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*Full Length Research Paper*

# Amelioration of heat stress induced disturbances of the antioxidant defense system in broilers

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**A comparative study on antistressor and antioxidative effects of synthetic vitamin C and polyherbal feed premix 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 and III) were supplemented with synthetic vitamin C (100 g/tonne of feed) and polyherbal feed premix (1 kg/tonne of feed) from day 0 to 6 weeks of age. Biochemical parameters were analysed after the 3rd and the 5th week and erythrocytic antioxidant enzymes were analysed after the 3rd and the 6th week of experiement. Hormonal and immunological parameters were analysed after the 6th week of the study. After the 3rd week, mean plasma glucose, cholesterol and antioxidant enzyme glutathione reductase (GSSG) were significantly (P ≤ 0.01) lower in treated groups (II and III) than control (I); however total protein, albumin to globulin ratio and antioxidant enzyme superoxide dismutase (SOD) were significantly (P ≤ 0.05) different in group II and III compared to group I. After the 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 the 6th week. Stress hormones namely cortisol and thyroxine ($T_4$) were observed to be significantly (P ≥ 0.05) higher in the untreated controls than the treated groups. Mean total immunoglobulin (Ig) level was significantly (P ≥ 0.01) higher in polyherbal premix and vitamin C treated birds than control birds after the 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 and the polyherbal antistressor, immunomodulator and adaptogenic feed premix.**

**Key words:** Antioxidants, glutathione, herbal, hormones, hypolipidaemic, immunity, stress.

## INTRODUCTION

Various environmental stressors such as high ambient temperature and relative humidity influence the 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 which results in oxidative stress induced immunosupression, predisposing birds to various infectious diseases and high mortality rates (Cahaner and Leestra, 1992; Maini et al., 2007). Variations in environmental temperatures stimulate the hypothalamo-

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hypophyseal-adrenocortical axis that in turn stimulates corticosterone release (Seigal, 1980). Higher levels of circulating corticosteroids have a 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). Reactive oxygen species (ROS) are generated at cellular level during normal bodily functions (Krauss et al., 2000), however, high ambient temperature has been shown to increase the free radicals and other ROS production in body fluids and tissues. Although, low levels of ROS are essential for many biochemical processes, 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). The body has its own defense mechanisms that protects the 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 and Reed, 1989). 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., 2000). Non-enzymatic antioxidants such as vitamin C (Sahin et al., 2003, 2004) and polyherbal products containing different immunomodulator (*Withania somnifera* and *Asparagus raecemosus*), antistressor (*Phyllanthus emblica* and *Mangifera indica*) and adaptogenic (*Ocimum sanctum* and *W. somnifera)* herbs have been used to protect tissues from superoxide radicals and enhance cell survival by stimulating antioxidative enzymatic systems (Davis and Kuttan, 2000; Rege et al., 1999 and Saravanan et al., 2007). Dietary modifications are among the most preferred and practical ways to alleviate the effect of high environmental temperature in poultry (Sahin et al., 2004). The objective of this study was to evaluate oxidative stress during the 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 animals 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 m and equipped with all poultry care facilities. The experiment was conducted during the summer months (June-July) with mean maximum daily temperature of 32.86 ± 0.68°C, relative humidity (RH) 83.57 ± 1.50% and temperature humidity index (THI) 84.74 ± 2.51. THI was calculated as per the formula proposed by Kelly and Bond, 1971. THI = $(T_{db})$ - (0.55 - 0.55RH). $(T_{db}$ -58) $T_{db}$ = Dry Bulb Temperature (°F) RH = Relative humidity expressed as fraction of 1

