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Research Article

EFFECT OF EUGENOL (CLOVE OIL) SUPPLEMENTATION ON HAEMATO-BIOCHEMICAL AND IMMUNOLOGICAL PARAMETERS IN BROILER CHICKS

NEETU NEHRA, DEEPIKA LATHER*, ASHMITA CHAHAL, VIKAS NEHRA and SARTHAK[1]
Department of Veterinary Pathology, [1]Department of Veterinary Physiology and Biochemistry,
College of Veterinary Sciences, Lala Lajpat Rai University of Veterinary and Animal Sciences (LUVAS),
Hisar-125004, Haryana, India

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ABSTRACT

The present study was envisaged with an aim to assess the hepatoprotective, antioxidative and immunostimulatory potential of eugenol, a monoterpene compound as feed additive in broiler chicken *via* its influence on haemato-biochemical indices, oxidative stress parameters and humoral and cell mediated immune response (CMI). A 42 days experimental study was conducted on 60 broiler chicks divided randomly into two groups; A (eugenol supplementation @5% in feed) and B (basal control feed). All the chicks at the age of 7 days were vaccinated intoanasally with F1 strain of NDV for humoral immune response study. Cell mediated immune response (CMI) was determined by delayed type hypersensitivity (DTH) skin test using dinitrochlorobenzene (DNCB) as an eliciting antigen. To study the parameters blood/serum samples were collected from randomly selected six chicks of each group after 7th, 14th 21st, 28th and 35th day post initiation of eugenol feeding. Haematological parameters i.e., haemoglobin (Hb), packed cell volume (PCV), total erythrocyte count (TEC) and total leucocyte count (TLC) revealed comparable values in both the groups. Analysis of oxidative stress parameters superoxide dismutase (SOD) and catalase activities revealed significantly higher values, while lipid peroxidase activity showed significantly lower values in group A as compared to control group. Biochemical parameters like total protein, serum albumin, liver function parameters (ALT, AST and GGT activities) and kidney function tests (serum uric acid and creatinine concentration) revealed significant improvement in serum albumin concentration and alanine transaminase (ALT) activity in eugenol supplemented group in comparison to control group. Immunological studies showed no significant difference in humoral immune response but improvement in CMI was noticed in group-A chicks. It was concluded that supplementing eugenol (0.5%) as feed additive not only enhances the antioxidative status in broiler chicks but also acts as a hepatoprotectant.

Keywords: Broiler chicks, Eugenol, Haemato-bichemical indices, Oxidative stress, Immune response

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Poultry industry has emerged as the fastest growing segment in the world. Feeding is the most important management aspect in poultry production since feed costs account for 60-70 percent of total poultry expense. Since most nations no longer allow the use of antibiotics as growth promoters, non-antibiotic substances with the same potential, such as probiotics, organic compounds, enzymes, and phytogenic chemicals, have been seen as effective replacements (da Silveira Deminicis *et al.*, 2021; Ghasemian *et al.*, 2022). The limitation was imposed due to the rapid growth of antibiotic-resistant strains of bacteria and their transmission through food chain (Gholami-Ahangaran *et al.*, 2021).

