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Investigating the Effects of Dietary Supplementation of Eucalyptus Camaldulensis Essential Oil on Haemato-Biochemical Indices, Immune Response and Oxidative Stress of Weaned Rabbits

Alagbe. J.O*

[*]Department of Animal Nutrition and Biochemistry, Sumitra Research Institute, Gujarat, India.

Corresponding Email: [*]dralagbe@outlook.com

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Abstract: ***The aim of this experiment was to investigate the effects of dietary supplementation of Eucalyptus camaldulensis essential oil on haemato-biochemical indices, immune response and oxidative stress of weaned rabbits. Sixty healthy weaned cross bred male rabbits (4 weeks of age) with initial body weight of 460.1 ± 0.25 g were grouped based on their body weight into four treatments containing treatment 1 (T1) basal diet with no Eucalyptus camaldulensis oil (ECO), T2, T3 and T4 were fed basal diet with 200 mg, 400 mg and 600 mg/kg respectively in a completely randomized design. Feed and clean water was supplied ad libitum throughout the experiment which lasted for 56 days. All haematological parameters (pack cell volume, red blood cell, haemoglobin, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentrations, white blood cells and its differentials) were higher (P<0.05) in T2, T3 and T4 relative to T1. Total protein, albumin, globulin, albumin/globulin ratio, cholesterol, aspartate transaminase, alanine transaminase and alanine phosphatase values were greater in ECO supplemented diets whereas urea nitrogen, creatinine, calcium, phosphorus, potassium, sodium and bicarbonate were influenced by the treatments. However, all values were within the optimal range for healthy rabbits. Immunoglobulins, melanodialdehyde, superoxide dismutase, glutathione peroxidase, catalase and total antioxidant capacity were higher (P<0.05) in T2, T3 and T4 than in other diet. Result revealed that ECO can be supplemented up to 600 mg/kg without negatively affecting the performance and health status of rabbits.***

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Keywords: Rabbits, Immune Response, Oxidative Stress, Phytochemical, Eucalyptus Camaldulensis Oil.

1. INTRODUCTION

A variety of plants, including herbs, spices, and essential oils and oleoresins generated from plants, are used as phytogenic feed additives (Christine, 2021). Numerous biological functions are expressed by essential oils, including antibacterial, anti-inflammatory, immune-stimulatory, hepatoprotective, and antifungal ones (Tobias, 2021). Numerous studies were done to investigate options after antimicrobial growth promoters were outlawed in the European Union and other nations in 2009 (Laura, 2022). The use of antibiotics in chickens must be reduced in order to lower the danger of antibiotic resistance globally (Manu, 2021) In particular plant components, such as leaves (eucalyptus), bark (cinnamon), seeds (Polyalthia longifolia, anis, and pepper), bulbs (garlic), roots (ginger), or fruit peels (lemon, orange), essential oils tend to collect. A number of active components in eucalyptus camaldulensis oil allow this phytogenic to have anti-inflammatory, antioxidant, anti-protozoal, and immuno-modulatory qualities in addition to its antibacterial action (Jan, 2020; Alagbe et al., 2023). Eucalyptus camaldulensis leaves and oils have been utilized to treat malaria, gastro-intestinal illnesses, diarrhea, cough, and dysentery (Suman et al., 2022). Natural phytogenic feed additives like Eucalyptus camaldulensis oil have been thoroughly researched, and the advantages of chicken feed containing essential oils are well established, all of which aim to improve the performance of birds (Santiago, 2020).

Numerous essential oils have been studied in rabbits, including those from thyme, rosemary, lemon grass, ginger, garlic, and turmeric (Ines, 2020). According to several scientific investigations, certain essential oils can cause cell toxicity when used at greater doses (Ahmed et al., 2005; Barra et al., 2010). According to research (Moataz et al., 2019; Farhadi et al., 2017), Eucalyptus camaldulensis oil may stimulate bile secretion in the intestinal tract, promote enzyme production, speed up digestion, and guard against the entry of pathogenic microorganisms into animals' bodies. The differences in efficacy of essential oils are explained by the chemical makeup of essential oils, which underlay some variance caused by their constituents and other influencing elements including climate, harvest, stage, and storage conditions (Ines, 2020). Despite being necessary, it has been demonstrated to have antibacterial characteristics and a natural antibiotic alternative. On the other hand, there are conflicting data regarding their effectiveness and the right dosage in animal feeds. Information on the impact of supplemental feeding of Eucalyptus camaldulensis oil on the physiological parameters of rabbits is scarce. Therefore this experiment aimed to investigate the effects of dietary supplementation of Eucalyptus camaldulensis essential oil on haemato-biochemical indices, immune response and oxidative stress of weaned rabbits.

