Biotechnology in Animal Husbandry 26 (5-6), p 361-381, 2010 ISSN 1450-9156
Publisher: Institute for Animal Husbandry, Belgrade-Zemun UDC 636.5
DOI:10.2298/BAH1006361S

AMELIORATION OF OXIDATIVE STRESS IN BROILERS DURING SUMMER

V. Sujatha[1], J. Pandurang Korde[2], S. K. Rastogi[1], A. K. Madan[1], S. Maini[3], K. Ravikanth[3]

[1]Department of Animal Physiology, College of Veterinary and Animal Sciences, Gobind Ballabh Pant University of Agriculture and Technology (GBPUAT), Pantnagar, Uttaranchal, India.
[2]Department of Animal Physiology, College of Veterinary and Animal Sciences, Seminary Hills, Nagpur, Maharashtra, India.
[3]Research and Development Division, Ayurvet Limited, Baddi, H.P. India, (M.VSc from GBPUAT, Pantnagar, India)
Corresponding author: shivi@ayurvet.in, drshivi29@yahoo.com

Abstract: A comparative study on antistressor and antioxidative effects of synthetic vitamin C and polyherbal feed premix Ayucee supplementation in broilers was conducted during the summer months of June-July when the mean temperature-humidity index was 84.74±2.51. Day old broiler chicks (n =60) were randomly divided into three groups. Control group I was given basal diet and treatment groups (II&III) were supplemented with Synthetic vitamin C (100g/tonne of feed) and Ayucee (100g/tonne of feed) from day 0 to six weeks of age. Biochemical parameters were analysed after 3rd & 5th week & erythrocytic antioxidant enzymes were analysed after 3rd & 6th week of experiement. Hormonal & immunological parameters were analysed after 6th of experiemental study. After 3rd week, mean plasma glucose, cholesterol & antioxidant enzyme glutathione reductase (GSSG) were significantly (P≤ 0.01) lower in treated groups (II & III) than control (I); however total protein, albumin to globulin ratio & antioxidant enzyme superoxide dismutase (SOD) were significantly (P≤ 0.05) different in group II & III compared to group I. After 5th week, mean plasma glucose, total protein, albumin globulin ratio were significantly (P≤ 0.05) different in both the treatments compared to control. Erythrocytic GSSG were significantly (P≤ 0.05) different in both the treatments than control, as observed after 6th week. Stress hormones namely cortisol & thyroxine (T_4) were observed to be significantly (P≥0.05) higher in untreated control than the treated groups. However, the two treatments did not differ significantly. Mean total immunoglobulin (Ig) level was significantly (P≤0.01) higher in AYUCEE & vit-C treated birds than control after 6th week of study. It can be concluded from the results that oxidative stress in broilers during summer could be ameliorated using antioxidant synthetic vitamin C & the polyherbal antistressor, immunomodulator & adaptogenic feed premix AYUCEE.

Key words: herbal, immunity, antioxidants, stress hormones, glutathione, hypolipidaemic

Introduction

High ambient temperature and relative humidity are major environmental stressors that influence performance of broilers by reducing feed intake, feed efficiency, nutrient utilization and feed conversion ratio *(Sahin et al., 2003)*. During the periods of heat stress, most of the production energy is diverted to thermoregulatory adaptations that results in to decreased weight gain, poor immunity, oxidative stress predisposing birds to various infectious diseases and high mortality rates *(Cahaner and Leestra, 1992; Maini et al., 2007)*. Both high and low environmental temperatures stimulate the hypothalamo-hypophyseal-adrenocortical axis will stimulate corticosterone release *(Seigal, 1995)*. Higher levels of circulating corticosteroids have catabolic effect through muscle wasting and retarded growth *(Hayashi et al., 1994)*. Adverse effect of heat stress is exhibited through the impairment of cellular functions by altering oxidative metabolism and thus damage to the cell membrane *(Mates et al., 1999)*. Cells generate small amount of reactive oxygen species (ROS) while performing their normal functions *(Krauss et al., 2000)*. High ambient temperature has been shown to increase the free radicals and other ROS in the body fluids and tissues. Although, the low level of ROS are essential for many biochemical processes but its accumulation due to over-production or a decreased antioxidant defense, leads to damage of biological macromolecules and disruption of normal cell metabolism and physiology *(Spurlock and Savage, 1993)*. Body has its own defense mechanisms that protects cell against cellular oxidants and prevent their accumulation *(Tainiguchi et al., 1992)*. Normally available antioxidants in the body are vitamin C, vitamin E, folic acid, zinc, and chromium *(Thomas et al., 1999)*. Furthermore, antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) play a vital role in protecting cellular damage from 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 in increases blood glucose and cholesterol concentrations is also evident *(Altan et al., 2000)*. Non-enzymatic antioxidants such as vitamin C *(Sahin et al., 1991)* and polyherbal formulation containing herbs namely have been used to protect tissues from superoxide radicals and enhance cell survival by stimulating antioxidative enzymatic system. Dietary modifications are among the most preferred and practical ways to alleviate the effect of high environmental temperature in poultry. The objective of this study was to evaluate oxidative stress during summer and to compare the efficacy of some antioxidants in

amelioration of heat stress and normalization of serum and erythrocytic stress markers in broilers.