Phytochemicals also known as phytobiotics, are broad subset of plant-based non-nutritive bioactive substances that exert protective or disease preventing effects by scavenging free radicals produced after oxidative stress and one such example is eugenol, a major phytogenic chemical found in clove oil. Inclusion of phytochemicals in the commercial diet of animals improves the quality of feed and thus promotes the production performance, increase productivity and enhancing the quality of the products obtained from these animals (Gholami-Ahangaran *et al.*, 2021a; Nagargoje *et al.*, 2023). Various essential oils like lime and oregano oils possess potent antioxidant and antimicrobial activity so can be used as a potent viable alternative to chemical preservative (Kumar *et al.*, 2023). In addition, several researches have demonstrated that phytobiotics can increase the activity of digestive enzyme and absorption potential. Additionally, some research findings suggested that phytogenic compounds could be able to increase intestinal mucus production, which might help the body to recover from pathogen pressure by preventing adhesion to the mucosa (Mohammadi and Kim, 2018). Eugenol (4-allyl-1-hydroxy-2-methoxybenzene), the active principle of clove (*Syzygium aromaticum*) and other herbs (like cinnamon oil, tulsi) is well known for its various pharmacological properties including anaesthetic, antioxidant, antimicrobial, anti-inflammatory, anti-neoplastic, neuroprotective, hypolipidemic and anti-diabetic effectiveness (Cui *et al.*, 2019; Garabadu and Sharma, 2019; Oroojan, 2020; Tandel *et al.*, 2021). Eugenol which is considered as GRAS (generally recognized as safe) and non-mutagenic by World Health Organization (WHO) and is widely used in pharmaceutical industry, health care and cosmetology (Nisar *et al.*, 2021).

*Corresponding author: drdeepika@luvas.edu.in

The present study is conducted to evaluate the hepatoprotective, antioxidative and immunostimulatory potential of eugenol for used as feed additive in broiler chicks.

MATERIALS AND METHODS

The experiment was conducted after the approval of Institutional Animal Ethics Committee (IAEC) vide letter no. VCC/IAEC/2022/1624+51 dated 10/05/2022. The Eugenol {2- Methoxy-4-(2-propenyl) phenol} used in the present study was procured from Sigma-Aldrich (Product Number W246719).

Experimental design: Sixty (60), day-old Cobb broiler chicks were purchased from a local commercial hatchery and reared in cages under strict hygienic conditions with optimum temperature and proper ventilation in the animal house of Department of Veterinary Pathology, LUVAS, Hisar. After 7 days of acclimatization, the chicks were randomly divided into two groups (n=30 each). On the same day, chicks were vaccinated with Newcastle disease vaccine (F1 strain) via intranasal route. Group A chicks were provided with eugenol as feed supplement (@0.5%) in basal feed, whereas chicks of group B were given basal control feed throughout the experiment. Blood and serum samples were collected from six chicks of each group on 7^{th}, 14^{th}, 21^{st}, 28^{th} and 35^{th} days post initiation of eugenol (DPIE) feeding in sterile ethylene diamine tetra acetate (EDTA) coated vials for haematological and oxidative stress parameter studies and in sterile tubes without anticoagulant for serum separation. Serum samples were used for assessing different biochemical parameters and humoral immune response.

Haemato-biochemical studies: Blood samples collected in EDTA coated sterile vials were analyzed for Haemoglobin (Hb), Packed Cell Volume (PCV), Total Erythrocyte Count (TEC) and Total Leukocyte Count (TLC) by using Automatic haematological analyzer (MS4Se-Melet Schloesing Laboratories, France). Serum samples were analysed for different biochemical parameters like total protein, serum albumin, liver function parameters (ALT, AST and GGT activities) and kidney function tests (serum uric acid and creatinine concentration) by using semi-automatic biochemistry analyzer (Erba Mannheim Chem-5 Plus, Transasia) and different kits procured from ERBA diagnostics Mannheim GmbH (Transasia Bio-Medicals Ltd.).

Oxidative stress parameters: 10% RBC lysate was prepared from blood samples collected in EDTA vials for analysis of different oxidative stress parameters like superoxide dismutase (SOD), catalase (Aebi, 1984) and assay of lipid peroxidase (LPO) (Shafiq-ur-Rehman, 1984).

Immunological studies: The humoral immune response in the serum samples was assessed by standard Hemagglutination inhibition (HI) test against New castle disease virus (NCDV) antigen and cellular immune response was determined by delayed type hypersensitivity (DTH) skin test using dinitrochlorobenzene (DNCB) as an eliciting antigen (Tiwary and Goel, 1985).

Statistical analysis: The data for various parameters were subjected to statistical analysis by using paired t-test. Individual means were compared at 5% confidence level for statistical analysis.

