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K Prashanth Kumar
Ph. D Scholar, Department of Poultry Science College of Veterinary Science, P. V. N. R Telangana Veterinary University, Rajendranagar, Hyderabad, Telangana, India

V Ravinder Reddy
Professor & University Head, Department of Poultry Science College of Veterinary Science, P. V. N. R Telangana Veterinary University, Rajendranagar, Hyderabad, Telangana, India

M Gnana Prakash
Principal Scientist, Poultry Research Station, P. V. N. R Telangana Veterinary University, Rajendranagar, Hyderabad, Telangana, India

Correspondence
K Prashanth Kumar
Ph. D Scholar, Department of Poultry Science College of Veterinary Science, P. V. N. R Telangana Veterinary University, Rajendranagar, Hyderabad, Telangana, India

Amelioration of heat stress induced oxidative damage in broilers by supplementing Ashwagandha (*Withania somnifera*) extract during summer

K Prashanth Kumar, V Ravinder Reddy and M Gnana Prakash

Abstract
An experiment was conducted to elucidate heat stress alleviating effect of supplementation of Ashwagandha extract (WS) on growth performance, carcass traits, stress parameters and intestinal *E. coli* counts in commercial broilers reared during hot summer. Day old broiler chicks (n=160) were randomly allocated to 4 groups consisting of 8 replicates with 5 chicks each from 1 to 42 day of age. The four groups were 1. Positive control (PC) (Basal diet +Vit-E 70mg/kg + Se 0.15 mg/kg), 2. Negative control (NC)-basal diet without antioxidant, 3. WS50 (Basal diet + 50 mg WS extract/kg diet) and 4. WS100 (Basal diet + 100mg WS extract/kg diet). Body weight gain, feed efficiency, feed intake, intestinal *E. coli* counts were affected ($P<0.05$) by supplementation of WS. Carcass traits were not affected with WS in broiler diets. Lipid peroxidation (LP) in serum decreased ($P<0.05$), while activities of glutathione peroxidase (GSH-Px), glutathione reductase (GSH-Rx) in serum increased ($P<0.05$) in WS fed groups. In liver and spleen activities of GSH-Px, GSH-Rx and SOD were not affected, while LP decreased ($P<0.05$) in WS supplemented groups. Present study results indicated that WS supplementation enhanced performance, antioxidant status and decreased intestinal *E. coli* counts. However, supplementation of WS did not influence carcass traits in present study.

Keywords: Anti-oxidant enzymes, Ashwagandha, broilers, heat stress, performance

1. Introduction
Heat stress can enhance the formation of reactive oxygen species (ROS), which are generated inside cells during normal bodily functions and cause oxidative stress (Krauss *et al*. 2000) [21]. This increase could cause an imbalance between host anti-oxidant defence and free radicals. Free radicals are highly unstable, reactive and are capable of damaging biological molecules such as DNA, proteins, lipids and cause lipid peroxidation (LPO). During the periods of heat stress, productive energy is diverted to thermoregulatory adaptations which results in oxidative stress induced immunosuppression, high mortality rates (Cahaner and Leestra, 1992 [12]). The body has its own defense mechanisms that protects the cell against cellular oxidants and prevent their accumulation (Tainiguchi M *et al*. 1992) [44]. The major antioxidant defence mechanisms of living cells include enzymatic system (Superoxide dismutase (SOD), Glutathione peroxidase (GSH-PX), Catalase) which prevents formation of free radicals and non-enzymatic system (Vit-A, E, C, Se and Glutathione (GSH)) which breaks the peroxidation chain. Increasing antioxidant status and decreasing LP are strategies to combat heat stress in broilers during summer (Lin *et al*. 2006) [25]. Dietary modifications are the most effective ways to alleviate heat stress in broilers by inclusion of anti-oxidants like Vit-E, Vit-C, Selinium. Inclusion of Natural anti-oxidants such as herbs can be suitable substitute for synthetic anti-oxidants and are believed to be safer and less hazardous for animals and human consumption which make them useful as natural animal feed additives (Faixova and Faix, 2008) [17]. Ashwagandha (*Withania somnifera*) commonly known as Indian ginseng has adoptogenic, immunomodulatory and antistress properties. The active constituents of Ashwagandha i. e. flavonoids, withanolides and polyphenolic compounds quenches free radicals and known to increase GSH, catalase and SOD (Uddin *et al*. 2012 [46]. The objective of this study was to evaluate the effect of supplementing broiler diets with different levels of Ashwagandha on performance, antioxidant responses and carcass traits.