### Dietary treatments

One hundred and twenty day old chicks were randomly divided into three groups consisting of forty chicks each, which were housed in a deep litter system. A basal diet of chick starter was offered from 0 - 21 days and a finisher diet from day 22 until the $6^{th}$ week (composition as described in Table 1). 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 (Stresroak) added at 1 kg/tonne of feed (supplied by Ayurvet Limited, Baddi, India). Stresroak is a polyherbal product containing natural vitamin C and bioflavonoids scientifically well known for their anti-oxidant and free radical scavenging activities (Pradhan, 1995). The product contains constituent herbs, *P. emblica* (fruit and leaves), *O. sanctum* (leaves), *Terminalia chebula* (fruit), *W. somenifera* (root) *and Shilajit.* All the constituents were grinded into a fine powder and mixed in fixed proportions. The antistressor and immunomodulating potential of constituent herbs; *W. somnifera* (Davis and Kuttan, 2000), *P. emblica* (Rege et al., 1999 and Kim et al., 2005), *O. sanctum* (Gupta et al., 2007) and *T. chebula* (Prasad et al., 2006; Sravanan et al., 2007) have been scientifically well established. Birds in Group III were given a basal diet supplemented with synthetic vitamin C at 100 g/tonne of feed as recommended by Aengwanisch et al. (2003). Feed and water was provided *ad libitum* to all groups. Chicks of all the groups were weighed at weekly intervals using a calibrated digital balance.

### Determination of antioxidant potential

To determine the antioxidant potential, the following procedure was carried out. First, an aqueous extract of antioxidant supplements (vitamin C and polyherbal premix) was prepared as per procedure described by Damien et al. (2003) and Radoslaw et al. (2006). Antioxidant supplement powder (10g) was resuspended in 100 ml of ultra pure deionized water and mixed for 24 h with continuous stirring using an electronic magnetic stirrer. The mixture was centrifuged for 15 min at 4,000 rpm and the supernatant was filtered with Whatman (Whatman International Ltd. Maidstone England) filter paper No. 42. The 100 ml filtrate was evaporated in a fan incubator at 37°C and the dried powder was stored at 4°C in a glass petri dish sealed with parafilm until further analysis. The antioxidative potential of the aqueous extract of the supplements was analyzed by employing an electron transfer reaction method namely total phenolics, 2,2-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity and chelating activity on $Fe^{2+}$. For analysis, 50 mg of extract was added to 50 ml deionized water to give an extract concentration of 1 mg/ml and further reconstitution was done performed according to the requirement of different tests. Ascorbic acid estimation was performed as described by Ranganna (1986). A sample of 2 mg of antioxidant (polyherbal premix Stresroak) was grinded with 25 ml of a Meta Phosphoric acid (MPA) acetic acid solution. The sample was pulverized by gentle grinding in MPA acetic acid solution and then filtered through Whatman filter paper no. 1. To 15 ml of filtrate, 0.75 g of acid treated charcoal was added and filtered through Whatman filter paper no. 1. The filtrate was titrated with indophenol dye solution. Ascorbic acid content in the sample is expressed as mg/100 g sample. Total phenolic compounds in the extract were determined according to the method

**Table 1.** Gross compositions of basal diets used during the 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-0.25 mg/g, Nicotinamide-1.0 mg/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.