RESULTS AND DISCUSSION

Haematological parameters (Table 1) revealed no significant ($P<0.05$) difference in mean Hb and TEC values and the values were comparable in the both groups. Values of TLC also revealed no significant ($P<0.05$) difference and were comparable in group A and B. In agreement to our findings, Yan and Kim (2012) in a 5 week trial investigated the effect of eugenol and cinnamaldehyde as feed additive in growing pigs and did not found any significant difference in TEC and TLC values except relative increased lymphocyte count. In contrast to our findings Bello *et al.* (2016) reported significant increase in TLC, monocyte and eosinophils concentrations in fowl supplemented with clove extract @ 400 and 600 mg/kg in diet. According to Ibrahim *et al.* (2022), birds given eugenol nanoemulsion at different doses (100, 250, and 400 mg/kg) exhibited increased RBCs count, whereas the Hb concentrations in different experimental groups did not differ significantly.

Biochemical studies (Table 2) revealed comparable total protein values in both groups A and B, although improved values were noticed in the eugenol supplemented group but the difference was significant ($P<0.05$) only at 21 DPIE. The present findings are in agreement with Tariq *et al.* (2015) who reported a nonsignificant effect of clove supplementation on serum protein in Japanese quail. In contrast Mahrous *et al.* (2017) observed significant increase in total protein and serum globulin following dietary supplementation of clove bud. Mean serum albumin concentration also data revealed improved serum albumin values in eugenol supplemented group A as compared to control group B but the difference was significant ($P<0.05$) only at 28 DPIE. Total protein and albumin are produced by the liver, which makes them valuable as indicators of the degree of hepatic damage. The findings of the current study demonstrated that eugenol's antioxidant capability enhanced the concentration of total protein and albumin. This might be accomplished by a mechanism combining increase protein synthesis and

Table 1. Effect of eugenol on blood hematological parameters (mean ± S.E.) in broiler chicks at different intervals

Parameters	7 DPIE	14 DPIE	21 DPIE	28 DPIE	35 DPIE
Hb (g/dl)					
Group A (E)	7.45^{a}±0.11	7.15^{a}±0.19	7.80^{a}±0.23	8.25^{a}±0.15	9.18^{a}±0.28
Group B (C)	7.65^{a}±0.1	7.45^{a}±0.08	7.63^{a}±0.28	8.53^{b}±0.19	9.07^{a}±0.24
TEC (million/µl)					
Group A (E)	2.29^{a}±0.02	2.34^{b}±0.04	2.41^{a}±0.04	2.64^{a}±0.03	2.93^{a}±0.11
Group B (C)	2.34^{a}±0.02	2.21^{a}±0.09	2.48^{a}±0.11	2.63^{a}±0.07	2.96^{a}±0.06
PCV (%)					
Group A (E)	32.87^{a}±0.31	33.03^{a}±1.04	31.01^{a}±0.43	34.23^{a}±1.11	35.12^{a}±0.67
Group B (C)	33.42^{a}±0.21	34.10^{a}±0.48	35.08^{b}±0.76	34.55^{a}±0.56	33.17^{a}±1.04
MCV (fl)					
Group A (E)	143.83^{a}±2.43	141.21^{a}±3.26	129.08^{a}±3.29	129.80^{a}±3.18	120.71^{a}±4.32
Group B (C)	138.4^{a}±0.82	155.29^{a}±5.43	142.48^{a}±6.09	131.84^{a}±4.59	112.33^{a}±5.11
MCHC (%)					
Group A (E)	22.68^{a}±0.47	21.72^{a}±0.77	25.19^{b}±0.86	24.22^{a}±0.86	26.19^{ab}±0.92
Group B (C)	22.55^{a}±0.32	21.88^{a}±0.49	21.8^{a}±0.87	24.72^{a}±0.61	27.41^{b}±0.78
TLC (10^3/mm^3)					
Group A (E)	17.79^{a}±0.48	14.99^{b}±0.46	16.31^{b}±0.54	19.37^{b}±0.65	20.23^{b}±0.43
Group B (C)	17.56^{a}±0.28	15.20^{b}±0.6	16.46^{b}±0.51	19.28^{b}±0.49	20.69^{b}±0.59