Materials and Methods

Experimental Animal and Climate. Day-old broiler chicks (Cobb Strain) were obtained from M/s Asam Hatcheries Private Limited, Haldwani, Uttaranchal, India and housed in a Students' Poultry Instructional Farm, Pantnagar located at latitude of 28°53'24" north, longitude of 74°34'27" with an altitude of 243.84 meters and equipped with all poultry care facilities. The experiment was conducted during summer months (June-July) with mean maximum daily temperature 32.86±0.68°C, relative humidity 83.57±1.50% and temperature humidity index (THI) 84.74±2.51.

Table 1. **Gross compositions of basal diets used during experiment.**

Ingredients (%)	Starter diet (0-21 days)	Grower/Finisher diet (22-49 days)
Maize	60.00	63.00
Ground nut cake	23.11	18.00
Fish meal	13.00	15.00
Common salt (NaCl)	0.22	0.33
Mineral mixture[1]	3.00	3.00
Vitamin A,B_2,D_3[2]	0.02	0.02
TM-100[3]	0.01	0.05
Amprosol[4]	0.05	0.05
Nuvimin[5]	0.05	0.55
Nutrient Composition		
Moisture (%)	6.29	6.22
Crude protein (%)	23.29	21.28
Total ash (%)	8.02	9.34

[1]Calcium-20%, Phosphorus-12%, Magnesium-5%, Iron-0.4%, Iodine-0.026%, Copper-0.1%, Manganese-0.12, Cobalt-0.12%, Flourine-0.07%, Zinc-0.08%, Sulphur-1.8-3.0%, Acid Insoluble Ash-3.0%, Lead-not more than 7.0 mg/kg.
[2]Vitamin A-82500 IU/g, Vitamin B-50 mg/g, Vitamin D_2-1200 IU/g.
[3]Oxytetracyclin-100 g/kg. [4]Amprolium HCl-20% w/w.
[5]Vitamin A-700IU/g, Vitamin D_3-70 IU/g, Vitamin E-0.25mg/g, Nicotinamide-1.0mg/g, Calcium-25.5%, Phosphorus-12.75%, Magnesium-6.0 mg/g, Iron-1.5 mg/g, Iodine-0.0325 mg/g, Copper-1.2 mg/g, Manganese-1.5 mg/g, Cobalt-0.15 mg/g, Zinc-9.6 mg/g, Sulphur-0.0072 mg/g, Selenium-0.1 mg/g.

Dietary Treatments. Sixty day old chicks were randomly divided into three groups consisting of twenty chicks in each, which were housed individually. Basal diet (Table 1) as chick starter was offered from 0 to 21 days and finisher diet

from day 22nd until 6th week. Birds in group I (control) were offered basal diet without any antioxidant supplement. Birds in group II were offered basal diet supplemented with polyherbal antistressor, adaptogenic and immunomodulator feed premix Ayucee @ 100g/tonne of feed (supplied by Ayurvet Limited, Baddi, India). Ayucee is a polyherbal formulation containing natural Vitamin C and bioflavonoids, scientifically well known for their anti-oxidant and free radical scavenging activities. The product contains constituent herbs, *Phyllanthus emblica (fruit & leaves), Ocimum sanctum (leaves), Terminalia chebula (fruit) and Withania somenifera (root).* All the constituent ingredients are grinded well to fine powder and mixed in fixed proportions. Antistressor & immunomodulating potential of constituent herbs; *Withania somnifera (Davis and Kuttan, 2000)*, *Phyllanthus emblica (Rege et al., 1999; Kim et al., 2005)*, *Ocimum sanctum (Gupta et al., 2007)* and *Terminalia chebula (Prasad et al., 2006; Sravanan et al., 2007)* have been scientifically well established. Birds in Group III were given basal diet supplemented with synthetic vitamin C@ 100g/tone of feed as suggested by *(Aengwanisch et al., 2003; Pardue et al., 1985)*. Feed and water was provided *adlibitum* to all groups. Chicks of all the groups were weighed at weekly intervals using calibrated digital balance.

Determination of antioxidant potential of vitamin C and Ayucee. The aqueous extract of antioxidant supplements was prepared as per procedure described by *Damien et al. (2003)* and *Radoslaw et al. (2006)* An amount of 10g of antioxidant supplement powder was suspended in 100ml of ultra pure deionized water and mixed for 24 hours with continuous stirring by an electronic magnetic stirrer. The mixture was centrifuged for 15 minutes at 4000 RPM and supernatant was filtered with Whatman filter paper No. 42. The 100 ml filtrate was evaporated in fan incubator at 37°C and dried powder was stored at 4°C in glass petridish packed with parafilm until analysis of antioxidative potential of extract. Antioxidative potential of aqueous extract of supplements was analyzed by employing electron transfer reaction methods namely total phenolics, 2,2-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity, chelating activity on Fe^{2+}. For analysis, 50mg of extract was added to 50 ml deionized water to form 1mg/ml of extract concentration and further reconstitution was done according to the requirement of different tests.