2. Materials and Methods

Day old *Cobb* broiler chicks (n=160) were randomly assigned to 32 replicates under 4 dietary treatment groups each with 5 chicks and kept in a closed, ventilated, wire-floor battery caged broiler house under uniform conditions of temperature, humidity, and ventilation. Birds were vaccinated against Newcastle (7th and 28th d) and infectious bursal diseases (15th d) as per the standard vaccination schedule. Maize and soybean meal-based diets were prepared to contain 3050 and 3,150 kcal ME/kg during the starter (0 to 21 d) and finisher (22 to 42 d of age) phases, with respective crude protein contents of 21.5 and 19.5 g/100 g feed (Table 1). The experimental treatment groups were as follows:

1. Positive control (PC)-Corn soybean based diet supplemented with Vit-E 70mg/kg + Se 0.15 mg/kg.
2. Negative control (NC)-Corn soybean based diet without any antioxidant.
3. WS50-Corn soybean based diet supplemented with 50 mg WS extract/kg.
4. WS100-Corn soybean based diet supplemented with 100mg WS extract/kg diet. Water and mash feed were provided adlibitum throughout 42 days trial period. The temperature and relative humidity of shed during trial period were 36.8oC±0.25-39.7oC±0.25 and 47-74% respectively.

Live body weight, feed consumption and feed conversion ratio was recorded at weekly intervals. At the end of 42 days one bird from each replicate was slaughtered and spleen, liver samples were collected. Ready to cook yield (RTC), breast meat yield, liver, heart, gizzard, spleen, abdominal fat and bursa were calculated and expressed as percent of live body weight. On 41st day approximately 4 mL of blood was drawn from the brachial vein of bird selected from each replicate into non-heparinized tubes and serum separated. Serum, spleen & liver samples were collected and stored-20oC until they further analysed for anti-oxidant responses.

2.1 Antioxidant responses

The antioxidant enzymes were estimated in serum, spleen and liver. GSHPx was estimated by Paglia and valentine (1967) [33] and GSHRx activity by method of Carlberg and Mannervik (1985) [13]. Levels of thiobarbituric acid reactive substance (TBARS), which is LP marker, in serum, liver and kidney were assessed by method following Ohkawa *et al*. 1979 [31] and expressed in nmol malondialdehyde/mg protein. SOD and FRAS (Ferric reducing ability of serum) activity by methods of Madesh and Balasubramanian (1998) [26] and Benzie and Strain (1996) [7] respectively.

2.2 Escherichia coli Counts

Two birds from each dietary treatment were slaughtered on 42day and 1 g of intestinal contents (duodeno-jejunal junction) collected aseptically suspended in 9ml of nutrient broth. Serial dilutions of each sample were made in nutrient broth and E. coli counts were recorded on EMB agar by surface spread method. The number of colonies was expressed as 1og10 value (Ahmed and yang, 2017) [3].

2.3 Statistical Analysis

The data were analysed using General Model procedure of Statistical Package for Social Sciences (SPSS) 15th version and comparison of means was done using Duncan's multiple range test and significance was considered at $P<0.05$. Data were subjected to statistical analysis under completely randomized design employing one-way analysis of variance.

3. Results

The effects of supplementation of different levels of Ashwagandha (*Withania somnifera*) extract (WS) on productive performance of broilers under summer conditions were presented in Table 2. There was no significant effect of WS observed on bodyweight gain (BWG) during starter phase (0-3 wks), as the study progresses WS increased bodyweight gain ($P<0.05$) during finisher phase (4-6 wks) and overall growth period. Among experimental groups WS100 recorded highest BWG and NC recorded lowest BWG, while WS50 and PC were comparable. The feed intake was highest in WS100 group, but comparable among WS50, PC and NC groups. Moreover, under heat stress conditions improved ($P<0.05$) feed efficiency was observed in WS100. Whereas, WS50 showed intermediary response to that of PC and NC. Highest mortality (5%) was recorded in NC group, while PC, WS50 and WS100 recorded 0%, 2.5% and 2.5% mortality respectively. The data on carcass traits (% live weight) are detailed in Table.3. No significant difference was observed among dietary treatments for ready to cook yield (%), liver weight (%), breast (%), heart (%), gizzard (%), spleen (%) and abdominal fat (%). Intestinal E.coli counts were significantly ($P<0.05$) decreased in WS50 and WS100 while, comparable among PC and NC (Table.4).

