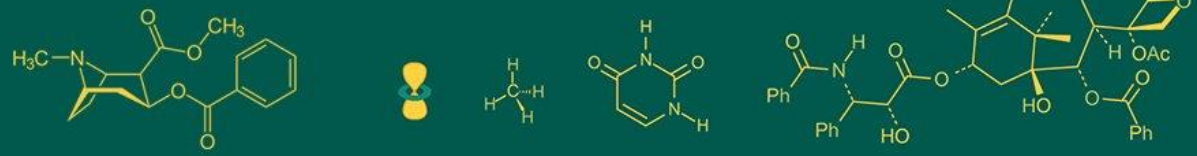
International Journal of Advanced Biochemistry Research



ISSN Print: 2617-4693
ISSN Online: 2617-4707
IJABR 2019; 3(2): 39-45
www.biochemjournal.com
Received: 21-05-2019
Accepted: 25-06-2019

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Haematological and serum biochemical indices of broiler starter chicks fed diets containing different levels of aqueous *Citrus aurantium* stem bark extract

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DOI: https://doi.org/10.33545/26174693.2019.v3.i2a.105

Abstract
The aim of this experiment was to evaluate some haematological and serum biochemical indices of broiler starter fed diets having different levels of aqueous *Citrus aurantium* stem bark (CASR). A total of 200 - 1-day old broiler chicks (Arbo acres) were randomly distributed into 5 groups, each with 4 replicates consisting of 10 birds each in a completely randomized design. The study lasted for 21 days during which clean feed and water were provided *ad libitum*. Treatment 1 (T1) basal diet + Ciprofloxacin 0.2 mL/litre of water, T2 (basal diet + 10 mL/litre CASB), T3 (basal diet + 20 mL/litre CASR), T4 (basal diet + 30 mL/litre CASR) and T5 (basal diet + 40 mL/litre CASR). Results on haematological parameters showed that pack cell volume (PCV), haemoglobin (Hb), red blood cell (RBC), monocytes, eosinophils and basophils were not significantly ($P> 0.05$) different among the treatment while white blood cell (WBC), lymphocytes and heterophils were significantly ($P< 0.05$) influenced by the treatments. Feeding birds CASR 10 mL to 40 mL/liters increased the WBC and lymphocyte counts. Total protein, albumin, globulin and creatinine values were similar across the treatments ($P> 0.05$) while cholesterol and urea values decreases as the level of CASR increased across the treatments ($P< 0.05$). It was concluded that CASR could be fed to broiler chicks up to 40 mL per liter of water without causing any deleterious effect on the blood profile of birds.

Keywords: *Citrus aurantium* extract, broiler chicks, haematology, serum biochemistry, feeding

Introduction
Blood is the fluid present in the body that consists of liquid containing numerous cells and protein suspended in it (William, 2003; Etim *et al*., 2014b) [38, 54]. It moves substances to and from tissue cells, nutrients such as proteins, glucose, vitamins, minerals, lipids into cells, transports oxygen by red blood corpuscles (oxyhaemoglobin), removes wastes products such as urea and carbon (iv) oxide from cells (Khan and Zafar, 2005) [56]. It is important in regulating temperature by altering the blood flow through the skin, provides immunity against infections and in the distribution of hormones to all parts of the body and contains substances that enhance clotting following a wound (Hauptmanova *et al*., 2006; Maxwell *et al*., 1990) [58].

Haematological values could serve as a standard for comparison in conditions of nutrient deficiency, toxicity and health status of farm animals (Oyawole and Ogunkunle, 2004; Daramola *et al*., 2005) [24, 25]. Serum biochemistry is used to monitor progression of disease before final evaluation such as pathology of arteries and organs (Church *et al*., 1984; Garacyk *et al*., 2003) [53, 55]. Haematological parameters consists of red blood cells, pack cell volume, haemoglobin, white blood cells or leucocytes, mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration while serum biochemical indices includes; albumin, globulin, cholesterol, urea, creatinine, bilirubin and truglycerides (Alagbe *et al*., 2018; Kurtoglu *et al*., 2005) [46, 47, 57].

Dietary intake has been recognized as the major factor affecting the blood composition of animals because blood transports nutrients and other materials to different parts of the body (Yeong, 1999; Olabanji *et al*., 2007) [60, 59]. Consequently, whatever affects the blood such as feeding will certainly affect the health of an animal (Oluwafemi *et al*., 2021; Abass *et al*., 2012) [18].

For instance, Adetola *et al*. (2021) reported a significant (*P*< *0*.05) difference in blood profile of birds fed aqueous extract of *Petiveria alliacea* root at 15 ml/litre of water. Similar result were recorded by Alagbe *et al*. (2018) [46, 47]; Nodu *et al*. (2016) [49] who recorded that *Azadirachta indica* leaf has a measurable effect on the blood components of broiler chickens. In another experiment conducted by Oni *et al*. (2018) [48] on the effect of garlic, ginger and chaya leaf on the haematology of broiler chickens, results showed that there was a significant (*P*< *0*.05) increase in pack cell volume, red blood cell, haemoglobin and white blood cell counts.

Therefore, the aim of this experiment is to evaluate the haematological and serum biochemical indices of broiler starter fed diets having different levels of aqueous *Citrus aurantium* stem bark (CASR).