2. MATERIALS AND METHODS

Experimental Site, Ethical Consent and Extraction of Eucalyptus Camaldulensis Oil

The study was carried out at the Livestock unit of Sumitra institute located between 23^{o} 13' N and 72^{o} 41' E. The study was carried out according to the rules and specifications of protocols

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approved by research ethic committee of Sumitra Research Institute, Gujarat, India. Fresh Eucalyptus camaldulensis leaves were harvested within the Research Institute and taken to the Department of Biological Sciences where a detailed identification and authentication was carried out. To eliminate dirt and other particles, leaves were washed. The samples were then divided into smaller pieces to minimize their surface area before being transported to the lab and kept in a sealed, tagged container to allow water to drain from the plant. Steam distillation technique is the extraction method used. The equipment needed for the lab procedure includes a steam generator, round bottom flask, beaker, condenser, safety tube, seperatory funnel thermometer, and a distillation flask connected to the steam generator by a delivery tube. 250 mL of distilled water was added after 80 grams of thinly cut Eucalyptus camaldulensis leaf was put into a 500 mL round bottom flask. Setting up the distillation flask, condenser, and receiver, the mixture in the round-bottom flask was heated to 70 ^{o}C for 15 minutes. The steam that was produced passed through the condenser, and when it was cold, oil was collected in the receiver (beaker). For the purpose of obtaining pure Eucalyptus camaldulensis essential oil (ECO), distillate is put through a seperatory funnel.

Animals and their Care

A respected breeding farm in Gujarat, India, sold sixty weaned male crossbreed rabbits with an initial body weight (BW) of 460.1 ± 0.25 g that were approximately 4 weeks old and in clinically good health. Two weeks before to the start of the experiment, galvanized cages were thoroughly disinfected using Morigad®. Nipple drinkers and automatic feeders were also included. The rabbits were thoroughly checked once more and placed in semi-housed open pens with individual cages that measured 35 cm x 71 cm x 50 cm (length x width x height). According to the nutritional requirements for rabbits (NRC, 1979), rabbits were confined for 14 days and fed a basal diet that was appropriate in all nutrients (Table 1). During their period of acclimation, they also received preventative treatment against parasites, including sulphadimidine sodium BP solution to prevent coccidiosis and Ivermectin® injection (subcutaneously administered at 0.1 mL/kg). In a perfectly randomized design, the body weight of the rabbits was used for splitting them into four treatment groups of fifteen rabbits each.

Experimental Arrangement

Treatment 1 (T1) utilizes a base diet without Eucalyptus camaldulensis essential oil (ECO); Treatment 2 (T2) used a base diet with 200 mg/kg ECO; Treatment T3 employs a base diet with 400 mg/kg ECO; and Treatment T4 includes a base diet with 600 mg/kg ECO.

Blood Samples Collection

On the last day of the experiment, blood samples was collected from 10 randomly selected rabbits per treatment for haematological and serum biochemical indices. Blood for haematological studies (2 mL) was collected from the marginal ear into a sample bottle with anticoagulants, another 2 ml was collected into bottles free from anticoagulant for serum biochemical analysis. All samples were placed in ice packs and transferred to the laboratory for further analysis. H360 fully automated 3 part haematology analyzer (model HEM0028, Czech Republic) was used to for haematological studies, this kit was employed because it is easy, efficient and reliable. It has the following technical specifications; Manual closed and

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open tube volume at 100 µL each, work station (intel pentium dual core 2.00 GHz 200 W desktop/tower), (3Gb/s 7200 RPM 16 MB Cache hard drive; 2 GB memory module CD-RW) and (11 inch torch screen with LCD monitor). Serum biochemical analysis was carried out using Pictus 700 automatic analyzer (model F1209-06A, Hungary) with the following technical specifications; photometric module (8 interference filters: 340, 405, 505, 546, 578, 630, 710 and 872 nm), measuring module (25 µL flow cell volume), 15 mm square cuvette, Minimum aspiration volume: 200 µL and analysis mode.