Ascorbic acid estimation. Ascorbic acid estimation was done as per the method described by *Ranganna (1986)*. 2gm sample of antioxidant was grinded with 25 ml of Meta Phosphoric acid (MPA) acetic acid solution. Sample was pulverized by gentle grinding in MPA acetic acid solution and then divided to a measure volume and filtered through Whatman filter paper no.1. To 15 ml of filtrate, 0.75 gm of acid treated charcoal was added and filtered through Whatman

filter paper no.1. Filtrate was titrated with indophenol dye solution. Ascorbic acid content in sample is expressed as mg/100gm sample.

Total phenolic compounds estimation. Total phenolic compounds in extract were determined according to the method described by *Germano et al. (2005)*. Aquous extract of product (10 mg/ml) was treated with Folin Cicalteu reagent (0.2ml) and incubated at 37°C for 5 min, the oxidation of phenols was measured spectrophotometrically at 765nm. For quantification of total phenolic extract, standard curve was prepared by plotting absorbance values on x-axis and different concentrations of gallic acid (50,100, 200, 500, 750 & 1000 µg) on y-axis. The regression equation was obtained from standard curve and total phenolic concentration was calculated as mg gallic acid equivalent/g of extract.

DPPH Activity. The scavenging effect of extract on DPPH radical was estimated according to the method described by *Yen and Duh (1993)* with slight modification suggested by *Singh et al. (2005)*. The hydrogen and electron donation ability of extract was measured from the bleaching of purple colour of methanolic solution of DPPH (0.04%) which was measured at an absorbance of 517nm. Radical scavenging activity of extract was compared with gallic acid at a concentration of 5, 10, 15, 20 & 25 µg/ml.

Chelating activity of aqueous extract on ferrous ions (Fe^{2+}). It was measured as per the method suggested by *Decker and Welch (1990)* and *Junctachote and Berghofer (2005)*. Inhibition of ferrozine-Fe^{2+} complex formation by extract was measured by decrease in colour intensity spectrophotometrically at 562nm.

Sample Collection

Blood samples were collected thrice during the study. For biochemical antioxidant enzyme estimations (approximately 1.5 ml/bird each) was collected aseptically from the wing vein (with 24 gauge needle), at the end of 3rd, & 5th week. For erythrocytic antioxidant enzyme estimations blood sample was collected aseptically from the wing vein (with 24 gauge needle), at the end of 3rd & 6th week. For hormonal assay & immunological analysis, blood samples were collected at the end of 6th week of experiement. The blood samples were immediately processed and centrifuged at 4000 RPM for separation of plasma and erythrocytes.

Laboratory Analysis. Cholesterol concentration (mg/dl) in plasma was done by cholesterol oxidase:p-aminophenazone (CHOD-PAP) enzymatic end point method with the help of MERCK diagnostic Kit (E. Merck India Ltd. Maharashtra) using spectrophotometer at 600nm wavelength against a blank reagent (Meiattni *et al*., 1978). Glucose (mg/dl) was estimated by Glucose oxidase peroxidase (GOD-

POD) method (using MERCK diagnostic Kit) at 546nm wavelength against a blank reagent. Estimation of total protein was done by Biuret method at 600nm wavelength against a blank reagent, concentration was expressed in g/dl. Total albumin was done by Bromocresol Green method at 540nm wavelength against a blank reagent. The albumin content was deducted from the total protein to obtain globulin level. Albumin and globulin ratio was calculated by dividing globulin from albumin content. For erythrocytic antioxidant enzyme analysis, erythrocyte pellet was washed thrice in ice-cool NaCl (0.9%). Packed erythrocytes were resuspended in phosphate buffer saline (PBS) **(**Yagi, 1999) and kept frozen at -20°C until estimation of erythrocytic enzymes. The 1:10 dilution of erythrocyte suspension in PBS was used for the estimation of superoxide dismutase (SOD). Hemoglobin concentration in erythrocyte suspension was determined by cyanmethemoglobin method *(Dacie and Lewis, 1974).* Glutathione reductase (GSSG) activity was assayed in erythrocytes as per the method described by Goldberg and Spooner (1933) and expressed as mM NADPH oxidized.min^{-1}.mg^{-1}. Hb. Erythrocyte SOD activity was estimated in haemolysate 1:10 as per the method described by Madesh and Balasubramanian (1998) and expressed as U.mg^{-1}.Hb. The blood samples collected at the end of 6^{th} week for hormonal estimation & humoral immune response studies. Estimation of cortisol, Total Tri-iodothyronine (T_3) and Total Thyroxine (T_4) was performed by Radio Immunoassay (RIA) technique using kits of Immunotech (A Bechman Countler Company procured from BARC, Mumbai). The total serum immunoglobulin was estimated by zinc sulphate turbidity test (McEvans *et al*., 1969). Delayed type hypersensitivity reaction to dinitrofluoro benzene (DNFB) was carried out by procedure described by *Phanuphak et al*. (1974)

Statistical Analysis. The results were expressed as mean ± standard error of mean. ANOVA was applied to compare the data of various treatment groups as described by Snedecor and Cochran (1994). Data was analyzed at minimum level of significance at 5% ($P \leq 0.05$) & at 1% level ($P \leq 0.01$).

