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Effect of Dietary Addition of Amla (*Emblica officinalis*) on Performance and HSP70 Gene Expression in Coloured Broiler Chicken during Extreme Summer

A.B. Mandal[1], Ram Kulkarni[2], J.J. Rokade[3]*, S.K. Bhanja[4] and Ram Singh[1]

[1]Division of Avian Nutrition and Feed Technology, ICAR-CARI, Izatnagar, U.P., INDIA
[2]Department of Poultry Science, College of Veterinary and Animal Sciences, Udgir, Maharashtra, INDIA
[3]Department of Avian Genetics Breeding, ICAR-CARI, Izatnagar, U.P., INDIA
[4]Section of Poultry Housing & Management, ICAR-CARI, Izatnagar, U.P., INDIA

**Corresponding author: JJ Rokade; Email: jaydeepvet@gmail.com*

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ABSTRACT

This study was designed to access performance of coloured broiler chickens (n= 112) fed diets with or without addition of *Amla* fruit powder during extreme summer (May-June, 38°C to 43°C). There were four dietary treatments with 0, 0.1, 0.2 and 0.3% *Amla* fruit dried powder respectively in broiler starting (0-3 wk) and finishing (3-6 wk) diets. Feed intake was lower (P<0.001) in broilers fed diets containing *Amla* fruit powder at any level in comparison to control. FCR during all phases improved (P<0.001) in treatment group compared to control and other dietary treatments. The feather loss, giblet, liver, gizzard, eviscerated yield and dressed yield differed significantly among various treatments. The yields of breast (P<0.01) and drum stick (P<0.001) increased at 0.1% or 0.2% level with reduction of cut of part-back yield (P<0.001). Cell-mediated immune response (CMI) improved (P<0.01) on addition of *Emblica* fruit powder. Lower levels of reduced glutathione (P<0.01) was estimated in treatment group and the values were lowest at 0.1% level. The m-RNA expression of HSP-70 in liver and bursa remain comparable while in spleen it was significantly down-regulated (P<0.001) on dietary addition of amla at 0.3% level (in comparison to control group) by a mean factor of 0.506. Use of Amla fruit powder 0.2% in diets was beneficial to improve FCR, CMI response while 0.3% was beneficial to improve HSP-70 expression during extreme summer.

Keywords: Coloured broiler, welfare, summer stress, *Emblica officinalis*

Various environmental stressors such as high ambient temperature and relative humidity influence the performance of broilers by reducing feed intake, feed conversion efficiency and hypophyseal-adrenocortical axis that in turn stimulates corticosterone which retarded growth (Dong *et al*., 2007). Reactive oxygen species (ROS) are generated at cellular level during normal bodily functions; however, high ambient temperature has been shown to increase the free radicals and other ROS production in body fluids and tissues. Their accumulation due to over-production or a decreased antioxidant defense, leads to damage of biological macromolecules and disruption of normal cell metabolism (Spurlock and Savage, 1993).

Dietary modifications are among the most preferred and practical ways to alleviate the effect of high environmental temperature in poultry. Furthermore, antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) play a vital role in protecting cellular damage from the harmful effects of ROS (Meister and Anderson, 1983). High ambient temperature depletes such antioxidants and induces oxidative stress. In addition to oxidative stress, marked elevation of temperature increases blood glucose and cholesterol concentrations (Altan *et al*., 2010). Non-enzymatic antioxidants such as vitamin C (Sahin *et al*., 2004; Sahin *et al*., 2003) and herbs containing different anti-stressor activity such as