Immune Response Analysis
Blood samples were collected from 10 random selected rabbits (same rabbits used for blood analysis) for immunological analysis. Blood were collected into a sterile labelled plain bottles early in the morning and placed in an ice pack before they are transferred to the laboratory for further studies. Analysis was carried out using Accu-Tell® elisa reader (Model: ABER-2, Belgium) with the following technical specifications; work environment temperature (0^oC – 40^oC), humidity (85%), standard wave length (405, 450, 492, 630 nm, substitute filters from 400 – 700 nm), abs range (0-3. 500A), measurement range (0-2.000A), accuracy (0-2.0A: ± 1.0% or ± 0.007A), precision (0-2.0A: ± 0.5% or ± 0.005A), power (a.c.110 V), reading speed (continuous mode < 5s) and fuses (T315AL250V).

Oxidative Stress Indices
Total antioxidant capacity and activities of superoxide dismutase, catalase, gluthathione peroxidase and melondialdehyde were measured using Getein 1180 immunofluorescence quantitative analyzer (Model 88-OL90D, Belgium). Calibration, buffering and other operations were strictly according to the manufacturer's recommendations.

Analysis of Experimental Diet
Analysis of experimental diet was carried out using Perkin Elmer near infra-red (Model DA 7250, England) which analyzes sample in 60 seconds. The machine has the following technical specifications; operating temperature range (5^oC to 40^oC), wavelength range (900 – 1700 rpm) and wavelength accuracy (<0.05 nm).

Statistical Analysis
All data obtained were subjected to statistical analysis of variance (ANOVA) using Statistical Analytical System (SAS, 2003). Treatment means were compared using Duncan multiple range test of the same software.
The Statistical model used is shown below:

$$Yi = \mu + Tij + eij$$

Where: Yi = the effect of the jth observation in the ith treatment µ = general mean of the population Ti = the effect of the ith treatment where i = 4 eij = random error associated with the jth observation in the ith treatment

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Table 1: Experimental diets' chemical composition

Feedstuffs	Quantity (kg)
Maize	32.00
Wheat offal	15.00
Palm kernel meal	20.10
Soya meal	25.00
Bone meal	4.00
Limestone	2.00
Lysine	0.20
Methionine	0.10
*Min/vit premix	0.25
Salt	0.35
Total	100.0
Calculated analysis (% Dry matter)	
Crude protein	14.81
Crude fibre	13.30
Ether extract	1.91
Calcium	1.91
Phosphorus	0.72
Energy (kcal/kg)	2410.7
Determined analysis (% Dry matter)	
Crude protein	15.73
Crude fibre	12.11
Ether extract	2.00
Calcium	1.93
Phosphorus	0.88
Energy (kcal/kg)	2513.6

*****Min/vitamin premix supplied per kg diet: - vitamin A, 6,800 I.U; vitamin E, 16.0 mg; vitamin D 2,000I.U, vitamin K, 5.00mg; vitamin B2, 5.0mg; Niacin, 65 mg; vitamin B12, 20 mg; choline chloride, 70 mg; Manganese, 3.0 mg; Zinc, 35.1mg; Copper, 2.0g; folic acid, 2.5mg; Iron, 7.1g; pantothenic acid, 18mg; biotin, 35.5g; antioxidant, 60mg

Hematological Parameters of Weaned Rabbits Fed Diet Supplemented with Eucalyptus Camaldulensis Oil (ECO)

Hematological parameters of weaned rabbits fed diet supplemented with Eucalyptus camaldulensis oil (ECO) is displayed in Table 2. The dietary treatments influenced ($P<0.05$) all the hematological parameters analyzed. However, the values were within the established ranges for healthy rabbits (Merck Veterinary Manual, 2011) recommending the absence of health challenges among rabbits. The pack cell volume, hemoglobin, red blood cells, mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentrations were greater ($P<0.05$) in T2, T3 and T4 than in T1. Higher pack cell volume, red blood cell and hemoglobin values could lead to an increased cell ability to transport oxygen