Results

Antioxidant Potential of treatments

Total Phenolics. Data pertaining to antioxidant potential of supplements is depicted in Table 2 to 4. The concentration of total phenolics per gram of aqueous extract of Ayucee and synthetic vitamin C was recorded to be 481.15 and 313.47 mg of gallic acid equivalent, which was higher than synthetic vitamin C group (Table 2), as calculated from regression equation (Y=3145.95X-58.48, R2=0.991).

Table 2 . Ascorbic acid and total phenolic content of antioxidant supplements (AYUCEE&synthetic vitamin C)

Antioxidant supplements	Ascorbic acid per 100 gm (mg)	Total phenolics per g of aqueous extract of antioxidant (mg of gallic acid equivalent)
Ayucee	32.7	481.15
Synthetic vitamin C	99.0	313.47

Table 3. 2,2-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity of antioxidant supplements (Ayucee &synthetic vitamin C)

Concentration of aqueous extract (µg/ml)	DPPH scavenging activity (%)		
	Gallic acid	Ayucee	Synthetic vitamin C
5	16.07	23.84	13.34
10	16.81	26.47	15.02
15	20.59	26.68	17.23
20	21.59	30.46	17.54
25	23.63	34.35	20.59
Regression equation	Y=2.369 X-31.922	Y=1.863X-37.841	Y=2.816X-32.150
R^2	0.960	0.932	0.959
IC_{50} DPPH (µg/ml)	86.53	55.31	108.65

DPPH= 2,2-diphenyl-2-picrylhydrazyl
R^2=Square of sample correlation
IC_{50}= 50% radical scavenging activity

DPPH scavenging activity. It was found to be concentration dependent for antioxidant supplements. The DPPH scavenging activity at 5 µg/ml of aqueous extract of Ayucee was found to be 23.84%, which was higher than the scavenging activity of gallic acid (16.07). The DPPH scavenging activity of synthetic vitamin C (13.34) was lower than gallic acid and Ayucee (Table 3). Similar results of DPPH scavenging activity was also recorded at 25 µg/ml of aqueous extract. The IC_{50} (50% radical scavenging activity) values of DPPH scavenging activity of aqueous extract of Ayucee (55.31 µg/ml) supplements was lower than the IC_{50} values of DPPH scavenging activity of gallic acid (86.53 µg/ml). Whereas, the IC_{50} value of DPPH scavenging activity of aqueous extract of synthetic vitamin C was recorded to be highest (108.65 µg/ml). The results indicate that synthetic vitamin C had poor DPPH scavenging activity and highest IC_{50} values.

Table 4. Chelating activity of antioxidant supplements (AYUCEE & synthetic vitamin C) on Fe^{2+} ions

Concentration of aqueous extract (mg/ml)	DPPH scavenging activity (%)	
	Ayucee	Synthetic vitamin C
0.10	48.24	1.18
0.25	54.12	7.06
0.50	55.29	16.47
0.75	67.06	23.53
1.00	68.24	34.12
EDTA (0.02 mM)	98.12%	

DPPH= 2,2-diphenyl-2-picrylhydrazyl

Fe^{2+}chelating activity. The chelating activity was compared with EDTA (0.02nM). The antioxidant supplements showed chelating activity on Fe^{2+} in a concentration dependent manner. The chelating activity of Ayucee supplements on Fe^{2+} at 0.1 mg/ml of aqueous extract was found to be 48.24, which was higher than synthetic vitamin C (1.18) (table 4). The EDTA had Fe^{2+} chelating activity of 98.12%. Ayucee (68.24%) had higher chelating activity compared to synthetic vitamin C (34.12%) although both were lower than the EDTA.

Body weight. The weekly body weight data are presented in Table 5.

Table 5. Weekly body weights (g) of broiler chickens after 6 weeks in antioxidant supplemented (AYUCEE & synthetic vitamin C) & control groups (mean ± S.E., n = 20).

Treatment / Week	CONTROL	Ayucee	Synth. Vit C
Day 0	44.40^{a} ±0.88	43.25^{a} ±0.53	43.40^{a} ±0.56
I	172.6^{a} ±3.38	165.05^{a} ±3.69	174.20^{a} ±3.05
II	427.85^{a} ±5.90	430.85^{a} ±11.12	437.45^{a} ±6.49
III	784.85^{a} ±9.98	809.55^{a} ±13.78	799.80^{a} ±11.15
IV	1141.40^{a}±19.18	1154.60^{a}±18.64	1110.80^{a}±18.12
V	1434.5^{a}±22.52	1470.00^{b}±24.36	1435.00^{a}±23.87
VI	1697.01^{a}±35.87	1746.02^{b}±42.53	1746.36^{b}±32.92

Means bearing different superscript in a row differ significantly at P≤0.05
S.E. = Standard Error

On day one of experimental trial, the average body weight (g/bird) of individual birds was almost similar with little variation. No significant difference in body weight gain & feed efficiency was observed among control & treated groups till 4th week of experiment, however body weight was observed to be significantly different in treated (II&III) groups compared to control (I) & the two treatments didn't significantly differ from each other.