described by Germano et al. (2005). An aqueous extract of the sample (10 mg/ml) was treated with 0.2 ml of Folin Cicalteu reagent, (Composition: 750 ml water, 100 g sodium tungstate, 25 g sodium molybdate, 50 ml of 85% phosphoric acid, 100 ml concentrated HCl, 150 g lithium sulfate "a few drops" of bromine)and incubated at 37°C for 5 min. The oxidation of phenols was measured spectrophotometrically at 765 nm. For the quantification of total phenolic extract, a standard curve was prepared by plotting absorbance values on the x-axis and different concentrations of gallic acid (50,100, 200, 500, 750 and 1000 µg/ml) on the y-axis. The regression equation was obtained from the standard curve and the total phenolic concentration was calculated as mg gallic acid equivalent/g of extract. The presence of total phenolics in the extract indicates the antioxidative potential of the constituent herbs (Damien et al., 2003). The scavenging effect of the extract on DPPH radical was estimated according to the method described by Yen and Duh (1993) with a slight modification suggested by Singh et al. (2005). The hydrogen and electron donation ability of the extract was measured from the bleaching of the purple colour of the methanolic solution of DPPH (0.04%) which was measured at an absorbance of 517 nm. For the quantification of $IC_{50}$ values (50% radical scavenging activity) of the DPPH scavenging activities of the aqueous extract of antioxidant supplements and gallic acid, a standard curve was prepared by plotting the DPPH scavenging activity (%) on the x-axis and different concentrations of aqueous extract of antioxidant supplements (vitamin C and polyherbal premix) and gallic acid (5, 10, 15, 20 and 25 µg/ml) on the y-axis. A regression equation was obtained from the standard curve and $IC_{50}$ values were calculated. The radical scavenging activity of the extract was compared with gallic acid at a concentration of 5, 10, 15, 20 and 25 µg/ml. The chelating activity of the aqueous extract on ferrous ions ($Fe^{2+}$) was measured using the method suggested by Decker and Welch (1990) and Junctachote and Berghofer (2005). Inhibition of ferrozine-$Fe^{2+}$ complex formation by the extract was measured spectrophotometrically by a decrease in colour intensity at 562 nm. Extracts rich in such components should be able to form complexes and stabilize metal ions thus hindering metal catalyzed initiation and hydroperoxide decomposition reactions (Gordon, 1990).

**Determination of immunological parameters**

Cholesterol concentration (mg/dl) in plasma was calculated using the cholesterol oxidase: p-aminophenazone (CHOD-PAP) enzymatic end point method with the help of MERCK diagnostic Kit (E. Merck India Ltd. Maharashtra) at 600 nm (Meiattni et al., 1978). Glucose (mg/dl) was estimated by the Glucose oxidase peroxidase (GOD-POD) method (using MERCK diagnostic Kit) at 546 nm. Estimation of total protein was done by the Biuret method at 600 nm wavelength against a blank reagent; concentration was expressed in g/dl. Total albumin was estimated by the Bromocresol Green method at 540 nm. The albumin content was deducted from the total protein content to obtain the globulin level. The albumin and globulin ratio was calculated by dividing globulin by albumin content. For erythrocytic antioxidant enzyme analysis, the erythrocyte pellet was washed three times in ice-cold NaCl (0.9%). Packed erythrocytes were resuspended in phosphate buffer saline (PBS) and kept frozen at -20°C until used for the estimation of erythrocytic enzymes. The 1:10 dilution of erythrocyte suspension in PBS was used for the estimation of superoxide dismutase (SOD). The haemoglobin concentration in erythrocyte suspensions was determined by the 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 diluted haemolysate

**Table 2.** Ascorbic acid and total phenolic content of antioxidant supplements (polyherbal premix and 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) |
|---|---|---|
| Polyherbal premix | 98.1 | 587.16 |
| Synthetic vitamin C | 99.0 | 313.47 |

**Table 3.** 2,2-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity of antioxidant supplements (polyherbal premix and synthetic vitamin C).

| Concentration of aqueous extract (µg/ml) | DPPH scavenging activity (%) | | |
|---|---|---|---|
| | Gallic acid | Polyherbal premix | Synthetic vitamin C |
| 5 | 16.07 | 28.15 | 13.34 |
| 10 | 16.81 | 41.07 | 15.02 |
| 15 | 20.59 | 57.25 | 17.23 |
| 20 | 21.59 | 67.33 | 17.54 |
| 25 | 23.63 | 76.58 | 20.59 |
| Regression equation | Y=2.369 X-31.922 | Y= 0.401X-6.708 | 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.

(1:10) as per the method described by Madesh and Balasubramanian (1998) and expressed as $U.mg^{-1}$.Hb.