Emblica officinalis have been used to protect tissues from superoxide radicals and enhance cell survival by stimulating anti-oxidative enzymatic systems. Amla (*Emblica officinalis*) or Indian gooseberry has been traditionally used for many chronic conditions including diabetes in ayurvedic medicine. It is rich in vitamin C (Sanjyal and Sapkota, 2011). Vitamin C has ability to sequester the singlet oxygen radical, stabilize the hydroxyl radical and regenerate reduced vitamin E back to the active state and to control reactive oxidant species formed during exercise (Kaminski and Boal, 1992). The active tannoid principles of *E. officinalis* are important hypolipidaemic agents that directly act upon the sympatho-adrenal axis and lower the synthesis of corticosterone (Sairam *et al.*, 2003). The hypolipidaemic effect of *E. officinalis* has been attributed to its potential in reducing lipid peroxidation. Impairment of immunological function in heat stress, such as T and B lymphocyte activity, has also been attributed to the effects of lipid peroxidation or oxidative damage in cell membranes (Pardue and Thaxton, 1986). Therefore, in the present experiment fruit powder of this plant was evaluated in coloured broilers during extreme summer months in the process of screening effective anti-heat stressors.

MATERIALS AND METHODS

All the experiments were carried out as per the code of practice approved by the Institute of Animal Ethics Committee at Central Avian Research Institute, Izatnagar, UP-24122, India (Permission no.: 452/01/ab/CPCSEA).

Experimental birds

Performance of colored broiler (CARI-DHANRAJA) chickens (n= 112, day-old) fed diets with or without addition of amla (*Emblica officinalis*) fruit powder during extreme summer (May-June, 38°C to 43°C) was assessed in open house rearing system. Fresh fruits were collected from the natural habitat away from the areal pollution, identified, authenticated and then were washed with distilled water. The fruits were then air dried under shed, powdered in an electrical grinder and stored in air tight container at room temperature for use. Four dietary treatments were prepared by adding different levels of dried fruit powder (0, 0.1, 0.2 and 0.3% of diet) in practical broiler starting (0-3 wk) and finishing (3-6 wk) diets (Table 1). Each diet was offered to 4 replicated groups of 7 birds each. The experimental birds were reared group wise in randomly allotted cabins of the electrically heated battery brooders with the provision of wire-mesh floor, feeder and waterer, located in the well ventilated room; with 24 hours light and uniform management following approved welfare practices. Weighed amount of each test diet used during starting (0-3 wk) and finishing (3-6 wk) period was offered daily in quadruplicate lot of 7 chicks each to ensure *ad libitum* feeding, but with care to avoid spillage and wastage. The fresh and wholesome water was always made available in suitable troughs to all the birds during the study period. Feed consumption was recorded on weekly basis while the body weight gain (BWG) was recorded on 21st (starting) and 42nd (finishing) days of age then feed conversion ratio (FCR, Feed: Gain) was calculated.

Table 1: Ingredients (%) and chemical composition of basal diet used during starting (0-3 wk) and finishing (3-6 wk) phases of the experiment

Ingredients	Starting (0-3 wk)	Finishing (3-6 wk)
Yellow maize	62.25	70.375
Soybean meal, solv.ext	34.2	26.4
Vegetable oil	0.3	0.00
Dicalcium phosphate	1.40	1.40
Limestone	1.20	1.20
Salt	0.3	0.3
DL-methionine	0.15	0.125
B-complex	0.02	0.02
Choline chloride	0.06	0.05
Trace mineral premix[1]	0.1	0.1
Vitamin premix[2]	0.1	0.1
Total	100.00	100.00
ME, kcal/kg	2951.175	3006.32
Crude protein	23.011	20.043

[1]Trace mineral mixture contained (mg/kg diet): KIO_3 2; $MnSO_4.H_2O$ 124; $CuSO_4.5H_2O$; $FeSO_4.7H_2O$ and $ZnSO_4.7H_2O$ 174.

[2] Vitamin premix supplied/kg diet Vit. A 8250 IU. Vit. D_3 1200 ICU, Vit. K 1 mg, Vit. E 40 IU, Vit. B_1 2 mg, Vit. B_2 5 mg, Vit. B_{12} 10 mcg, Choline 500 mg, Niacin 60 mg and Pantothenic acid 10 mg.