Biochemical Parameters. Estimation of blood biochemicals revealed that the total plasma cholesterol concentration in Ayucee followed by synth. Vit. C groups was found to be significantly (P≤0.01) lower compared to untreated control after 3rd & 5th week (Tables 6, 7). The plasma glucose concentration (mg/dl) in control group was significantly (P≤0.05) higher after 3rd & 5th week in comparison to the treatment groups, however no significant difference was observed among treatments (table No.6,7). Plasma protein & total globulin concentrations (g/dl) were significantly (P≤0.05) higher in treated groups compared to untreated control.

Table 6. Biochemical profile in broiler chickens in antioxidant supplemented (Ayucee & synthetic vitamin C) & control groups after 3 weeks of treatment (mean ± S.E., n =20).

Parameter / Treatment	Plasma cholesterol (mg/dl)	Plasma Glucose (mg/dl)	Plasma total protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)	A:G ratio
CONTROL	124.67^{b} ±4.67	238.54^{b} ±2.97	3.63^{a} ±0.06	1.96^{a} ±0.03	1.68^{a} ±0.08	1.20^{b} ±0.07
Ayucee	86.33^{a} ±2.50	213.39^{a} ±4.91	4.42^{b} ±0.06	1.80^{a} ±0.02	2.62^{b} ±0.07	0.69^{a} ±0.02
Synth. Vit C	108.33^{b} ±4.51	208.26^{a} ±2.83	4.63^{b} ±0.07	1.80^{a} ±0.02	2.84^{b} ±0.06	0.64^{a} ±0.02
C.D. (Significance level)	**15.89 (1%)**	**20.91 (1%)**	**0.37 (5%)**	**0.16 (5%)**	**0.33 (5%)**	**0.15 (5%)**

Means bearing different superscript in a column differ significantly at (P≤0.05) or at (P≤0.01).
A:G: Albumin globulin ratio
S.E.: Standard Error
C.D.: Critical Difference

Table 7. Biochemical profile in broiler chickens in antioxidant supplemented (Ayucee & synthetic vitamin C) & control groups after 5 weeks of treatment (mean ± S.E., n = 20).

Parameter / Treatment	Plasma cholesterol (mg/dl)	Plasma Glucose (mg/dl)	Plasma total protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)	A:G ratio
CONTROL	131.96^{b} ±2.91	249.52^{b} ±8.12	3.87^{a} ±0.19	2.11^{b} ±0.08	1.76^{a} ±0.13	1.24^{a} ±0.06
Ayucee	90.21^{a} ±3.11	182.18^{a} ±4.23	4.10^{a} ±0.25	1.90^{a} ±0.08	2.21^{b} ±0.18	0.91^{b} ±0.07
Synth. Vit C	108.93^{b} ±5.07	202.93^{b} ±11.44	4.33^{b} ±0.13	2.10^{b} ±0.06	2.23^{b} ±0.16	1.01^{b} ±0.31
C.D. (Significance level)	**15.89 (1%)**	**15.81 (5%)**	**0.37 (5%)**	**0.16 (5%)**	**0.33 (1%)**	**0.20 (1%)**

Means bearing different superscript in a column differ significantly at (P≤0.05) or at (P≤0.01)
A:G: Albumin globulin ratio
S.E.: Standard Error
C.D.: Critical Difference

Antioxidant Enzymes. The status enzymatic (SOD & GSSG) antioxidants in erythrocytes of broilers at 3rd and 6th week study are depicted in Table, 8. After 3rd week, the SOD erythrocytic enzyme activity was significantly (P≤0.05) higher than Ayucee & control group. However after 6th week, the SOD activity was recorded to be significantly higher (P≤0.01) in Ayucee than vit C treated & control groups. GSSG enzymatic activity in both the treatment groups was found to be significantly higher than control after 3rd week (P≤0.01) & after 6th week (P≤0.05), but the two treatments were non-significantly different from each other.

Immunological Parameters. Data pertaining to the serum total immunoglobulin (Ig) concentration at the end of 6th week study is presented in Table, 9. Antioxidant supplemented and treated groups II & III showed significantly (P≤0.01) higher serum total Ig concentration (g/l) compared to untreated control birds (2.79±0.06). However, the two treatments were non-significantly different from each other. DTH (skin thickness in cm) response after post challenge with Dinitrofluoro benzene (DNFB) is presented in Table 9. At 72 hours of initial sensitization, the average increase in skin thickness was significantly (P≤0.01) higher birds in synthetic vit.C (1.39±0.08 cm) and Ayucee (1.06±0.02 cm) groups than the control and both the treatments were non-significantly different from each other. Average skin leisions were significantly

(P≤0.01) severe in synthetic vit C treated birds compared to those in Ayucee & control group.

Table 8. Serum antioxidant enzyme profile in broiler chickens treated with different antioxidants after 3rd and 6th weeks of treatment (mean ± S.E., n =20).