Blood samples were collected at the end of the 6th week for hormonal estimation and humoral immune response studies. Estimation of cortisol, total Tri-iodothyronine (T3) and total Thyroxine (T4) was performed by the Radio Immunoassay (RIA) technique using kits from Immunotech (BARC, Mumbai). The total serum immunoglobulin was estimated by the zinc sulphate turbidity test (McEvans et al., 1969). A delayed type hypersensitivity reaction to dinitrofluoro benzene (DNFB) was carried out by the procedure described by Phanuphak et al. (1974).

For the different biochemical and immunological estimations, blood samples were collected three times during the study from 20 randomly selected representative birds per group, given specific identification marks. Sampling was done from a similar batch of 20 randomly selected birds at 3rd and 6th week per group. However after the 5th week all remaining birds from each group were subjected to sampling. For biochemical antioxidant enzyme estimations (approximately 1.5 ml/bird each), blood was collected aseptically from the wing vein (with a 24 gauge needle) at the end of the 3rd and the 5th week. For erythrocytic antioxidant enzyme estimations blood samples were collected aseptically from the wing vein (with 24 gauge needle) at the end of the 3rd and the 6th week. For the hormonal assay and immunological analysis, blood samples were collected at the end of the 6th week. The blood samples were immediately processed and centrifuged at 4000 rpm for separation of plasma and erythrocytes.

### Statistical analysis

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

## RESULTS

### Antioxidant potential

Data pertaining to antioxidant potential of supplements is depicted in Tables 2 - 4. The concentration of total phenolics per gram of aqueous extract of Stresroak and synthetic vitamin C was recorded as 587.16 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). DPPH scavenging activity was found to be concentration dependent for antioxidant supplements. The DPPH scavenging activity at 5 ∝g/ml of aqueous extract of the polyherbal premix was found to be 28.15%, 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 the polyherbal premix Stresroak (Table 3). Similar results of DPPH scavenging activity were also recorded at 25 ∝g/ml of aqueous extract. The $IC_{50}$ (50% radical scavenging activity) values of the DPPH scavenging activity of the

**Table 4.** Chelating activity of antioxidant supplements (polyherbal premix and synthetic vitamin C) on $Fe^{2+}$ ions

| Concentration of aqueous extract (mg/ml) | DPPH scavenging activity (%) | |
|---|---|---|
| | Polyherbal premix | Synthetic vitamin C |
| 0.10 | 11.76 | 1.18 |
| 0.25 | 27.06 | 7.06 |
| 0.50 | 50.59 | 16.47 |
| 0.75 | 55.29 | 23.53 |
| 1.00 | 71.76 | 34.12 |
| EDTA (0.02 mM) | 98.12 | |

DPPH= 2,2-diphenyl-2-picrylhydrazyl.

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

| Treatment / Week | Control | Polyherbal premix | Synth. vitamin C | C.D. |
|---|---|---|---|---|
| Day 0 | $44.40^{a}$ ±0.88 | $44.60 \pm 0.60^{a}$ | $43.40^{a}$ ±0.56 | NS |
| I | $172.6^{a}$ ±3.38 | $168 \pm 2.86^{a}$ | $174.20^{a}$ ±3.05 | NS |
| II | $427.85^{a}$ ±5.90 | $422.00 \pm 5.27^{a}$ | $437.45^{a}$ ±6.49 | NS |
| III | $784.85^{a}$ ±9.98 | $812.40 \pm 12.22^{a}$ | $799.80^{a}$ ±11.15 | NS |
| IV | $1141.40^{a}$ ±19.18 | $1192.90^{bc}$ ±15.14 | $1110.80^{a}$ ±18.12 | 52.83 |
| V | $1434.5^{a}$ ±22.52 | $1520.65^{bc}$ ±17.67 | $1435.00^{a}$ ±23.87 | 66.74 |
| VI | $1697.01^{d}$ ±35.87 | $1892.00^{bc}$ ±28.08 | $1746.36^{b}$ ±32.92 | 101.54 |

