

## SHORT COMMUNICATION

# Effect of dietary supplementation of phytogenic feed additive on performance traits, serum neopterin, and cutaneous basophil hypersensitivity response in heat-induced stress model of broiler chickens

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### ABSTRACT

**Objective:** The trial was aimed at assessing the effect of phytogenic feed additive (PFA), a natural adaptogen, on growth performance, serum neopterin level, and cutaneous basophil hypersensitivity (CBH) response in heat-induced stress model of broilers.

**Materials and Methods:** One-day-old Ross 308 chicks ( $N = 360$ ) were randomly distributed among normal control (NOR), heat-stress control (HSC), and PFA treatment (HSC plus PFA at 200 gm/ton of feed) group. HSC and PFA groups were subjected to heat stress (HS) ( $32^{\circ}\text{C}$ – $36^{\circ}\text{C}$ ) from 9:00 a.m. to 5:00 p.m. for 35 days. The impact of HS on growth performance, serum neopterin level, and CBH response was assessed.

**Results:** High ambient temperature worsened the performance traits [bodyweight ( $p < 0.05$ ) and feed conversion ratio] and significantly lowered the serum neopterin level and CBH response in the HSC group when compared to the NOR group. However, supplementation of PFA at 200 gm/ton of feed to birds mitigated the detrimental effects of HS.

**Conclusion:** PFA at 200 gm/ton demonstrated the immunomodulatory effect through the restoration of serum neopterin level, CBH response, and growth performance traits in heat-stressed broiler chickens. Thus, PFA can be used as a natural adaptogen to increase the stress resistance and mitigate the negative *consequences* of various stressors in broiler chickens.

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Broiler, heat stress, neopterin, cutaneous basophil hypersensitivity, phytohemagglutinin-P, cell-mediated immune response.



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## Introduction

Poultry production is one of the rapidly evolving segments in the livestock industry in developing countries [1], but its sustainability is affected by several environmental factors [2]. Modern breeds of broiler chickens are highly susceptible to different climatic conditions such as temperature, humidity, and radiation that directly affect the thermoregulatory system, eventually causing stress in broilers [3]. Stress is a nebulous stimulus of an organism that disturbs the normal physiological equilibrium or homeostasis [4]. Stress due to high ambient temperature has become a substantial economic burden to the livestock industry during the summer

season [5], and the broiler chickens are highly sensitive to heat stress (HS) due to the feathering and absence of sweat glands [6]. Moreover, HS remarkably contributes to the distress of animals leading to changes in pulse rate, respiration rate, and metabolism, thus affecting their growth, production, and immune system [7]. It is an essential unit of the defense machinery to safeguard the livestock in response to pathogenic infection. Indeed, invading pathogens are neutralized by the innate and adaptive immune system [8]. Typically, exposure to an erratic temperature pattern modified several components of the immune function in

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the blood, namely, T-cell counts, lymphocyte activation, and cytokine secretion in heat-stressed chickens [9]. However, an extended period of HS can overwhelm the thermoregulatory mechanism and result in reactive oxygen species (ROS)-mediated immunosuppression, increasing the susceptibility of birds to invading pathogens, and inferior production performance [10]. Therefore, particular attention should be given to enhance the bird's immune system, which further diminishes the vulnerability of HS in broilers.

Erratic temperature pattern activates the pituitary-adrenal axis by altering the hypothalamic neuroendocrine functions of the poultry [11]. This, in turn, releases the peptide hormone corticotropin, which further acts on the anterior part of the pituitary gland to produce another polypeptide tropic hormone known as adrenocorticotropin hormone [12]. Then, it stimulates the cortex of the adrenal gland to secrete the glucocorticoids, a reliable stress indicator in poultry birds [13]. Elevated levels of circulating corticosteroids contribute to the re-establishment of homeostasis by varying the oxidative metabolic pathway, compromising the essential cellular functions, and thus cell membrane damage, loss of muscle mass, and growth retardation [14]. In chickens, as in mammals, T cells play a critical role in the regulatory mechanism of cell-mediated immunity, and it is critically associated with CD4+ T helper ( $T_h$ ) cells [15]. When the body encounters an antigen,  $T_h$  cells secrete predominantly interferon-gamma (IFN- $\gamma$ ), a key pro-inflammatory molecule that provides essential signals to activate the macrophages differentiated from monocytes [16], which further exclusively produces the neopterin [17]. The determination of neopterin concentration in body fluids is an innovative tool for monitoring stress-related pathologies [18]. Phytohemagglutinin-P (PHA-P) is a mitogenic agent which predominantly stimulates the proliferation of  $T_h$  cells in vertebrates [19]. In chickens, intradermal injection of PHA-P leads to activation of basophil infiltration by PHA-P stimulated  $T_h$  cells [20]. Basophil infiltration actively contributes to the immediate hypersensitivity reactions [21]. Thus, cutaneous basophil hypersensitivity (CBH) response can be used as a tool to assess cell-mediated immunity in broilers.