Sampling and analysis

Haematological and biochemical indices

These parameters were performed at the end of experiment (6th week age) in eight birds per treatment group. Packed cell volume (PCV,%) in whole blood was determined by capillary microhaematocrit method by centrifugation at 10000 rpm for 15 min. Haemoglobin concentration (g/dl) in the whole blood was estimated by cyanomethemoglobin method (Vankampen and Zinglstra, 1961). Blood smears were prepared from fresh blood was stained by Geimsa stain (1:9 Dilution for 45 min) for differential leucocyte count (DLC). Reduced glutathione (GSH) level in serum was estimated using the method (Lin Hu *et al*., 1988**)** with some modifications. Lipid peroxide level in serum was measured by determining the malondialdehyde (MDA) production using thiobarbituric acid (TBA) with slight modifications (Beuge and Aust 1978; Suleiman *et al*., 1996). Oxidative stress factors (LPO and GSH were multiplied and then divided by PCV) were then calculated.

Humoral and Cell mediated Immunity

The humoral immune response (haemagglutination-HA titre to sheep red blood corpuscles-SRBC) of broiler chicks fed diet with or without addition of *Emblica* fruit powder were analyzed (Siegal and Gross, 1980; Vander Zijpp, 1983). The cell mediated immune (CMI) response (foot web index to Phytohaemagglutinin, lectin from *Phaseolus Vulgaris*-PHAP) was studied on 21st day (Corrier and Deloach, 1990). For immune study, a total of 32 chicks (8 chicks/ treatment) were selected each for cell mediated and humoral immune-response. The weight of lymphoid organs (bursa of Fabricius and spleen) was taken at the end of experiment and expressed as per cent of live weight.

Heat shock protein 70 expression

Six experimental birds [three from control (0% amla) and three from dietary treatment (0.3% amla)] were scarified at the end of 6th wk. The liver and spleen tissues were collected aseptically and preserved for estimation of HSP 70 expression. Total RNA was isolated from the tissues (Liver and spleen) following established TRIzol method. The purity of RNA checked before the preparation of first-strand cDNA. Prepared cDNA stored frozen at -20°C and was used for the HSP 70 expression studies. Expression of HSP 70 was quantified by using specific primer pairs for genes of interest (GOI) in Real-Time PCR. GAPDH used as a reference gene. Oligo-nucleotide sequence of gene primers has been given in table 2.

Table 2: Oligo-nucleotide sequence of gene primers

Primer	Oligonucliotide sequence	Annealing Temp (°C)	Size (Bp)	Accession No.
HSP 70	F - GGCACCATCACTG GGCTT R-TCCAAGCCATAGGC AATAGCA	56°C	74	HM587997
GAPDH	F - GTGTGCCAACCCCCA ATGTCTCT R-GCAGCAGCCTTCAC TACCCTCT	65°C	45	NM-204305

The data pertaining to various parameters were analyzed statistically by the methods of (Snedecor and Cochran, 1983). The significant mean differences were attributed according to Duncan's multiple range test (DMRT) (Duncan, 1955). The mRNA expression levels (expression ratio) of genes of interest were analyzed by REST 2009 software. The difference in mean values was considered as significant at the level of 95% ($P < 0.05$).

RESULTS

The overall body weight gain due to dietary (Table 3) addition of *Emblica* fruit powder during 0-3, 3-6 and 0-6 wk of age remained statistically unchanged (1239 to 1289 g). No mortality was observed during experimental period at any treatment. Feed intake was significantly ($P<0.001$) reduced in starter (846 *vs* 730, 730, 757 g/bird), finisher (1799 *vs* 1676, 1506, 1716g/bird) and overall growth phase (2627 *vs* 2406, 2236, 2474 g/bird) in the broilers fed with diet containing 0, 0.1, 0.2 and 0.3% *Emblica* fruit powder

Table 3: Growth performance of broilers on dietary addition of Amla at different level