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

aqueous extract of the polyherbal premix (13.34 ∝g/ml) supplements was lower than the $IC_{50}$ values of the DPPH scavenging activity of gallic acid (86.53 ∝g/ml). The $IC_{50}$ value of the DPPH scavenging activity of the aqueous extract of synthetic vitamin C was the highest recorded (108.65 ∝g/ml). The results indicate that synthetic vitamin C had poor DPPH scavenging activity and the highest $IC_{50}$ values. The $Fe^{2+}$ chelating activity was compared with EDTA (0.02nM). The antioxidant supplements showed chelating activity of $Fe^{2+}$ in a concentration dependent manner. The chelating activity of $Fe^{2+}$of the polyherbal premix supplements at 0.1 mg/ml of aqueous extract was found to be 11.76, which was higher than synthetic vitamin C (1.18) (Table 4). The EDTA had an $Fe^{2+}$ chelating activity of 98.12%. The polyherbal premix (71.76%) had higher chelating activity than synthetic vitamin C (34.12%) although both were lower than the EDTA.

## Body weight

The weekly body weight data are presented in Table 5. On day one of the trial, the average body weight (g/bird) of individual birds showed little variation. No significant difference in body weight gain and feed efficiency was observed among the control and treated groups until the 3rd week of the experiment. Body weight was observed to be significantly different in treated groups (II and III) compared to the control group (I) during the 4th and 6th week. The final mean body weight (g/bird) of the polyherbal product treated group (1,892.00 g ± 28.08) was significantly (P ≤ 0.05) higher than the control (1697.01 g ± 35.87) and the synthetic vitamin C treated group (1,746.36 g ± 32.92).

## Biochemical parameters

Estimation of blood biochemical parameters revealed that the total plasma cholesterol concentration in the Stresroak treated group (80.50 ± 3.84) followed by the synthetic vitamin C group (108.33 ± 4.51) were significantly (P ≤ 0.01) lower compared to the control group (124.67 ± 4.67) after the 3rd week (Table 6). A similar trend was observed after the 5th week of the experiment (Table 7). After the 3rd and 5th week, plasma glucose concentration (mg/dl) in the control group I (238.54 ± 2.97 and 249.52 ± 8.12) was significantly (P≤0.05) higher than the treatment group II

**Table 6.** Biochemical profile in broiler chickens taking antioxidant supplement (polyherbal premix and synthetic vitamin C) and control groups after 3 weeks (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 | $124.67^{a}$ ± 4.67 | $238.54^{a}$ ± 2.97 | $3.63^{a}$ ± 0.06 | $1.96^{a}$ ± 0.03 | $1.68^{a}$ ± 0.08 | $1.20^{a}$ ± 0.07 |
| Polyherbal premix | $80.50^{b,c}$ ± 3.84 | $208.81^{b}$ ± 3.50 | $4.54^{b}$ ± 0.14 | $1.54^{a}$ ± 0.04 | $3.01^{b}$ ± 0.16 | $0.53^{b}$ ± 0.04 |
| Synth. Vit. C | $108.33^{b}$ ± 4.51 | $208.26^{b}$ ± 2.83 | $4.63^{b}$ ± 0.07 | $1.80^{a}$ ± 0.02 | $2.84^{b}$ ± 0.06 | $0.64^{b}$ ± 0.02 |
| C.D. | 15.89 | 20.91 | 0.37 | 0.16 | 0.33 | 0.15 |
| (Significance level) | (1%) | (1%) | (5%) | (5%) | (5%) | (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 taking antioxidant supplement (polyherbal premix and synthetic vitamin C) and 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 | $131.96^{a}$ ± 2.91 | $249.52^{a}$ ± 8.12 | $3.87^{a}$ ± 0.19 | $2.11^{a}$ ± 0.08 | $1.76^{a}$ ± 0.13 | $1.24^{a}$ ± 0.06 |
| Polyherbal premix | $91.24^{b}$ ± 5.21 | $181.33^{b}$ ± 4.57 | $4.59^{b}$ ± 0.08 | $1.82^{b}$ ± 0.08 | $2.77^{b}$ ± 0.07 | $0.67^{b}$ ± 0.04 |
| Synth. vitamin C | $108.93^{b}$ ± 5.07 | $202.93^{b}$ ± 11.44 | $4.33^{b}$ ± 0.13 | $2.10^{a}$ ± 0.06 | $2.23^{b}$ ± 0.16 | $1.01^{b}$ ± 0.31 |
| C.D. | 15.89 | 15.81 | 0.37 | 0.16 | 0.33 | 0.20 |
| (Significance level) | (1%) | (5%) | (5%) | (5%) | (1%) | (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.