Parameter / Treatment	SOD (U/mg Hb)		GSSG (mM NADPH oxidized /gm Hb/min)	
	3rd Week	6th Week	3rd Week	6th Week
Control	58.48[a] ±3.80	47.84[a] ±5.55	4.90[a] ±0.65	10.99[a] ±2.34
Ayucee	60.97[a] ±3.99	69.67[b] ±3.37	24.15[b] ±0.76	17.75[b] ±0.64
Synth. Vit C	72.61[b] ±3.46	48.99[a] ±5.63	14.83[b] ±0.45	14.96[b] ±0.85
C.D	**11.76 (5%)**	**15.56 (1%)**	**4.89 (1%)**	**3.67 (5%)**

Means bearing different superscript in a column differ significantly at (P≤0.05) or at (P≤0.01)
SOD: Superoxide Dismutase
GSSG: Glutathione Reductase
NADPH: Nictotinamide adenine dinucleotide phosphate
Hb: Haemoglobin
S.E.: Standard Error
C.D.: Critical Difference

Stress hormones. Total cortisol, T_3 & T_4 were estimated at the end of study after 6th week and data is presented in table, 10. At the end of 6th week, total plasma cortisol levels were significantly (P≤0.05) lower among treatment groups [Ayucee (2.81±0.30 nM/l) and synthetic vit.C (3.84±0.42 nM/l)] compared to untreated control broilers (4.93±0.4 nM/l). Total T_3 concentration (nM/l) was non-significantly higher in Ayucee (2.18±0.13) followed by synthetic vit.C (2.12±0.15) as compared to the control group (1.83±0.18). The plasma total T_4 (nM/l) concentration in control group (21.46±1.36), synthetic vit. C (22.82±0.88) groups were significantly lower than Ayucee (28.73±0.56) supplemented groups.

Table 9. Effect of immunological profiles in broiler chickens treated with different antioxidant after 6 weeks of treatment (mean ± S.E., n =20)

Parameter / Treatment	Total Ig (g / l)	DTH (skin thickness in cm)						
		0 h	6 h	12 h	24 h	48 h	72 h	Average
Control	2.79^{a} ±0.06	0.37^{a} ±0.01	0.45^{a} ±0.02	0.52^{a} ±0.04	0.85^{a} ±0.08	1.03^{a} ±0.03	0.88^{a} ±0.10	0.68^{a} ±0.11
Ayucee	3.83^{b} ±0.07	0.39^{a} ±0.01	0.51^{b} ±0.02	0.64^{b} ±0.01	1.06^{b} ±0.02	1.14^{b} ±0.04	1.06^{b} ±0.06	0.80^{a} ±0.13
Synth. Vit C	3.37^{b} ±0.29	0.49^{b} ±0.01	0.68^{b} ±0.01	0.87^{b} ±0.03	1.39^{b} ±0.08	1.23^{b} ±0.03	1.12^{b} ±0.04	0.96^{b} ±0.14
CD	**0.56 (1%)**	**0.03 (5%)**	**0.06 (5%)**	**0.09 (5%)**	**0.21 (5%)**	**0.13 (5%)**	**0.17 (5%)**	**0.48 (1%)**

Means bearing different superscript in a column differ significantly at (P≤0.05) or at (P≤0.01)
Ig: Immunoglobulin
DTH: Delayed test for Hypersensitivity
S.E.: Standard Error
C.D.: Critical Difference

Discussion

Antioxidant potential of Ayucee & Vitamin C

Total Phenolics. The presence of total phenolics in the extract indicates the antioxidative potential of constituent herbs *(Damien et al., 2003).* The higher concentration of total phenolics in Ayucee (Ayucee) might be due to presence of *Withania somnifera*, *Emblica & Terminalia chebula* as ingredients. The total phenolics content of *Mangifera indica and Terminalia chebula* were reported to be 166.33±18.01 and 135.00±9.54 mg/g in aqueous extract, respectively *(Farrukh et al., 2006).* The phenolic content of *Emblica officinalis (Adhikari, 2007)* and

Withania somnifera (Ashvin and Mishra, 2007) were reported to be higher than ascorbic acid.

Table 10. Hormonal profile in broiler chickens treated with different antioxidants after 6 weeks of Treatment (mean ± S.E., n = 20).

Parameter / Treatment	Cortisol (nmol/L)	T_3 (nmol/L)	T_4 (nmol/L)
CONTROL	4.93±0.49[a]	1.83±0.18[a]	21.46±1.36[a]
Ayucee	2.81±0.30[b]	2.18±0.13[a]	28.73±0.56[b]
Synth. Vit. C	3.84±0.42[b]	2.12±0.15[a]	22.82±0.88[a]
C.D. (Significance level)	**0.98 (5%)**	**1.42 5%**	**3.68 (5%)**

Means bearing different superscript in a column differ significantly.
T_3: Tri-iodothyronine
T_4: Thyroxine
S.E.: Standard Error
C.D.: Critical Difference

DPPH scavenging activity . The higher DPPH scavenging activity of herbal antioxidants viz. *Emblica officinalis, Withania somnifera, Ocimum sanctum and Terminalia chebula* has been due to presence of gallic acid and phenolic compounds as their active ingredients whereas in *Mangifera indica* due to presence of flavonoids and glycosides *(Farrukh et al., 2006)*. *Khopde et al. (2001)* reported that the total antioxidant capacity in terms of the ascorbic acid equivalents is 94 mg/g of amla extract, which is ~ 9.4% and hence *Emblica officinalis* is a more potent antioxidant than vitamin C. Free radicals are involved in the process of lipid peroxidation leading to pathological conditions *(Damien et al., 2003)*. Ayucee was found to have greater DPPH scavenging activity with lower IC_{50} values indicated their better DPPH scavenging activity compared to gallic acid and synthetic vitamin C. The phenoxide group of deprotonated phenolic compound possesses a high charge density which can bind a suitably highly charged cation *(Hider et al., 2001)*.