*Means±S.E, with unlike superscript in the same column differ significantly (P 0.05), DPIE=Days post administration of eugenol; E=eugenol; C=control

Table 2. Effect of eugenol on serum biochemical parameters (mean ± S.E.) in broiler chicks at different intervals

Parameters	7 DPIE	14 DPIE	21 DPIE	28 DPIE	35 DPIE
Total Protein (g/dl)					
Group A (E)	3.53^{a}±0.02	3.70^{a}±0.03	4.01^{a}±0.04	4.31^{a}±0.09	4.66^{a}±0.02
Group B (C)	3.57^{a}±0.01	3.73^{a}±0.04	4.15^{b}±0.06	4.36^{a}±0.09	4.58^{a}±0.03
Serum Albumin (g/dl)					
Group A (E)	1.85^{a}±0.02	2.05^{a}±0.04	1.99^{a}±0.03	2.00^{a}±0.03	2.15^{a}±0.06
Group B (C)	1.87^{a}±0.02	2.06^{a}±0.04	1.96^{a}±0.03	1.89^{b}±0.02	2.13^{a}±0.03
Serum AST (IU/L)					
Group A (E)	179.65^{a}±0.63	180.53^{a}±0.72	182.29^{a}±0.79	187.92^{a}±1.28	189.16^{a}±0.71
Group B (C)	179.83^{a}±0.87	182.67^{a}±1.32	183.24^{a}±0.98	187.56^{a}±1.16	190.28^{a}±0.85
Serum ALT (IU/L)					
Group A (E)	8.03^{a}±0.03	7.09^{a}±0.02	6.08^{a}±0.03	5.05^{a}±0.03	4.44^{a}±0.01
Group B (C)	8.45^{a}±0.16	7.11^{a}±0.02	6.16^{a}±0.02	5.15^{b}±0.02	4.48^{a}±0.03
Serum GGT (IU/L)					
Group A (E)	26.49^{a}±0.14	26.58^{a}±0.17	27.36^{a}±0.13	27.42^{a}±0.11	27.54^{a}±0.08
Group B (C)	26.52^{a}±0.09	26.53^{a}±0.17	27.44^{a}±0.15	27.40^{a}±0.11	27.52^{a}±0.07
Creatinine (mg/dl)					
Group A (E)	0.44^{a}±0.01	0.46^{a}±0.01	0.47^{a}±0.02	0.48^{a}±0.01	0.46^{a}±0.02
Group B (C)	0.44^{a}±0.01	0.46^{a}±0.01	0.45^{a}±0.02	0.48^{a}±0.01	0.45^{a}±0.02
Uric acid (mg/dl)					
Group A (E)	6.47^{a}±0.02	6.84^{a}±0.09	6.81^{a}±0.07	5.54^{a}±0.04	5.45^{a}±0.03
Group B (C)	6.46^{a}±0.01	6.86^{a}±0.08	6.87^{a}±0.09	5.51^{a}±0.08	5.47^{a}±0.06

*Means±S.E, with unlike superscript in the same column differ significantly (P 0.05), DPIE=Days post administration of eugenol; E=eugenol; C=control

protection against macromolecular damage via its antioxidant capacity.