Antioxidant responses of serum and tissue were shown in Table.5 & 6. Supplementation of WS linearly decreased lipid peroxidation (LP) of serum. Activities of GSH-Px, GSH-Rx and SOD were significantly ($P<0.05$) higher in WS100 and was comparable to PC. The activities of GSH-Px, GSH-Rx and SOD in WS50 group were similar to that of NC. No significant effect of WS on activities of GSH-Px, GSH-Rx and SOD was observed in spleen and liver. LP of spleen and liver was significantly ($P<0.05$) reduced in WS50 & WS100 groups.

4. Discussion

4.1 Performance

In the present study supplementation of Ashwagandha extract in broiler diets during hot summer has improved bodyweight gain and feed efficiency. Similar findings were reported by jadhav *et al*. (2008) [18] by feeding ashwagandha in broilers during summer (40oC-45oC) and also similar reports observed by Ansari J *et al*. (2013) [6], Ahmed *et al*. (2014) [2], Ottalwar *et al*. (2015), Vasanthakumar *et al*. (2015) [47]. This improvement could be related to quenching of free radicals that generated during high temperatures by polyphenols in ashwagandha and stimulant effect of Withanine, Withanolides 1 & 2 on antioxidant enzymes, digestive enzymes in intestine and pancreas that might have improved digestion and performance (Lilja 1983) [24]. Ashwagandha also have anabolic effects on liver and body tissues through increasing thyroid hormone activity (More *et al*. 1980 [29]; Analagan and Sadique, 1981) [5]. Present experiment revealed WS supplemented groups had lower mortality compared to NC group. Results were inconsistent with Biswas *et al*. (2012) [11]; Kumari *et al*. (2015) [22, 23]; Joshi *et al*. (2015) [20] and singh *et al*. (2017) [40]. The death of chicks during starter phase is due to unabsorbed yolks. Better livability might be due to health restorative activity, adoptogenic and antistress properties of withanolides in ashwagandha (Ansari J *et al*. 2013) [6].

4.2 Carcass traits

Carcass traits in terms of breast meat yield %, liver %, gizzard %, heart %, spleen %, abdominal fat % and ready to cook yield (RTC) % were not affected with (P>0.05) supplementation of WS Table 3. Similar results have been reported, wherein feeding Ashwagandha revealed insignificant effects on carcass traits (Javed *et al*. (2009) [19], Ansari J *et al*. 2013) [6] in broilers. On the contrary Saini *et al*. (2014) [39], Abdul Abass *et al*. (2015) [1] and Vasanthakumar *et al*. (2015) [47] reported significant effect on carcass traits.

4.3 Anti-oxidant responses of serum and tissue (Liver & Spleen)

In the present study supplementation of WS linearly (P<0.05) decreased the LPO levels in serum Table 5. Which were in consistence with the findings of Battacharya A *et al*. (2001) [9]; Pandey *et al*. (2009) [35]. On the contrary, the activities of glutathione peroxidase (GSHPx), glutathione reductase (GSHRx), and superoxide dismutase (SOD) (Visavadiya *et al*. 2010) increased (P<0.05) linearly with increasing doses of WS (Bharavi *et al*. 2010 [8]; Sujatha *et al*. 2010 [40]; Vasanthakumar *et al*. 2015) [47] in broiler diets compared to NC group, while serum FRAS levels were unaffected (P>0.05) by feeding WS. Concentrations of GSHPx, GSHRx and SOD in liver and spleen were unaffected Table 6. Whereas, LPO in spleen and liver were significantly (P<0.05) decreased in groups fed with WS than other groups (Ansari J *et al*. 2013 [6]. SOD and GSHPx, GSHRx catalyses the reduction of super oxide ions (O2-) to hydrogen peroxide (H2O2-) and H2O2- to water (H2O) respectively and protects biological cells from oxidative damage (Papp *et al*. 2007) [36]. LPO is a process, which damages the cell membranes and accumulation of ROS during summer induces LPO and oxidative stress. Withanolides, Sitoindosides VII-X and Withaferin-A in Ashwagandha increases the activities of GSHPx, GSHRx, CAT and SOD (Uddin *et al*. 2012) [46] and decreases the LPO of biological cell membranes by quenching ROS that accumulated during hot summer and exhibits antistress activity (Battacharya *et al*. 1987) [10]. Flavonoids, natural polyphenolic compounds present Ashwagandha donates their H + ions to free radicals and reduces oxidative stress by stabilising ROS (Pandey and Rizvi 2009) [35].