(208.81 ± 3.50 and 181.33 ± 4.57) and III (208.26 ± 2.83 and 202.93 ± 11.44), respectively, although no significant difference was observed among treatments (Table 6 and 7). Plasma protein and total globulin concentrations (g/dl) were significantly (P ≤ 0.05) higher in treated groups compared to the untreated control group. After the 3rd and 5th week, albumin to globulin ratio in the treatment group
II (0.53 ± 0.04 and 0.67 ± 0.04) followed by treatment group III (0.64 ± 0.02 and 1.01 ± 0.31) was significantly (P ≤ 0.05) lower than the untreated control (1.20 ± 0.07 and 1.24 ± 0.06), respectively.

## Immunological parameters

The status of enzymatic (SOD and GSSG) antioxidants in erythrocytes of broilers at the 3rd and the 6th week of study are shown in Table 8. After the 3rd week, the SOD erythrocytic enzyme activity (U/mg Hb) of both the treatment groups II (71.17 ± 4.94) and III (72.61 ± 3.46) was significantly (P ≤ 0.05) higher than the control group (48.48 ± 3.80). No significant difference was evident between the two treatments. However after the 6th week, the SOD activity was significantly higher (P ≤ 0.05) in the polyherbal premix treated group (93.60 ± 3.31) than the vitamin C treated (48.99 ± 5.63) and the control groups (47.84 ± 5.55). Similarly, GSSG enzymatic activity in both treatment groups was found to be significantly higher than the control group after the 3rd week (P ≤ 0.05) and 6th week. No significant difference was evident among the two treatments after the 3rd week, however the GSSG enzymatic activity of the polyherbal premix treated group was significantly higher than the synthetic vitamin C and the control group.

Data pertaining to the serum total immunoglobulin (Ig) concentration at the end of the 6th week of the study is presented in Table 9. Antioxidant supplemented and treated groups III (4.93 ± 0.16) showed significantly (P ≤ 0.05) higher serum total Ig concentration (g/l) than group II (3.37 ± 0.29) compared to untreated control birds (2.79 ± 0.06). The DTH (skin thickness in cm) response after post challenge with dinitrofluoro benzene (DNFB) is presented in Table 9. At 72 h of initial sensitization, the average increase in skin thickness of both the treated group II (1.05 ± 0.11) and group III (0.96 ± 0.14) was significantly (P ≤ 0.01) higher than the control (0.68 ± 0.11). The treatments groups were non-significantly different from each other.

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

| Parameter | SOD (U/mg Hb) | | GSSG (mM NADPH oxidized /gm Hb/min) | |
|---|---|---|---|---|
| Treatment | 3rd week | 6th week | 3rd week | 6th week |
| Control | 48.48[a] ± 3.80 | 47.84[a] ± 5.55 | 4.90[a] ± 0.65 | 10.99[a] ± 2.34 |
| Polyherbal Premix | 71.17[b] ± 4.94 | 93.60[b] ± 3.31 | 42.66[b] ± 2.04 | 29.89[b] ± 2.02 |
| Synth. vitamin 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, $T_3$ and $T_4$ were estimated at the end of study after the 6th week and data are presented in Table 10. At the end of the 6th week, total plasma cortisol levels were significantly (P ≤ 0.05) lower in the polyherbal premix treated group [Stresroak (2.11 ± 0.22 nM/l) followed by the synthetic vitamin C (3.84 ± 0.42 nM/l)] and untreated control group (4.93±0.4 nM/l). Total $T_3$ concentration (nM/l) was not significantly higher in either the polyherbal premix (2.22 ± 0.21) of the synthetic vitamin C (2.12 ± 0.15) group as compared to the control group (1.83 ± 0.18). The plasma total $T_4$ (nM/l) concentration in the control group (21.46 ± 1.36) and the synthetic vitamin C (22.82 ± 0.88) groups were significantly (P ≤ 0.05) lower than the polyherbal premix (31.58 ± 2.13) supplemented groups.