Minimizing immunosuppression is an important alternative strategy to reduce the burden of HS in the broiler industry [22]. However, strategies to control the immunosuppression are mainly dependent on the vaccination programs for poultry management to minimize HS [23]. The use of natural adaptogens as immunomodulators in poultry is a unique approach to boost the resistance of animals and to reduce the vulnerability to invading pathogens [24]. These adaptogens are known to increase the resistance of the body against a variety of the infection by triggering both humoral and cell-mediated immune pathways [25]. Unfortunately, a

limited number of studies have demonstrated the immunomodulatory effect of herbal adaptogens in broilers. Hence, there is an obvious need to explore the evidence for the efficacy of these herbal adaptogens in target animal species. Phytocee, a phytogetic feed additive (PFA) formulated by Natural Remedies Private Limited, Bengaluru, India, was reported to have an anti-stress effect in broiler chickens [26]. However, this PFA is not scientifically validated for its herbal adaptogenic effect to boost the immune system, to nullify the detrimental impact of stress caused by high ambient temperature in broilers. Therefore, the current study was aimed to assess the immunomodulatory property of PFA through evaluating the effect of HS on growth performance, serum concentrations of neopterin, and cutaneous basophil hypersensitive response in broiler chickens.

## Materials and Methods

### Chemical and reagents

PHA-P was purchased from Sigma Aldrich, USA. Neopterin Enzyme-linked Immunosorbent Assay (ELISA) Kit was procured from IBL GmbH, Germany. All other chemicals used were of analytical grade.

### Phytogetic feed additive

Phytocee is a PFA formulated by Natural Remedies Private Limited, Bengaluru, India, containing *Embllica officinalis* fruits, *Ocimum sanctum* whole plant, and *Withania somnifera* roots.

### Study plan and ethical approval

All animal procedures were performed according to the ethical norm of Natural Remedies Private Limited, with the approved study protocol (IEC No: - AHS/PLT/03/2018). An experiment of 42 days duration was conducted using 1-day-old Ross 308 chicks ( $N = 360$ ) randomly distributed among normal control (NOR), heat-stress control (HSC), and PFA treatment (HSC plus PFA at 200 gm/ton) groups, with six replicates of 20 chicks each. HSC and PFA groups were subjected to HS (32°C–36°C) from 9:00 a.m. to 5:00 p.m. for 35 days (day 8 to day 42) using incandescent bulbs. Upon arrival, a sucrose solution (4%) was provided for the first 4 h to revive the dehydrated chicks. After randomization, the chicks were evenly distributed to the individual semi-closed pen and tagged with a wing band. House temperature, humidity, and photoperiod were maintained using brooder (Table 1). The birds were fed with poultry mash feed (Higain Feeds & Farms India Pvt. Ltd., Bengaluru) *ad libitum* with the help of a chick feeder/jumbo feeder (Table 2). The chicks were administered with Marek's disease vaccine at hatching, infectious bursal

**Table 1.** Environmental conditions.

Day	Temperature at chick level	Relative Humidity (%)	Photoperiod
1–3	29°C–30°C	60–70	23 h light and 1 h darkness
4–7	28°C–30°C	50–60	
8–9	27°C–28°C	50–60	22 h light and 2 h darkness
10–11	27°C–28°C	50–60	21 h light and 3 h darkness
12–26	25°C–27°C	50–60	20 h light and 4 h darkness
> 27	24°C–27°C	50–60	