4.4 *Escherichia coli* counts

In the present study birds supplemented with WS in diets have reduced E. coli counts than other groups (Table 4). Significantly lower E. coli counts were observed in WS100 group while, all other groups were comparable. Similar results were reported by El-Boshy *et al*. (2013) [16] and Sundaram *et al*. (2011) [41]; Alam *et al*. (2012) [4]; and Kumari and Gupta *et al*. (2015) [22, 23]. Antibacterial effect of WS is attributed to their immunopotentiating compounds (Mishra *et al*. 2000 [27]; Dhuley. 1998) [15] and increase in phagocytosis and intracellular killing by macrophages (Threlfall *et al*. 1996 [42]; Dhuley.1998) [15]. Phenolics and Anthocyanins cause hyper acidification at the plasma membrane interface of microbes and disrupts H+-ATPase required for ATP synthesis (Vattem *et al*. 2004) [45].

5. Conclusion

The conclusion of present study indicated that supplementation of Ashwagandha extract at 100mg/kg diet as natural antioxidant had improved the growth performance and increased the anti-oxidant status (Decreases lipid peroxidation, increases the activities of GSH-Px, GSH-Rx and SOD), which means reduced effects of oxidative damage during summer.

List of Tables

Table 1: Ingredient and nutrient composition of basal diet

Ingredients (%)	Starter (0-21d)	Finisher (22-42d)
Maize	56.9	61.31
Soya bean meal	35.43	30.60
Vegetable oil	3.355	4.287
Salt	0.423	0.422
Dicalcium phosphate	1.602	1.542
Limestone powder	1.343	1.177
DL-methionine	0.278	0.217
L-Lysine	0.241	0.076
$AB_2D_3K^*$	0.015	0.015
B-complex**	0.015	0.015
Trace mineral mixture***	0.100	0.100
Nutrient composition		
ME (kcal/kg)	3050	3150
Crude protein (%)	21.5	19.5
Lysine (%)	1.25	0.98
Methionine (%)	0.56	0.48
Calcium (%)	0.9	0.82
Available Phosphorous (%)	0.42	0.40
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*AB2D3K provided per kg diet: Vitamin A 20000 IU, Vitamin B2 25 mg, Vitamin D3 3000IU, Vitamin K 2mg.

**Riboflavin 25mg, Vitamin B1 1mg, Vitamin B6 2mg, Vitamin B12 40mg, and Niacin 15mg.

***Trace mineral provided per kg diet: Manganese 120mg, Zinc 80mg, Iron 25mg, Copper 10mg, Iodine 1mg

Table 2: Effect of Ashwagandha extract on performance and mortality (%)

Treatment	Feed intake (g/bird)			Feed intake (g/bird)			Feed efficiency (feed intake/body weight gain)			Mortality (%)		
	Phase			Phase			Phase			Phase		
	Starter (0-3 wk)	Finisher (4-6 wk)	Overall (0-6 wk)	Starter (0-3 wk)	Finisher (4-6 wk)	Overall (0-6wk)	Starter (0- 3wk)	Finisher (4-6 wk)	Overall (0-6 wk)	Starter (0-3 wk)	Finisher (4-6 wk)	Overall (0-6 wk)
PC	618.7	1140b	1759^{b}	825	2,160	2,986^{b}	1.334	1.895^{b}	1.697^{b}	0	0	0
NC	626.9	1095^{c}	1722^{b}	845	2,135	2,981^{b}	1.349	1.950^{a}	1.731^{a}	0	5	5
WS50	612.7	1138^{b}	1751^{b}	831	2,177	3,008^{ab}	1.356	1.913^{ab}	1.718^{ab}	2.5	0	2.5
WS100	640.1	1201^{a}	1842^{a}	848	2,218	3,067^{a}	1.326	1.846^{c}	1.664^{c}	2.5	0	2.5
SEM	5.0	9.5	10.9	5.13	11.34	12.36	0.004	0.009	0.006			
P-Value	0.257	0.000	0.000	0.323	0.060	0.046	0.075	0.000	0.000			

Each mean represents data from eight replicates.
a,b,c Means bearing different superscripts in a column differ significantly (P<0.05)
PC-Positive controls; NC-Negative control; WS50-Ashwagandha extract @ 50mg/kg, WS100-Ashwagandha extract 100mg/kg

Table 3: Effect of Ashwagandha extract on various carcass traits (% live weight) in broier chicken (0-42 d)