## DISCUSSION

### Antioxidant potential total phenolics

The higher concentration of total phenolics in the polyherbal premix might be due to the presence of *W. somnifera*, *Emblica officinalis and T. chebula* as ingredients. The total phenolics content of *M. indica* and *T. chebula* have been 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 *E. officinalis* (Adhikari, 2007) and *W. somnifera* (Ashvin and Mishra, 2007) were reported to be higher than ascorbic acid.

### DPPH scavenging activity

The higher DPPH scavenging activity of the herbal antioxidants in *E. officinalis, W. somnifera, O. sanctum and T. chebula* is due to the presence of gallic acid and phenolic compounds in their active ingredients, and the presence of flavonoids and glycosids in *M. indica* (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 is approximately 9.4% and hence *E. 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). The polyherbal premix was found to have greater DPPH scavenging activity with lower $IC_{50}$ values which indicated their better DPPH scavenging activity compared to gallic acid and synthetic vitamin C.

### Iron chelating activity

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 available to compete with ferrozine for iron (II) ions is less when compared to EDTA.

### Body weight gain and feed efficiency

Stress in broilers results in a decline in body weight, feed consumption and overall feed efficiency. However, supplementation of antioxidants along with the basal diet has been scientifically well proven to improve growth and performance in birds (Sahin et al., 2003). The results of body weight gain after the 3rd week corroborates with those of Sapcota et al. (2006) and Maini et al. (2007), who reported an increase in body weight gain when *P. emblica* was fed to broilers at the end of the 6th week. Sahin et al. (2003) and Njoku (1986), in their studies,

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

| Parameter | Total Ig | DTH (skin thickness in cm) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Treatment | ( g / l) | 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 |
| Polyherbal premix | $4.93^{b}$ ± 0.16 | $0.55^{b}$ ± 0.02 | $0.97^{b}$ ± 0.02 | $1.09^{b}$ ± 0.03 | $1.37^{b}$ ± 0.10 | $1.23^{b}$ ± 0.07 | $1.12^{b}$ ± 0.05 | $1.05^{b}$ ± 0.11 |
| Synth. vitamin 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 | 0.03 | 0.06 | 0.09 | 0.21 | 0.13 | 0.17 | 0.48 |
| | (1%) | (5%) | (5%) | (5%) | (5%) | (5%) | (5%) | (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.

found increased body weight gain in an ascorbic acid supplemented group compared to a control group of broilers under heat stress. Mujeeb Ather (1995) and Pradhan (1995) also observed that Stresroak (polyherbal formulation) supplemented birds showed increased body weight gain when compared to a 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 and lipid catabolism in turn elevating plasma cholesterol concentration. Sahin et al. (2004) reported that exposure of Japanese quails to a temperature of 34°C elevated plasma cholesterol concentrations to 4.51 mM/l and supplementation with vitamin C (150 mg), resulted in a 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 with vitamin C decreased these changes at the end of the 3rd week. Sairam et al. (2003) also suggested that active tannoid principles of *E. officinalis* are an important hypolipidaemic agent that directly acts upon the sympatho-adrenal axis and lowers the synthesis of corticosterone. The hypolipidaemic and hypocholesterolaemic effect of *E. officinalis* has been attributed to its potential in reducing lipidperoxidation and enhancing clearance of endogenous cholesterol (Mathur et al., 1996). The efficacy of polyherbal premix (Stresroak) in lowering serum cholesterol in the present study can be well correlated to the hypocholesterolemic and hypolipidaemic activity of constituent herbs.