**Table 2.** Ingredients and nutrient composition of the basal diet.

Ingredients (Kg/ton)	Pre-starter	Starter	Finisher
Corn	550.0	596.4	580.0
Rice bran	24.5	-	45.2
Soya bean meal CP 46.5%	340.0	290.0	274.0
Corn gluten meal CP 60%	-	19.6	-
Deoiled rice bran	17.0	16.2	17.3
Limestone fine	11.0	9.0	8.0
Dicalcium phosphate	14.5	14.5	12.0
Common salt common	2.3	2.5	2.8
Sodium bicarbonate	3.0	3.2	2.6
Blended oil (Veg)	21.0	33.0	44.0
L-Threonine 98.5%	1.10	1.00	0.80
L-Lysine	3.00	2.80	1.65
DL-Methionine 99%	3.40	2.90	2.60
Choline chloride 60%	0.65	0.60	0.50
Trace mineral mixture <sup>a</sup>	1.20	1.20	1.20
Organic trace mineral	-	-	0.30
Betaine	0.75	0.75	0.75
Mycotoxin binders	1.00	1.00	1.00
Organic selenium	0.06	0.06	0.06
Sodium butyrate	0.50	0.25	0.25
Premix broiler <sup>b</sup>	5.00	5.00	5.00
Metabolizable Energy (Kcal)	3000	3125	3150
Crude Protein	21	20	18.5

<sup>a</sup>The trace mineral premix provided the following per kilogram of TM premix: Fe, 40 g; Cu, 10 g; Mn, 100 g; Zn, 100 g; Se, 0.25 g; and I, 1.5 g.

<sup>b</sup>The broiler premix provided the following per kilogram of broiler premix: antioxidants, 0.125 g; emulsifier, 0.500 g; phytase, 0.100 g; acidifier, 1.00 g; vitamin premix<sup>c</sup>, 0.500 g; deoiled rice bran, 2.775 g.

<sup>c</sup>The vitamin premix provided the following per kilogram of vitamin premix: vitamin A, 25 MIU; vitamin D3, 5 MIU; vitamin E, 24 IU; vitamin K, 3 g; vitamin B1, 3 g; vitamin B2, 10 g; vitamin B6, 4 g; vitamin B12, 0.015 g; niacin, 30 g; pantothenic acid, 20 g; folic acid, 1 g.

disease (IBD-MB) vaccine on day 12, and Newcastle disease (ND-VH) vaccine on days 5, 18 (first booster dose), and 30 (second booster dose).

### Zootechnical parameters

Each group was observed for mortality once daily for six weeks. The individual chick body weight was recorded at 1, 21, and 42 days. Feed conversion ratio (FCR) is the ratio of feed intake and total body weight, which was calculated at 21 and 42 days.

### Cutaneous basophil hypersensitivity test

CBH test was performed by the previously described method [27]. Briefly, the bird's right and left foot were intradermally injected with PHA-P (0.1 ml) and saline, respectively. The thickness of the skin (mm) on both feet was measured using a digital micrometer (Mitutoyo, Tokyo, Japan) before inoculation and after 24 h of inoculation. The PHA (right foot) and saline response (left foot) were calculated by deducting the pre-injection skin thickness (mm) from post-injection skin thickness (mm). The percentage of CBH response was calculated using the following formula:

$$= \left[ \frac{\text{PHA response/pre-injection thickness, right foot} - \text{saline response/pre-injection thickness, left foot}}{\text{pre-injection thickness, right foot} - \text{pre-injection thickness, left foot}} \right] \times 100.$$

### Serum neopterin level

The neopterin concentration was estimated by the previously described method [28]. Briefly, serum samples (15 µl) were mixed with the conjugated enzyme (120 µl) and neopterin antiserum (60 µl) in microtiter well plate and kept at room temperature for 120 min on a mechanical shaker. Then, it was washed using wash buffer and subsequently incubated with a substrate solution (180 µl) for 15 min at room temperature. Finally, the stop solution (150 µl) was added to each well to stop the substrate reaction and measured the optical density (within 60 min) at 450 nm using a Tunable Microplate reader (VersaMax, Molecular Devices Inc., Sunnyvale, CA).

### Statistical analysis

The mean data were calculated and statistically analyzed using Statistical Package for the Social Sciences software [26]. One-way analysis of variance (using location as a blocking factor) was employed to calculate the statistical difference. Least Significant Difference (LSD) multiple comparison test with the significance level of 0.05 was used to separate the means between the groups.

## Results

### Performance traits

The birds exposed to high ambient temperature (32°C–36°C) displayed a drastic reduction ( $p < 0.01$ ) in body weight gain when compared to NOR birds on day 42.

However, PFA supplementation had numerically improved the body weight gain in birds subjected to HS. FCR was found to be significantly worsened in HSC as compared to NOR, whereas it was improved by PFA addition in heat-stressed birds (Table 3).