Data from liver function parameters revealed better values of ALT in eugenol fed group A as compared to control group B, but the values were found to be significant (P<0.05) only at 28 DPIE. Similarly, Abd El-Hack *et al.*

Table 3. Effect of eugenol on oxidative stress parameters (mean ± S.E.) in broiler chicks at different intervals

Parameters	7 DPIE	14 DPIE	21 DPIE	28 DPIE	35 DPIE
SOD (U/mg Hb)					
Group A (E)	17.67^{a}±0.01	18.73^{a}±0.02	20.13^{a}±0.04	21.56^{a}±1.04	22.95^{a}±1.1
Group B (C)	17.67^{a}±0.02	18.85^{b}±0.02	19.50^{b}±0.05	20.55^{a}±0.08	22.43^{a}±0.06
Catalase (U/mg Hb)					
Group A (E)	18.38^{a}±0.04	19.67^{a}±0.06	22.76^{a}±0.04	23.44^{a}±0.06	25.6^{a}±0.03
Group B (C)	18.41^{a}±0.05	20.34^{b}±0.06	20.85^{b}±0.04	22.31^{b}±0.07	23.47^{b}±0.07
LPO (nM MDA/ml)					
Group A (E)	3.40^{a}±0.03	3.61^{a}±0.03	3.71^{a}±0.02	3.81^{a}±0.01	3.97^{a}±0.02
Group B (C)	3.44^{a}±0.02	3.68^{b}±0.02	3.74^{a}±0.02	3.88^{a}±0.02	4.06^{b}±0.03

*Means±S.E, with unlike superscript in the same column differ significantly (P 0.05), DPIE=Days post administration of eugenol; E=eugenol; C=control

Table 4. Effect of eugenol on mean HI titre (log_2, mean ± S.E.) against NDV (New Castle Disease Virus) in broiler chicks at different intervals

Groups	Days Post Initiation of Eugenol (DPIE)				
	7 DPIE	14 DPIE	21 DPIE	28 DPIE	35 DPIE
A (E)	6.18^{a}±0.08	6.70^{a}±0.07	7.08^{a}±0.08	7.01^{a}±0.07	6.50^{a}±0.12
B (C)	6.07^{a}±0.06	6.78^{a}±0.05	7.02^{a}±0.14	6.99^{a}±0.08	6.58^{a}±0.07

*Means±S.E, with unlike superscript in the same column differ significantly (P 0.05), E-Eugenol, C-Control

(2020) also observed decreased ALT values after feeding cinnamon essential oil (CEO) in broilers. Mahrous *et al.* (2017) also noticed decrease in ALT following dietary supplementation of clove bud. GGT and AST values of group A were numerically improved in comparison to the values of control group but difference was not found significant (P<0.05) at any interval. Our results are consistent with Mehr *et al.* (2014) findings who also reported the effect of varying concentrations of clove essential oil on serum AST in broilers and found no significant (P<0.05) difference among different groups. The activity of transaminases such as ALT and AST is routinely used to assess liver functions, while GGT, a membrane bound enzyme is well recognised biomarker for tissue injury caused by various contaminants. Rapid elevation in the levels of serum transaminases is generally considered as a sign of hepatocellular injury caused by generation of reactive oxygen species by some pathogen or toxicant. The alleviating action of eugenol suggests its hepatoprotective ability due to the inhibitory effect of eugenol against reactive oxygen species and nitric oxide production (Abd El-Hack *et al.*, 2020). Previous workers have also shown that eugenol has anti-hepatotoxic activity and is therefore a promising dietary supplement for the treatment of hepatic damage (Marchese *et al.*, 2017; Andjelkovic *et al.*, 2019).

Values of kidney function parameters (serum uric acid and creatinine concentration) were comparable in both groups (A and B) and revealed no significant (P<0.05)

Table 5. Effect of eugenol on Dinitrochlorobenzene (DNCB) response (mean increase in skin thickness in mm, mean±S.E.) in broiler chicks at different intervals

Groups	Left Abdominal Skin Thickness (in mm) after DNCB application	
	After 24 hrs	After 48 hrs
Group A (E)	3.49^{a}±0.05	3.44^{a}±0.06
Group B (C)	3.36^{a}±0.12	3.14^{a}±0.13

*Means±S.E, with unlike superscript in the same column differ significantly (P 0.05), E-Eugenol, C-Control

differences (Table 2). In contrary, Mahrous *et al.* (2017) studies revealed non-significant increase in serum creatinine concentration at 3rd week while significant decrease at 5th week in clove treated broiler chicken as compared to control birds. Nephroprotective potential of eugenol was reported by earlier workers who reported that treatment with eugenol (@100 mg/kg) not only improved the carbon tetrachloride induced kidney damage but also rectified the altered functional parameters and histological structure of kidney (Fathy *et al.*, 2022). Beneficial effects of eugenol on kidney functions through its powerful antioxidant properties have also been demonstrated by Barhoma (2018).