AMLA (% of diet)		T1 0%	T2 0.1%	T3 0.2%	T4 0.3%	SEM	P value
BWG (g)	0-3 wk	438.4	447.9	439.2	442.9	4.49	NS
	4-6 wk	850.9	813.3	825.9	796.4	10.91	NS
	0-6 wk	1289.3	1261.3	1265.1	1239.3	14.06	NS
FI (g/b)	0-3 wk	846.8^{a}	730.3^{c}	730.5^{c}	757.4^{b}	5.72	P<0.001
	4-6 wk	1779.8^{a}	1675.9^{c}	1506.4^{d}	1716.6^{b}	10.66	P<0.001
	0-6 wk	2626.5^{a}	2406.1^{c}	2236.9^{d}	2474.1^{b}	15.15	P<0.001
FCR (kg feed/ kg gain)	0-3 wk	1.95^{b}	1.64^{a}	1.68^{a}	1.73^{a}	0.02	P<0.001
	4-6 wk	2.15^{b}	2.09^{b}	1.85^{a}	2.18^{b}	0.03	P<0.001
	0-6 wk	2.07^{c}	1.93^{b}	1.78^{a}	2.01^{bc}	0.02	P<0.001

Values bearing different superscripts differ significantly (P<0.05) NS= Non significant

Table 4: Carcass trait and cut-up parts of broilers on dietary addition of Amla at different level

AMLA (% of diet)		T1 (0%)	T2 (0.1%)	T3 (0.2%)	T4 (0.3%)	SEM	P value
Carcass trait (% live weight)	Shrinkage	3.92	2.65	3.52	3.88	0.21	NS
	Blood loss	1.96	2.88	2.78	2.52	0.17	NS
	Feather loss	7.06^{a}	4.32^{a}	5.10^{bc}	5.98^{ab}	0.29	P<0.001
	Giblet	4.73^{b}	5.28^{ab}	5.66^{a}	4.86^{b}	0.13	P<0.031
	Heart	0.54	0.49	0.49	0.48	0.02	NS
	Liver	1.94^{b}	1.93^{b}	2.56^{a}	2.14^{b}	0.08	P<0.011
	Gizzard	2.25^{b}	2.86^{a}	2.61^{ab}	2.24^{b}	0.09	P<0.023
	Eviscerated yield	69.51^{ab}	70.86^{a}	68.76^{b}	68.30^{b}	0.34	P<0.027
	Dressed yield	74.25^{b}	76.14^{a}	74.42^{b}	73.16^{b}	0.32	P<0.004
Cut of parts	(% BW) Breast	14.96^{bc}	16.58^{a}	15.84^{ab}	14.50^{c}	0.26	P<0.010
	Drum stick	9.26^{b}	11.16^{a}	11.56^{a}	9.63^{b}	0.29	P<0.003
	Thigh	10.28	10.36	10.27	9.79	0.17	NS
	Wing	9.08	9.46	9.46	9.18	0.11	NS
	Neck	3.68^{b}	4.88^{a}	4.97^{a}	3.31^{b}	0.24	P<0.016
	Back	21.98^{a}	18.09^{b}	16.52^{b}	21.64^{a}	0.56	P<0.001

Values bearing different superscripts differ significantly (P<0.05) NS= Non significant

respectively. The feed conversion ratio (kg feed per kg weight gain) during 0-3 wk, 3-6 wk and 0-6 wk phase (1.93, 1.78, 2.01 *vs* 2.07) were better (P<0.001) in diet with 0.2% *Emblica* fruit powder compared to other dietary treatments including control. Carcass traits *viz*. shrinkage-loss, blood loss and relative weight of heart remained comparable but feather-loss, giblet, liver, gizzard, eviscerated yield and dressed yield differed among various treatments (Table 4). The yields of wing and thigh remained similar but that of breast (P<0.01), drum stick (P<0.001) and neck (P<0.01) increased with reduction of back yield (P<0.001) on dietary addition of *Emblica* fruit powder. The humoral immune response against sheep red blood cells, relative weights of bursa and spleen (Table 5) remained comparable, but cell-mediated immune response improved (P<0.01) on dietary addition of *Emblica* fruit powder at any level

Table 5: Immune organ weight and immune response of broilers on dietary addition of Amla at different level

AMLA (% of diet)		Control T1 (Mean)	0.1% T2 (Mean)	0.2% T3 (Mean)	0.3% T4 (Mean)	SEM	P value
Immune organ weight (%BW)	Bursa	0.07	0.09	0.06	0.06	0.01	NS
	Spleen	0.19	0.16	0.25	0.16	0.02	NS
Immune response	CMI	0.22^{a}	0.31^{b}	0.29^{b}	0.29^{b}	0.01	P<0.01
	HA	3.17	5.15	4.56	5.74	0.44	NS