## Immunological parameters

Oxidative stress leads to the production of ROS and decrease in erythrocytic enzymes activity. However, supplementation of antioxidants, synthetic vitamin C and the polyherbal premix significantly improved erythrocytic enzymatic activity, after the 3rd and 6th week respectively. The results of the present study can be correlated with the justification given by Irshad and Chaudhary (2002). According to these authors, the antioxidant defense mechanism scavenges ROS produced by lipid peroxidation under stress-ful conditions. This finding is further supported by investigations by Bhattacharya et al. (1999) who reported that supplementation of antioxidant herbs e.g. active tannoid principles of *E. officinalis,* markedly increased free radical scavenging enzyme(SOD, GSSG) along with a decrease in lipid peroxidation. Macardle and Jackson (2000) also reported that supplementation of antioxidants to birds under heat stress resulted in significant increase in values of SOD and NADPH as observed in the present study at the 3rd and 6th week in the treatment groups. In the present study, total Ig concentration was higher in the antioxidant supplemented groups (polyherbal premix followed by synthetic vitamin C when compared to the control group owing to the adaptogenic and immunomodulator potential of

**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] |
| Polyherbal premix | 2.11±0.22[b] | 2.22±0.21[a] | 31.58±2.13[b] |
| Synth. Vitamin C | 3.84±0.42[b] | 2.12±0.15[a] | 22.82±0.88[a] |
| C.D. | 0.98 | 1.42 | 3.68 |
| (Significance level) | (5%) | 5% | (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.

the polyherbal formulation and vitamin 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 immunopotentiating efficacy of Stresroak premix can be well correlated to the findings of Mujeeb Ather (2000) that supplementation of Stresroak in parent broiler flock increased the maternal antibody titre against infectious bursal disease. The reduced mean delayed hypersensitivity (DTH) response after 72 h 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 those of Murray et al. (1988), where an increase in corticosterone levels and a decrease in antibody titer to the vaccines administered 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 and Thaxton, 1986).

## Stress hormones

High ambient temperature induces production and release of corticosteroids (Siegal, 1980), exerts catabolic effects (mobilization of proteins and lipids) through muscle wasting and reduces growth rate (Odedra et al., 1983 and Hayashi et al., 1994). Similar results were obtained in the present study, where serum cortisol levels were significantly ($P \leq 0.05$) higher in the control compared to the treatment groups. Higher cortisol in thermal stressed birds might be mediated through enhanced CRH-ACTH-corticosteroid activity acting through the hypothalamo-pituitary-adrenal cortex axis. In the present study, T4 levels are significantly higher in treated groups than control groups which can be correlated to suppression in plasma thyroid hormone concentration in heat stressed birds possibly 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 women (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 Stresroak has been seen to reverse the trend exhibiting a stress ameliorative effect.

## Conclusion

It can be concluded that the concentration of total phenolics, DPPH scavenging activity and chelating activity of the polyherbal premix was higher than vitamin C. Estimation of biochemical parameters revealed that vitamin C was comparatively more efficient in normalizing the biochemical parameters, namely plasma glucose, total protein and erythrocytic antioxidant enzyme GSSG. In contrast, supplementation of the polyherbal premix was efficacious in normalizing values of plasma cholesterol and the antioxidant enzyme SOD after 3 weeks. Both the antioxidants were found to significantly improve the cell mediated and humoral immune response, decrease the total plasma cortisol level and increase the total thyroxine level as estimated at the end of the 6$^{th}$ week.

The polyherbal premix (Stresroak) (1 kg/tone) 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 a larger number of birds to determine the most economical dose of these herbal antioxidants required for heat stress amelioration.

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