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which translates to efficient distribution of nutrients in the body of animals (Kerr, 1989). This observation agreed with the findings of Algae (2023) where increased in pack cell volume, hemoglobin and red blood cell was reported in weaner pigs supplemented with Eucalyptus oil. Mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentrations were within the normal ranges by Alessandro (2007) suggesting that there was acute inflammation in the cells of rabbits. White blood cells, lymphocytes, monocytes, neutrophils and eosinophils were highest ($P<0.05$) in T2, T3 and T4 relative to T1 indicating that the animal's immune system is strengthened (Adele et al., 2021; Singh et al., 2021). White blood cell is vital in body defense and immunity, it also detects foreign organisms and destroys those (Shetty et al., 2022). Rising white blood cell in the blood stream is usually indicates a physiological problem/infection (Ross, 2021). Neutrophils are indices for stress and they also protect the body against bacterial infection such as acute inflammation (Harcourt and Baker, 2001). Elevation in the number of basophils occurs during acute hypersensitive reaction or allergy (Queensberry et al, 2003). Eosinophils plays special role in the elimination of parasites such as worms and allergic infections (Silva et al., 2005). Monocytes and lymphocytes are indices for immune response (Algae et al., 2023).

Table 2: Hematological parameters of weaned rabbits fed diet supplemented with Eucalyptus camaldulensis oil (ECO)

Parameters	Treatment 1	Treatment 2	Treatment 3	Treatment 4	SEM	Range
Pack cell volume (%)	34.18^{b}	40.70^{a}	40.92^{a}	41.00^{a}	0.82	31- 51
RBC ($\times10^{6}$/L)	9.12^{b}	12.33^{a}	12.85^{a}	12.90^{a}	0.03	8 – 18
Hemoglobin (g/L)	100.6^{b}	127.6^{a}	129.5^{a}	131.6^{a}	3.18	100 – 180
MCH (pg.)	19.35^{b}	27.71^{a}	28.83^{a}	29.96^{a}	0.39	16 - 30
MCV (flu)	49.50^{b}	59.40^{a}	60.85^{a}	61.23^{a}	0.97	30 – 60
MCHC (%)	35.01^{b}	41.57^{a}	43.08^{a}	44.26^{a}	0.80	29 – 50
WBC ($\times10^{9}$/L)	8.42^{b}	11.55^{a}	12.50^{a}	12.58^{a}	0.02	5 – 21
Lymphocytes (%)	51.48^{b}	67.62^{a}	67.95^{a}	70.10^{a}	0.91	20 – 85
Neutrophils (%)	23.70^{b}	32.18^{a}	33.04^{a}	33.86^{a}	0.65	21 - 50
Monocytes (%)	1.77^{b}	2.03^{a}	2.19^{a}	2.85^{a}	0.01	1 - 5
Eosinophil's (%)	1.45^{b}	2.99^{a}	3.02^{a}	3.08^{a}	0.01	1 - 4

^{a,b,c}Means in a row without a similar superscripts differ ($P<0.05$); SEM: standard error of the mean

Serum Biochemical Indices of Weaned Rabbits Fed Diet Supplemented with Eucalyptus Camaldulensis Oil (ECO)

The dietary treatment influenced the serum biochemical indices with the exception of creatinine, urea nitrogen, calcium, potassium, sodium, biocarbonate and phosphorus ions. Total protein, albumin and globulin values were maximum ($P<0.05$) in T2, T3 and T4 relative to T1. However, all values were within the optimum range for healthy rabbits. The increase in globulin levels amongst rabbits fed ECO suggests efficient transportation of essential metals

