipids and triglycerides levels ($p<0.05$) in the liver which was evidenced from lipid infiltration in histopathological observations as well. This was substantiated by multiple authors who observed low feed intake and body weight,^[5] high abdominal fat weight^[18] and high liver weight^[4] in birds fed HELP diet. However, supplementation of PHF improved the performance parameters (body weight gain (15.6%) ($p<0.05$), ADFI and FCR (159g less feed per unit of body weight gain), liver triglycerides and total lipids levels in birds raised on HELP diet. The results of the present study were in agreement with the findings of several reports which stated that choline supplemented diets increased the growth performance^[19, 20] and decreased the liver fat content in broilers^[21-24]. Carcass

characteristics were significantly affected by HELP diet, but it was numerically improved by PHF supplementation. These results disagreed with Khosravinia *et al.* (2015)^[25] findings who reported no difference in the carcass yield of birds fed on diets with and without lipotropic agents. The histopathological examinations of the liver revealed the infiltration of lipid in birds fed with HELP diet while it was reversed in the birds supplemented with PHF. This was supported by Rozenboim *et al.* (2016)^[5] who observed high lipids vacuolization in low protein high- fat diet fed group, but Yalu Song *et al.*, (2017)^[26] found these abnormalities were alleviated by soybean lecithin (another form of choline) supplementation. These findings indicated the restoration of the liver to a fully functional status by dietary administration of PHF in broiler chicken raised on HELP diet. The liver is the principal organ in avian metabolism, but its function can be impaired by HELP diet. Thus, we have selected the polyherbal formulation that was already demonstrated to have a choline-like function in broilers^[8]. The individual plants such as *Acacia nilotica* and *Curcuma longa* used to prepare PHF are the rich source of polyphenols and curcuminooids respectively were proved to have hepatoprotective activity in rats. This statement was supported by Narayanan Kannan *et al.* (2013)^[27] who reported the hepatoprotective effect of *A. nilotica* on acetaminophen-induced hepatotoxicity in rats and Tranchida *et al.*, (2015)^[28] who found that the *C. longa* extracts played a significant role in liver fat metabolism. These findings strongly suggest that improvement in zootechnical parameters (body weight gain and FCR) and alleviation of liver histopathology changes and lipid content in the PHF supplemented group could be attributed to its hepatoprotective and lipotropic activity.

5. Conclusion

In conclusion, HELP diet caused growth depression, excessive abdominal fat deposition and fatty liver in broiler chickens. Further, it was confirmed that PHF at 400 g/ton has the potential to reverse the detrimental effects in broiler chickens induced by HELP diets, which were evidenced by the improved growth performance and feed efficiency, attenuation of excessive abdominal fat deposition and amelioration of abnormal histopathological changes in the liver.

6. Conflict of Interest

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

7. Acknowledgement

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