Treatment	Breast	liver	heart	gizzard	spleen	RTC	Abdominal fat
PC	17.98	1.8380	0.48	1.83	0.09	66.83	1.14
NC	18.36	1.6868	0.44	1.99	0.08	68.98	1.25
WS50	18.69	1.9141	0.46	1.85	0.09	66.97	1.38
WS100	19.12	1.5419	0.42	1.76	0.07	66.91	1.12
SEM	0.314	0.065	0.012	0.035	0.007	0.591	0.077
P-Value	0.639	0.180	0.370	0.135	0.774	0.531	0.619

Each mean represents data from eight replicates.
A, b, c Means bearing different superscripts in a column differ significantly (P<0.05)
PC-Positive controls; NC-Negative control; WS50-Ashwagandha extrac @ 50mg/kg; WS100-Ashwagandha extract 100mg/kg

Table 4: Effect of Ashwagandha extract on Escherichia coli counts in broier chicken (0-42 d)

Treatment	*Escherichia coli* (log_{10} of cfu/ml count)
PC	6.684^{a}
NC	6.725^{a}
WS50	6.446^{ab}
WS100	6.234^{b}
SEM	0.066
P-Value	0.010

Each mean represents data from eight replicates.
A, b, c Means bearing different superscripts in a column differ significantly (P<0.05).
PC-Positive controls; NC-Negative control; WS50- Ashwagandha extract @ 50mg/kg, WS100-shwagandha extract 100mg/kg; NDV-Newcastle disease virus; PHA-P-Phytohaemagglutinin-phosphate.

Table 5: Effect of Ashwagandha extract on serum anti-oxidant responses in broiler chicken (1-42 d)

Treatment	GSH-Px (units/ml)	GSH-Rx (units/ml)	FRAS (µ mol/ml)	LP (nmol MDA/mg protein)	SOD (units/mg protein)
PC	350.6^{bc}	412.3^{ab}	1198	6.275^{b}	3.018^{b}
NC	277.3^{a}	380.5^{a}	1096	8.663^{c}	1.418^{a}
WS50	301.1^{ab}	381.1^{a}	1148	6.736^{b}	1.631^{a}
WS100	372.2^{c}	416.1^{b}	1213	4.438^{a}	2.760^{b}
SEM	10.86	6.06	27.4	0.323	0.176
P-Value	0.003	0.045	0.443	0.000	0.000

Each mean represents data from eight replicates.
A, b, c Means bearing different superscripts in a column differ significantly (P<0.05)
PC-Positive controls; NC-Negative control; WS50-Ashwagandha extrac @ 50mg/kg; WS100-Ashwagandha extract 100mg/kg; GSH-Rx-Glutathione peroxidase; GSH-Rx-Glutathione reductase; FRAS-Ferric reducing ability of serum; LP-Lipid peroxidation; SOD-Superoxide dismutase

Table 6: Effect of Ashwagandha extract on spleen and liver anti-oxidant responses in broiler chicken (1-42 d)

	Spleen				Liver			
Treatment	**GSH-Px (units/ml)**	**GSH-Rx (units/ml)**	**SOD (units/mg protein)**	**LP (n mol MDA/mg protein)**	**GSH-Px (units/ml)**	**GSH-Rx (units/ml)**	**SOD (units/mg protein)**	**LP (n mol MDA/mg protein)**
PC	424.2	396.3	3.55	5.60[a]	409.9	400.6	3.36	5.02[bc]
NC	392.5	369.0	2.65	6.96[b]	387.7	375.1	2.35	5.47[c]
WS50	407.6	392.5	2.84	5.93[a]	384.9	378.1	2.21	4.45[b]
WS100	425.0	418.9	3.37	5.05[a]	421.2	406.1	2.75	3.75[a]
SEM	8.03	11.37	0.297	0.206	9.91	11.30	0.213	0.173
P-Value	0.456	0.523	0.706	0.002	0.536	0.729	0.227	0.000

Each mean represents data from eight replicates.

A, b, c Means bearing different superscripts in a column differ significantly ($P<0.05$)

PC-Positive controls, NC-Negative control, WS50-Ashwagandha extrac @ 50mg/kg, WS100-Ashwagandha extract 100mg/kg; GSH-Rx-Glutathione peroxidase; GSH-Rx-Glutathione reductase; FRAS-Ferric reducing ability of serum; LP-Lipid peroxidation; SOD-Superoxide dismutase

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