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via the blood stream to all parts of the rabbit's body (Adelaja et al., 2020). Animals with severe infections frequently exhibit high globulin levels as a result of unusually elevated antibody production (Adelaja et al., 2020). Increased total protein and albumin/globulin ratio in T2, T3 and T4 suggests that rabbits' ability to use protein is uninhibited. Cholesterol values were higher ($P<0.05$) in T1 than in other diet, this reveals the ECO has hypocholesterolmic potential which could promote food safety and prevent the risk of heart diseases (Alagbe and Anuore, 2023; Shittu and Alagbe, 2022). Urea nitrogen and creatinine levels were within the normal range for healthy rabbits reported by (Ozkan et al., 2012), this suggest the absence of kidney or liver damage and other metabolic disorders (Omokore and Alagbe, 2019). Aspartate transaminase, alanine transaminase and alanine phosphatase are indices for liver damage, these values were within the optimal ranges for healthy rabbits (Saunder and Davis, 2005) indicating absence of chronic liver inflammation, bile ducts, cholestatis as well as muscle damage in rabbits (Fudge, 2000; Reavil and Schmidt, 2000). Calcium, potassium, sodium, phosphorus and bicarbonate levels were not influenced ($P>0.05$) by the treatments. However, all values were within the optimum ranges for rabbits reported by Haghjooyjavan mard et al. (2009) suggesting that the experimental diets were able to regulate chemical reaction, maintain fluid balance and blood volume as well as energy processing in deoxyribonucleic acid and adenosine triphosphate (Kozma, 1974; Betancourt et al., 2011). Harcourt-Brown (2002) reported that a normal serum bicarbonate level is a definite sign that kidney infections are absent.

Table 3: Serum biochemical indices of weaned rabbits fed diet supplemented with Eucalyptus camaldulensis oil (ECO)

Parameters	Treatment 1	Treatment 2	Treatment 3	Treatment 4	SEM	Reference val.
Total protein (g/L)	3.78^{b}	4.93^{a}	4.95^{a}	5.02^{a}	0.02	5.4 – 7.5
Albumin (g/L)	1.84^{b}	2.50^{a}	2.51^{a}	2.52^{a}	0.01	2.7 – 5.0
Globulin (g/L)	1.94^{b}	2.43^{a}	2.44^{a}	2.50^{a}	0.01	1.5 – 2.7
Albumin/globulin ratio	0.95^{b}	1.02^{a}	1.03^{a}	1.01^{a}	0.03	0.5 – 1.8
Cholesterol (mmol/L)	76.81^{a}	54.50^{b}	53.28^{b}	50.91^{b}	1.02	11 – 70
Urea nitrogen (mmol/L)	3.21	3.27	3.08	3.11	0.01	1.0 – 4.0
Creatinine (mmol/L)	2.09	2.11	2.08	2.21	0.01	0.91 – 3.0
AST (U/L)	115.7^{a}	100.6^{b}	98.12^{b}	98.00^{b}	1.23	35 – 130
Alanine transaminase (U/L)	72.56^{a}	60.80^{b}	60.11^{b}	60.02^{b}	1.01	45 - 80
Alanine phosphatase (U/L)	54.12^{a}	48.04^{b}	44.16^{b}	44.05^{b}	0.09	12 – 96
Calcium (mmol/L)	2.50	2.68	2.80	2.88	0.01	1.0 -3.0
Potassium (mmol/L)	1.51	1.55	1.61	1.66	0.02	1.0 – 4.0
Sodium (mmol/L)	130.6	134.7	138.5	139.1	2.93	100 – 141
Bicarbonate (mmol/L)	27.4	28.5	29.1	29.6	0.09	15 - 40

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Phosphorus (mmol/L)	1.40	1.49	1.51	1.55	0.01	1.0 – 3.5

AST: Aspartate transaminase; [a, b,c]Means in a row without a similar superscripts differ (P<0.05); SEM: standard error of the mean

Immune Response of Weaned Rabbits Fed Diet Supplemented with Eucalyptus Camaldulensis Oil (ECO)

Table 4 revealed the immune response of weaned rabbits fed diet supplemented with Eucalyptus camaldulensis oil (ECO). Immunoglobulin A, immunoglobulin G and immunoglobulin M values were altered (P<0.05) by supplementing ECO in the diets of rabbits. Immoglobulins (A, G and M) were higher (P<0.05) in T2, T3 and T4 relative to T1 suggesting that ECO contains bioactive compounds especially eucalyptol and α-pinned which have immune-stimulory properties (Algae, 2023). Oluwafemi et al. (2020) also explained that phytochemicals or bioactive compounds have several pharmacological activities including; hepato-protective, antimicrobial, antioxidants, antiviral, anti-helminthic, antifungal amongst others. Phenols are antioxidants or immune boosters which prevents the body against the invasion of pathogenic organisms (Oloruntola et al., 2021). The results obtained in this study corroborated with the findings by Oloruntola et al. (2016) where increased immunoglobulin was reported in rabbits fed diet supplemented with Alchornea cord folia leaf. Similar results was recorded when phytogenic (Gliricidia leaf) was supplemented in the diets of weaned rabbits (Oloruntola et al., 2018).