Table 6: Oxidative stress profile of broilers on dietary addition of amla at different level

AMLA (% of diet)	Control T1 (Mean)	0.1% T2 (Mean)	0.2% T3 (Mean)	0.3% T4 (Mean)	SEM	P value
Hb (g %)	13.82	15.94	14.08	14.37	0.65	NS
LPO (n mole/ml)	59.99	57.91	39.57	43.22	4.32	NS
GSH (mg% of blood)	22.66^{a}	19.36^{b}	22.43^{a}	18.59^{b}	0.59	P<0.01
OSF (LPOXGSH/PCV)	33.29	24.39	23.35	19.07	3.01	NS
H:L ratio	0.43	0.41	0.38	0.36	0.01	NS

Values bearing different superscripts differ significantly (P<0.05) NS= Non significant

owing to the adaptogenic and immunomodulator potential of the polyherbal formulation and vitamin C.

The haemoglobin level, lipid peroxidase activities, oxidative stress factor and H: L ratio (Table 6) remained comparable but lower levels of reduced glutathione (P<0.01) was estimated in broiler chickens fed diets with *Emblica* fruit powder being lowest at 0.1% level. The mRNA expression of HSP-70 in liver and bursa remain comparable to control group (Table 7). However, the mRNA expression level of HSP-70 in spleen was significantly down-regulated (P<0.001) in dietary supplemented amla at 0.3% level (in comparison to control group) by a mean factor of 0.506 (Table 7 and Fig. 1). Expression of HSP70 is tissue and age specific.

DISCUSSION

Reports on *Amla* incorporation in broiler diet were scarce hence discussions were made with vitamin C as when required. *Emblica officinalis* belongs to Citrus group, which is rich source of ascorbic acid (Vitamin C) (Jain and Khurdiya, 2002). Findings of the present study was in line with earlier reports (Tuleun and Njoku, 2000; Pena *et al*., 2008; Patil *et al*., 2014) who also reported no beneficial effect of ascorbic acid/ amla supplementation on weight gain in broilers as an anti-stress agent during high environmental temperature. In contrary reports (Njoku, 1986; Vathana, 2002) were shown an increased weight gain in broilers fed ascorbic acid supplemented diet. The incorporation of *Emblica* at 0.4 and 0.8% resulted in higher body weight gain in broilers than the respective un-supplemented group (Patel *et al*., 2016). Similar positive observations in broilers were also reported by (Patil *et al*., 2014; Kumar *et al*., 2012; Kumar *et al*., 2013). The higher body weights observed in *E. officinalis* supplemented groups may be attributed to anabolic and antioxidant effect of ascorbic acid, gallic acid and tannic acids present in *E. officinalis* (Mcdowell, 1989). The insignificant effect of *Emblica* in the current study might be due to lesser level of incorporation compared to the earlier levels. The reduction in broiler feed intake fed with *Emblica* was also observed (Tuleun and Njoku, 2000; Lin *et al*., 2003). While, no effect of amla or ascorbic acid supplementation on feed consumption was also reported (Kumar *et al*., 2013; Abdel Raheem and Ghaffar, 2004). In contrary, significantly higher feed consumption was also recorded (Patil *et al*., 2014; Jadhav, 2005). The results of this experiment

Table 7: Effect of dietary addition of amla fruit powder at 0.3% level on expression of HSP 70 in broiler chicken

Tissue	Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P (H1)	Result
Spleen	HSP70	TRG	1	0.506	0.392-0.650	0.347 – 0.70	P<0.001	DOWN
	GAPDH	REF	1	1				
Liver	HSP70	TRG	1	1.072	0.683-1.579	0.654-1.692	P<0.649	NS
	GAPDH	REF	1	1				
Bursa	HSP70	TRG	1	0.742	0.483-1.560	0.458-1.690	P<0.375	NS
	GAPDH	REF	1	1				

are in agreement with available reports (Vathana, 2002; Njoku and Nwazota, 1989), who reported improved feed conversion with ascorbic acid during high temperature. The incorporation of *E. officinalis* in broiler diet tends to positively alter the yield composition of the broiler carcass. However, the dietary supplementation of ascorbic acid did not cause any significant differences among the analyzed treatments on carcass and cut-up part yields of 33-day-old birds (Pena *et al*., 2008).