Results of oxidative stress parameters conducted in the present experimental trial have been shown in Table 3. Eugenol supplemented group (A) revealed significant (P<0.05) increase in catalase activity from 14DPIE onwards till the end of experiment as compared to control group B. The mean values of SOD activities at different

intervals also revealed higher values in group A as compared to control group but difference was significant (P<0.05) at 14 and 21 DPIE only. The findings of the present study are in agreement with Mahrous *et al.* (2017) who reported significant increase in SOD and GSH activity following clove bud supplementation in broilers. The current findings are also consistent with Bello *et al.* (2016) who observed that increase in dietary clove concentration in Japanese Silkie fowls resulted in linear increase in antioxidant enzymes (SOD, Catalase and Glutathione Peroxidase) activity. In the present study for LPO activity estimation, MDA levels were detected in serum and lower values were noticed in eugenol supplemented group A in comparison to control group B but difference was significant (P<0.05) only at 14 and 35 DOA. Similar findings were reported by Abd El-Hack *et al.* (2020) who showed significant impact of dietary cinnamon essential oil (having eugenol) on serum malondialdehyde (MDA) levels in broiler chicken. Bello *et al.* (2016) studies results also showed that supplementation of clove extract @ 400 and 600 mg/kg in diet caused decrease in serum MDA levels in black-meated fowls as compared to control birds. The possible mechanism of reduced MDA formation by essential oils is due to its ability in limiting and preventing cell membrane destruction caused by oxidative stress and production of free radicals. The anti oxidant potential of eugenol is reported to be due to its ability to donate the hydrogen atoms or electrons and scavenge the free radicals produced, thereby terminating the mechanism of peroxide chain reaction (Bonilla *et al.*, 2018).

Mean HI titre (log_2, mean ± S.E) against New Castle disease virus (NDV) in different experimental groups at different intervals (Table 4) revealed that no significant (P 0.05) difference existed in mean HI titres between group A and the control group (B). The mean skin thicknesses of broiler chicks measured after dinitrochlorobenzene (DNCB) application in different groups (Table 5) indicated increase in skin thickness in group A as compared to group B at both 24 and 48 hrs post challenge which was also supported by histopathological examination of skin tissue. The difference was although non-significant at both the intervals. Many plants and their extracts have been discovered as potential substitutes for antibiotic growth promoters in broilers since it has been demonstrated that they have an impact on production of cytokines, T cells, and antibodies at cellular as well as at molecular levels. In agreement to our findings Carrasco *et al.* (2009) investigated the in vivo impact of clove essential oil (CEO) containing >98% eugenol in mice on both humoral and cell-mediated immune responses and reported that CEO might increase immune responses by stimulating the hematopoietic system and raising the number of lymphocytes in peripheral blood. In contrary, some researchers observed repressed cell-mediated responses and fortified humoral immune responses in immunocompetent rats after feeding 0.1 ml/kg CEO (comprised of 87.34% eugenol) daily for a period of two weeks (Halder *et al.*, 2011). These results indicate mixed immune response pattern of essential oils. The immunomodulatory activities of essential oils are mediated through multiple mechanisms involving specific and non-specific immunity. They can fortify the immune system by increasing the number leucocytes (leucocytosis) and enhancing their phagocytic power. They can even suppress the inflammatory responses and diminish cytokine generation by interfering with inflammatory mediators (Sandner *et al.*, 2020).

It was concluded from the results of present study that eugenol administration in broiler feed elicits a hepatoprotective, antioxidant, mild immunostimulatory effect.

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