Iron Chelating Activity. Extracts rich in such components should be able to form complex with metal ions and stabilize the form of metal ion thus hindering metal catalyzed initiation and hydroperoxide decomposition reactions *(Gordon, 1990)*. Although the antioxidant supplements exhibited an ability to chelate iron (II) ions in a dose dependant manner, the aqueous extract of antioxidant

supplements possessed poor iron (II) chelating activity at both lower and higher concentration as compared to EDTA. This indicates that the amount of compound in antioxidant supplements to compete with ferrozine for iron (II) ions was less as compared to EDTA.

Body weight gain & feed efficiency. Stress in broilers results into decline in body weight, feed consumption and overall feed efficieny. However, supplementation of antioxidants alongwith basal diet is scientifically well proven to improve growth and performance in birds *(Sahin et al., 2003)*. The results of body weight gain after 5th week corraborates with those of *Sapcota et al. (2006)* and *Maini et al. (2007)*, who reported increase in body weight gain when *Phyllanthus emblica* was fed to the broilers at the end of 6th week. *Sahin et al. (2003)* and *Njoku (1986)*, in their studies found increased body weight gain in ascorbic acid supplemented group than the control broilers under heat stress. *Mujeeb Ather (1995)* and *Pradhan (1995)* in their study observed that Stresroak (polyherbal formulation) supplemented birds showed increased body weight gain as compared to control group.

Biochemical Parameters. Significant deviation from normal biochemical values as well as hormonal disturbances is the outcome of stress in birds. Increased stress induced sympatho-adrenal activity further leads to protein & lipid catabolism in turn elevating plasma cholesterol concentration. *Sahin et al. (2004)* reported that exposure of Japanese quails to 34°C temperature elevated plasma cholesterol concentration to 4.51 mM/l and supplementation of vitamin C @ 150mg, resulted in decline in its concentration to 2.98 mM/l. The findings of the present study are well supported by the findings of *Donkoh (1989)* who reported an increase in serum cholesterol upon heat exposure while supplementation of vitamin C decreased these changes at the end of 3rd week. *Sairam et al. (2003)* also suggested that active tannoid principles of *Emblica officinalis* to be an important hypolipidaemic agent that directly acts upon sympatho-adrenal axis and lowers the synthesis of corticosterone. This hypolipidaemic and hypocholesterolaemic effect of *Emblica officinalis* has been attributed to its potential to reduce lipid peroxidation and enhances clearance of endogenous cholesterol *(Mathur et al. 1996)*.

Antioxidant Enzymes. Oxidative stress leads to production of ROS and decrease in erythrocytic enzymes activity. However, supplementation of antioxidants, synthetic vit. C & Ayucee significantly improved erythrocytic enzymatic activity, after 3rd & 6th week respectively. The results in present study can be correlated with the justification given by Irshad and Chaudhary (2002). According to them, the antioxidant defense mechanism scavenges ROS, produced by lipid peroxidation under stressful conditions. This study is further supported by investigations of *Bhattacharya et al. (1999)* who reported that supplementation of antioxidant herbs viz. active tannoid principles of *Emblica officinalis* markedly

increased free radical scavenging enzyme (SOD, GSSG) along with decrease in lipid peroxidation. *Macardle and Jackson (2000)*, reported that supplementation of antioxidants to birds under heat stress resulted in significant increase in these values and were found to be 89.83±4.24 U/g Hb for SOD and 34.71±1.74 mM NADPH oxidized / g Hb/min for GSSG in haemolysate.

Immunological parameters. In present study, total Ig concentration was higher in antioxidant supplemented groups (Ayucee followed by synthetic vit. C) as compared to control group owing to the adaptogenic & immunomodulator potential of polyherbal formulation & vit. C. The findings are in congruence with those of *Savic et al., (1993)* that heat stress reduces immune response. *Tuekam et al. (1994)* also reported that there was a positive correlation between antibody titer and ascorbic acid supplementation. The reduced mean delayed hypersensitivity (DTH) response after 72 hrs in control birds in comparison to treated ones could be due to decreased immune function in heat stress and these findings are in agreement with that of *Murray et al., (1988)*, where increase in corticosterone level and decrease in the antibody titer to the vaccines given to the bird was reported during heat stress. 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 et al., 1985)*.