Table 4: Immune response of weaned rabbits fed diet supplemented with Eucalyptus camaldulensis oil (ECO)

Parameters	Treatment 1	Treatment 2	Treatment 3	Treatment 4	SEM
Immunoglobulin A (µg/mL)	1.77^{b}	2.97^{a}	3.05^{a}	3.11^{a}	0.02
Immunoglobulin G (µg/mL)	3.00^{b}	4.07^{a}	4.14^{a}	4.58^{a}	0.13
Immunoglobulin M (µg/mL)	0.92^{b}	1.26^{a}	1.55^{a}	1.57^{a}	0.01

[a,b,c]Means in a row without a similar superscripts differ (P<0.05); SEM: standard error of the mean

Oxidative Stress Indices of Weaned Rabbits Fed Diet Supplemented with Eucalyptus Camaldulensis Oil (ECO)

Oxidative stress indices of weaned rabbits fed diet supplemented with Eucalyptus camaldulensis oil (ECO) is presented in Table 5. Total antioxidant capacity values varied from 3.00 – 4.74 (nmol/L), superoxide dismutase (28.61 – 42.15 nmol/L), glutathione peroxidase (21.88 – 36.04 nmol/L), catalase (11.50 – 21.01 nmol/L) and melanodialdehyde (58.07 – 71.87 nmol/L). Total antioxidant capacity, superoxide dismutase, glutathione peroxidase, catalase and malonodialdehyde values were greater (P<0.05) in T2, T3 and T4 relative to T1. This result

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suggests that ECO has antioxidant properties (α-pinene and eucalyptol) which protects against toxic effects of free radicals (Olafadehan et al., 2021). According to Oloruntola et al. (2016); Alagbe et al. (2022), the protective mechanisms of antioxidants can occur at different levels; preventing the initiation of chain reactions by removing free radicals, interrupting chain sequence of scavenging free radicals generated in the chain reactions and removing the peroxidases, thereby preventing further generation of reactive oxygen species. The results obtained in this study agreed with the findings by Awad et al. (2020) where the activities of free radicals was scavenged in animals fed diet supplemented with probiotics. Similar results was reported by Manuelian et al. (2021) who recorded a positive response when natural plant extract was supplemented in the diet of rabbits.

Table 5: Oxidative stress indices of weaned rabbits fed diet supplemented with Eucalyptus camaldulensis oil (ECO)

Parameters	Treatment 1	Treatment 2	Treatment 3	Treatment 4	SEM
Total antioxidant capacity (nmol/mL)	3.00^{b}	4.52^{a}	4.68^{a}	4.74^{a}	0.02
Superoxide dismutase (nmol/mL)	28.61^{b}	38.90^{a}	40.11^{a}	42.15^{a}	0.17
Glutathione peroxidase (nmol/mL)	21.88^{b}	30.14^{a}	35.49^{a}	36.04^{a}	0.05
Catalase (nmol/mL)	11.50^{b}	19.60^{a}	20.56^{a}	21.01^{a}	0.02
Melondialdehyde (nmol/mL)	71.87^{a}	59.93^{b}	59.34^{b}	58.07^{b}	0.08

^{a,b,c}Means in a row without a similar superscripts differ ($P<0.05$); SEM: standard error of the mean

3. CONCLUSION

In conclusion, ECO expresses a wide range of biological activities including antimicrobial, antiviral, immune-stimulatory, hepato-protective, antioxidants amongst others. ECO is safe at 600 mg/kg because it does not pose any detrimental effect on haemtological and serum biochemical parameters as well as the immune response of rabbits. Its ability to protect against toxic effects of free radicals will prevent diseases/mortality and promote food safety.

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