Apart from CMI response to PHA-P, the humoral immunity and immune organs weights were remained unaffected by *E. officinalis* feeding. The present findings was in line with earlier works in broilers (Lin *et al*., 2003; Abdel Raheem and Ghaffar, 2004) who also reported that improved immune function with dietary addition of ascorbic acid during heat stress. The ascorbic acid can improve immunity of bird during heat stress as compared to the birds exposed to heat stress without supplementation (Farooqi *et al*., 2003). Ascorbic acid also takes part in the synthesis of leukocytes especially phagocytes and neutrophils which play a part in the defense system of the chickens (Null, 2011).

The hemoglobin (g%), heterophil: lymphocyte ratio and lipid peroxidase enzyme activity (n mol/ml) were remained unaffected by different levels of amla feeding. The observations of present study were similar to previous studies where no change in H: L ratio due to dietary addition of ascorbic acid was reported (Campo and Davila, 2002). In contrary of present findings, increased haemoglobin concentration in broiler (Njoku and Nwazota, 1989) reported with dietary addition of ascorbic acid. A decrease in lipid peroxidation was recorded when dietary supplementation of antioxidant herbs e.g. active tannoid principles of *E. officinalis* (Bhattacharya *et al.,* 1999). The practical poultry production imposes multiple and concurrent stressor effects and reported that heterophil: lymphocyte ratios increased linearly and additively as a function of the imposition of single or multiple stressors (McFarlane *et al*., 1989a; McFarlane *et al*., 1989b). Ascorbic acid supplementation showed positive effects on the reduction of heterophil/lymphocyte ratio in birds exposed to heat stress (33°C) and supplemented with 150 and 300 ppm ascorbic acid in the diet (McKee and Harrison, 1995). Feeding of broilers with 0.5% of amla in combination with 0.5% graph seed powder reduced the plasma cortisol levels in broiler breeders (Priya *et al*., 2010).

High ambient temperatures can potentially induce patho-physiological alterations spatially in gastrointestinal tract during heat stress. Heat stress lead to expression of a small group of highly conserved polypeptides, known as heat shock proteins (HSP) in different tissues, by prokaryotic and eukaryotic cells is clearly observed (Leandro *et al*., 2004). HSP are thought to play a role in cellular protection under high ambient temperature, with a proposed relationship between the development of thermo-tolerance and HSP synthesis, especially HSP70 (Lindquist and Craig 1988). Exposure of chicken to high temperature increased the induction of HSP70 in brain (Zulkifli *et al*., 2009), liver (Mahmoud and Edens, 2003), lungs and heart (Mahmoud and Edens, 2005) and in quail ovary and brain (Sahin *et al*., 2009). The present findings got support from earlier work, where supplementation

of ascorbic acid leads to decreased HSP70 expression compare to control non-supplemented group under heat stressed environments (Mahmoud *et al*., 2003). The mechanism of this beneficial effect is not yet clear, but it is believed that supplementation of amla enhance potentially release of bioactive substances that could prevent oxidative damage and ultimately lowers expression of HSP. Also dietary ascorbic acid supplementation reduced the circulating levels of corticosterone as well as serum cholesterol in the heat-stressed chickens this could result in a decrease in the HSP70 expression (Pardue and Thaxton 1986; Wang and Edens, 1993; Mahmoud *et al*., 2004).

CONCLUSION

Finally it is concluded that, Amla (*Emblica officinalis*) fruit powder 0.2% in diets of coloured broiler chickens (0-42 d of age) was beneficial to improve feed conversion efficiency, cell-mediated immune response while 0.3% was beneficial to improve HSP-70 expression during extreme summer.

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CONFLICT OF INTEREST

The authors do not have any conflict of interest.

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