### Serum neopterin

In our study, NOR birds showed the baseline level of neopterin production ( $7.23 \pm 1.73$  nmol/l), while the serum neopterin level was significantly ( $p < 0.05$ ) lowered in birds exposed to HS. Nevertheless, serum neopterin level was restored in birds fed with diet containing PFA (200 gm/ton), which was at par with the NOR level as well (Table 3).

### CBH response

Heat-stressed broilers exhibited a very low CBH (%) response when compared to NOR birds, whereas the CBH response was significantly increased at 24 h of PHA-P challenge in the PFA group when compared to both HSC and NOR (Table 3).

### Discussion

Birds are continuously exposed to biological/production stressors (cold, heat, and stocking density) and nutritional stressors (fasting, feed restriction, and dietary protein deficiency). More specifically, thermal stressors are considered a severe health problem in the poultry industry [6,7], as it disrupts the thermoregulatory mechanism of birds and makes it difficult to dissipate excess heat [26,29]. Therefore, the addition of nutritional supplements in the poultry diet is considered as a solution to improve the growth and immunity of the birds, especially under HS. Many natural adaptogenic agents are known to afford the protection against diverse stressors by increasing the resistance of the body to a variety of infections [25]. Hence, in the current study, we used the PFA as a natural

adaptogen to alleviate the burden of HS through boosting the immune system in broilers.

In the current study, the stress model was developed in broilers by exposing them to high temperature ( $32^{\circ}\text{C}$ – $36^{\circ}\text{C}$ ) from 9:00 a.m. to 5:00 p.m. for 35 days, as high ambient temperature caused an unfavorable outcome on the well-being of the birds and reduced the performance traits [26]. Similarly, we found the broiler chickens raised under HS conditions gained low bodyweight gain with high feed intake, indicated that HS caused a negative impact on the zootechnical parameters of broilers. Likewise, Sohail et al. [30] and Niu et al. [31] demonstrated that broilers subjected to HS had significantly lowered the feed intake, lowered the body weight gain, and increased the FCR at 42 days of age. PFA in the current scenario is gaining importance as it improved the growth performance in poultry. The broilers fed with *W. somnifera* root powder rich diet significantly showed the enhanced performance than other groups, which was evidenced in terms of body weight gain [32]. Several lines of evidence also showed an improvement in the body weight gain in broilers fed with PFA containing *E. officinalis* fruit powder [33,34]. Similarly, in the current study, PFA was found to improve the zootechnical parameters, which was consistent with the previous findings. This indicates that the negative impact due to heat-stress could be ameliorated by PFA supplementation, which was mainly due to the presence of *W. somnifera* and *E. officinalis*. Thus, PFA can be used as a supplement to improve productivity in heat-stressed broiler chickens.

CBH skin test is a diagnostic tool to investigate the cell-mediated immune responses [27]. It is well established that CBH reactivity is determined by the level of basophil infiltration at PHA-P injected site of skin [35]. This study revealed that CBH response was dramatically reduced in broilers under HS, which was a coincidence with the results of Gao et al. [36]. However, PFA supplementation increased the CBH response to PHA-P injection

**Table 3.** Effect of PFA on growth performance, serum neopterin, and CBH test in broiler chickens.

Parameters	Time Points	NOR	HSC	HSC + PFA (200 g/ton)
Bodyweight (gm) (n = 117–120)	Day 1	44.11 ± 0.31	45.00 ± 0.31	44.36 ± 0.33
	Day 21	<sup>a</sup> 750.84 ± 11.58	<sup>a</sup> 748.08 ± 12.47	<sup>a</sup> 746.64 ± 11.29
	Day 42	<sup>a</sup> 2415.76 ± 36.78	<sup>b</sup> 2275.19 ± 33.36	<sup>b</sup> 2294.29 ± 32.76
FCR (n = 6)	Day 21	<sup>a</sup> 1.632 ± 0.04	<sup>a</sup> 1.609 ± 0.03	<sup>a</sup> 1.571 ± 0.04
	Day 42	<sup>a</sup> 1.831 ± 0.02	<sup>a</sup> 1.878 ± 0.01	<sup>a</sup> 1.834 ± 0.02
Neopterin (nmol/l) (n = 7–10)	Day 42	<sup>a</sup> 7.23 ± 1.73	<sup>b</sup> 3.83 ± 0.50	<sup>a</sup> 7.22 ± 1.14
CBHT (% Response) (n = 11–12)	24 h	<sup>a</sup> 21.48 ± 4.32	<sup>a</sup> 15.94 ± 2.70	<sup>bc</sup> 34.94 ± 4.63