Stress hormones. High ambient temperature induced production and release of corticosteroids *(Siegal, 1995)*, exerted catabolic effects (mobilization of proteins and lipids) through muscle wasting and reduces growth rate *(Odedra et al., 1983; Hayashi et al., 1994)*. In the present investigation, the control group showed significantly higher than normal cortisol concentration as compared to treated groups. Higher cortisol in thermal stressed birds might be mediated through enhanced CRH-ACTH-corticosteroid activity acting through hypothalamo-piyuitary-adrenal (HPA) cortex axis. The normal value of tri-iodothyronine (T_3) was reported to be 0.5-4.0 ng/ml in poultry *(Sturkie, 2000)*. In the present study, T4 levels are significantly higher in treated groups than control which can be correlated to suppression in plasma thyroid hormone concentration in heat stressed birds might be due to suppression of hypothalamo-pituitary–thyroid axis as a result of high cortical (ACTH/CRH) activity as observed earlier in present investigation due to direct influence of temperature on hypothalamic TRH release *(Benker et al., 1990)*. Supplementation of ashwagandha capsules in thyrotoxicoxis affected woman *(Hooft et al., 2005)*; an aqueous extract of *W. somnifera* in cockrels *(Panda et al., 1997)* and ascorbic acid in broilers *(WeiLong et al., 2000)* have been shown to increase thyroid hormone concentration in serum and supplementation of polyherbal formulation Ayucee has been observed to reverse the trend exhibiting a stress ameliorative effect.

Conclusion

It can be concluded that the concentration of total phenolics, DPPH scavenging activity & chelating activity of Ayucee was numerically higher than vitamin C. Estimation of biochemical parameters revealed that vitamin C was comparatively more efficacious in normalizing the biochemical parameters namely plasma glucose, total protein & erythrocytic antioxidant enzyme GSSG after 5 weeks treatment. In contrast, supplementation of Ayucee was efficacious in normalizing values of plasma cholesterol & antioxidant enzyme SOD after 3 weeks. Both the antioxidants were found to significantly improve cell mediated & humoral immune response, decrease total plasma cortisol & increase total thyroxine level as estimated at the end of 6^{th} week. Ayucee @ 100g/tonne of feed could be used to minimize heat stress in broilers during summer months. It is also suggested that these herbal antioxidants could replace synthetic vitamin C supplementation which is economically expensive. Further investigation would be required on larger number of birds to work out most economical dose of these herbal antioxidants required for heat stress amelioration.

Acknowledgment

The authors would like to place on records the assistance rendered by Incharge, Instructional Poultry Farm and Dean, College of Veterinary and Animal Sciences, GBPUA&T, Pantnagar for providing necessary facilities and financial assistance for conducting the research.

Poboljšanje oksidativnog stresa kod brojlera u letnjem periodu

V. Sujatha, J. Pandurang Korde, S. K. Rastogi, A. K. Madan, S. Maini, K. Ravikanth

Rezime

Komparativno je ispitivan uticaj sintetičkog vitamina C kao antistresora i anitoksidansa i poliherbalnog premiksa Ayucee dodatih u obrok za brojlere tokom letnjih meseci (jun i juli) kada je indeks srednje temperature i vlažnosti bio 84.74±2.51. Jednodenvni brojelri (n =60) su metodom slučajnog uzroka podeljeni u tri grupe. Kontrolna grupa I je dobijala osnovni obrok, a ogledne grupe (II i III) su dobijale obrok dopunjen sintetičkim vitaminom C (100g t^{-1} hraniva) i Ayucee

(100g/tona hraniva) od prvog dana do šest nedelja uzrasta. Analizirani su biohemijski parametri posle treće i pete nedelje, kao i eritrocitni antioksidantski enzimi posle treće i šeste nedelje ogleda. Hormonalni i imunološki parametri su analizirani posle šeste nedelje ispitivanja. Posle tri nedelje ogleda, glukoza u plazmi, holesterol i antioksidantski enzim glutation reduktaza (GSSG) su bili signifikantno niži ($P \leq 0.01$) kod pilića u oglednim grupama (II i III) nego u kontroli (I); međutim, ukupni protein, odnos albumina i globulina i antioksidantski enzim superoksid dismutaza (SOD) su bili signifikantno različiti ($P \leq 0.05$) u grupama II i III u poređenju sa grupom I. Posle 5. nedelje, srednja vrednost glukoze u plazmi, ukupnog proteina, odnosa albumina i globulina su se signifikantno razlikovali ($P \leq 0.05$) u oba tretmna u poređenju sa kontrolom. Eritrocitni GSSG je bio signifikantno ($P \leq 0.05$) različit u oba tretmana u odnosu na kontrolu, što je registrovano u 6. nedelji. Hormoni stresa, kortizol i tiroksin (T_4) su bili signifikantno ($P \geq 0.05$) viši kod netretiranih pilića u kontrolnoj grupi nego u oglednim. Međutim, dva tretmana se nisu signifikantno razlikovala. Srednja vrednost nivoa imunoglobulina (Ig) je bila signifikantno veća ($P \leq 0.01$) kod brojlera tretiranih AYUCEE i vitaminom C nego kod kontrolnih pilića posle 6. nedelje isptitivanja. Može se zaključiti na osnovu dobijenih rezultata da je oksidativni stres kod pilića tokom letnjih meseci mogao da bude poboljšan korišćenjem antioksidansa sintetičkog vitamina C i poliherbalnog antistresora, imunomodulatora i adaptogenskog premiksa AYUCEE.

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Received 17 September 2010; accepted for publication 11 October 2010