NOR, normal control; HSC, heat stress control; PFA, phytochemical feed additive; FCR, feed conversion ratio; CBHT, cutaneous basophil hypersensitivity test; Values are expressed as mean ± standard error of the mean; <sup>a,b</sup>Means bearing different superscripts in the same row differs significantly ( $p < 0.05$ ) by one-way analysis of variance (ANOVA) followed by LSD.

in heat-stressed birds, which was even higher than NOR. The obtained response could be due to the presence of herbal ingredients, which was reinforced by Singh et al. [37], who demonstrated that a higher level of CBH response in broilers fed with *W. somnifera* and vitamin E rich diet. Similar to our results, Kamboh et al. [27] demonstrated the increase in cutaneous basophils hypersensitivity response followed by supplementation with genistein and hesperidin in broilers. Furthermore, it was confirmed that dietary supplementation of *O. sanctum* leaf extracts showed a protective effect in attenuating both antibody and cellular mediated immune reaction in rats exposed to restraint stress [38].

Several studies showed that HS could disrupt the physiological and immunological status of broilers [5]. Stress in birds is followed by an increase in glucocorticoids, which further promotes the apoptosis of both lymphocytes' cells and, therefore, suppresses the immunity [13]. Glucocorticoids, the primary regulator of the stress response, demonstrates a key role in influencing the synthesis of neopterin. Neopterin is produced by macrophages upon stimulation by IFN- $\gamma$  derived from activated T<sub>H</sub> cells under stressful conditions [16,17]. In our study, decreased neopterin production was observed in broilers when exposed to HS, which could be linked to the inhibition of cell-mediated immune response caused by the high ambient temperature [39]. Our data also revealed that dietary supplementation of PFA significantly increased the serum neopterin level, equivalent to NOR bird's level, in heat-stressed broiler chickens. The elevated serum neopterin strongly suggested that PFA can boost the cell-mediated immune response by activating macrophages triggered by T<sub>H</sub> cells [17,19]. Similar to our result, Mandal et al. [40] demonstrated that the dietary addition of *E. officinalis* fruit powder significantly boosted the cell-mediated immunity in broilers reared under high ambient temperature. Similarly, *O. sanctum* was reported as a potent adaptogen as it augments the T-cell mediated immunity in birds [41]. Moreover, *W. somnifera* extracts were suggested to use as an immunomodulatory agent as it enhances the cell-mediated immune response in chicks [42].

The adaptogenic potential of PFA could be understood by examining its ingredients, such as *E. officinalis*, *W. somnifera*, and *O. Sanctum*. Hasan et al. [43] found that *E. officinalis* fruit's pulp extract stimulated the cell-mediated immune response in mice. *W. somnifera*, one of the ingredients of PFA, modulates the effects on hypothalamus-pituitary-adrenal axis and thereby enhances the resistance to stress in adults suffering from mild stress [44]. Another ingredient of PFA, *O. sanctum* is a well-known immunomodulator as it improves the adaptation to stress and is proven as an herbal adaptogen in rats as well [45]. The above literature

support substantiates that improvement in cutaneous basophil hypersensitivity test (CBHT) response and serum neopterin level in HS birds upon supplementation of PFA signifies the stimulation of cell-mediated immunity leading to better performance traits in broilers. However, further studies required to strengthen our current findings and to identify the underlying molecular mechanisms of action of PFA on stimulation of cell-mediated immunity and amelioration of HS in broiler chickens.

## Conclusion

In the current study, the HS model was developed by exposing the birds to high ambient temperature (32°C–36°C), which was characterized by worsening of performance parameters and cell-mediated immunity (decreased serum neopterin level and cutaneous basophil response). The supplementation of PFA improved the cell-mediated immune reaction and mitigated the negative impact of HS on broilers, and hence it can be used as a natural adaptogen to boost immunity against heat-induced stress in broilers chickens.

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## Conflict of interest

The authors confirm that there is no conflict of interest.

## Authors' contribution

Arigesavan Kaninathan prepared the document. Saravanakumar Marimuthu critically revised the document, performed the statistical analysis, and interpreted the compiled data. Ramasamy Selvam designed the trial, interpreted the compiled data, critically reviewed, and proofread the document. Prashanth D'Souza designed the trial, interpreted the compiled data, and reviewed the document.

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