Materials and methods

Experimental site

The experiment was carried out at Division of Animal Nutrition, Sumitra Research Institute, Gujarat, India with a coastline of 1,600 Km, 23º 13'N 72º41'E.

Source and extraction of *Citrus aurantium* stem bark (CASR)

Fresh stem of *Citrus aurantium* were obtained within Sumitra Research Institute, India in the month of March, 2022. It was authenticated by a qualified taxonomist Dr. Kumar, cut into pieces and thoroughly washed with running tap water to remove sand and other dirt's. It was then shade dried for 10 days and the dried samples were pulverized into powder using pestle and mortar, thereafter 200 grams of the sample was soaked into 1000 litres of water for 72 hours and stirred 3 hours interval and kept in the refrigerator at 4 ºC. All mixtures were filtered using Whatman filter paper and the filtrates (CASR) were collected into a clean labeled plastic container.

Gas chromatography –mass spectrometry (GC-MS) of *Citrus aurantium* stem bark

GC-MS analysis of aqueous CASB was carried out using a Perkin-Elmer GC clarus 500 system and gas chromatograph interfaced to a mass spectrometer equipped with an Elite-I fused silica capillary column (30m × 0.25 mm × ID × 1µm). Injection temperature was maintained at 25 ºC, helium flow rate as 1.5ml/min and ion source temperature at 230 ºC. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. Identifications of the compounds were based on mass spectral matching with standard compounds in National Institute of Standard and Technology (NIST) having more than 62000 patterns.

Management of experimental birds and design

200, one-day old broiler chicks (Cobb 500) were purchased from a commercial hatchery in India in the month of April, 2022 and randomly distributed into 5 groups, each with 4 replicates consisting of 10 birds each. Prior to the arrival of the birds, pens and cages were properly disinfected with Cid 2000 at the rate of 10 ml to 20 litres of water. Birds were given anti-stress on arrival; feeds were formulated based on NRC (1994) requirements for broilers. A battery cage system placed in a semi closed well ventilated pens with dimension 200 cm × 100 cm × 80 cm (length × breath × height) of 100 cm above the ground was used for the experiment. Feed and water were offered *ad libitum* all other management practices were strictly adhered to throughout the experimental period which lasted for 21 days. Completely randomized design was used in the experiment.

Data collection

The treatments were assigned to receive treatment diet as follows:

Treatment 1 (T1) basal diet + Ciprofloxacin 0.2 mL/litre of water, T2 (basal diet + 10 mL/litre CASR), T3 (basal diet + 20 mL/litre CASR), T4 (basal diet + 30 mL/litre CASR) and T5 (basal diet + 40 mL/litre CASR).

Blood collection

At the end of the 21st day of the experiment blood samples (2 ml) were collected from the wing vein of 2 birds in each replicate of the treatments into two different bottles for heamatological and serum biochemical analysis. Bottles used for haematological investigations contain an anticoagulant (EDTA) while those used for serum analysis contain no anticoagulant. Haematological parameters covers: pack cell volume, haemoglobin, red blood cell, white blood cell and its differentials (lymphocytes, monocytes, neutrophils and eosinphils) were determined using commercial kit Auto haematology analyzer (HEMA – D6051 Mini, Netherlands) with Advanced Sweep – Flow technology which uses diluent, LH lyse, LD Lyse and probe cleanser as reagents and ≤ 20 ul as sample volume.

The serum biochemical indices examined include: total protein, albumin, globulin, creatinine, urea and cholesterol values determined using commercial kit XL 1000® China with sample and reagent volume 2-60 µl and 120 µl respectively.

Statistical analysis

All data were subjected to one -way analysis of variance (ANOVA) using SPSS (25.0) and significant means were separated using Duncan's test of the same statistical package.

The model: $Tij = \mu + \alpha i + \beta ij$ was used in this experiment:

Where Tij = any of the response variables; i = the overall mean; αi = effect of the xth treatment and βij = random error due to experimentation.

Table 1: Composition of experimental diets (0-21 days)

Materials	Quantity
Maize	50.00
Wheat offal	6.00
Soya meal	30.55
Groundnut cake	10.00
Fish meal (72%)	2.00
Bone meal	0.60
Limestone	0.30
Lysine	0.20
Methionine	0.20
*Premix	0.25

/

Salt	0.30
Total	**100.0**
Determined analysis	
Crude protein (%)	23.64
Ether extract (%)	5.03
Crude fibre (%)	3.17
Calcium (%)	0.98
Phosphorus (%)	0.46
Lysine (%)	1.17
Meth+Cyst (%)	0.87
ME (Kcal/kg)	2940.5

*****Starter premix supplied per kg diet: - vit A, 13,000 I.U; vit E, 5mg; vit D3, 3000I.U, vit K, 3mg; vit B2, 5.5mg; Niacin, 25mg; vit B12, 16mg; choline chloride, 120mg; Mn, 5.2mg; Zn, 25mg; Cu, 2.6g; folic acid, 2mg; Fe, 5g; pantothenic acid, 10mg; biotin, 30.5g; antioxidant, 56mg.

Table 2: GC-MS analysis on the bioactive compounds in *Citrus aurantium* stems bark (CASR)

Compounds	Area (%)	R.T (min)
Myrcene	3.44	9.21
Linalool	6.02	13.16
γ-terpinene	1.10	13.73
β-fenchol	1.40	15.74
Cis-4-thujanol	1.67	10.33
Carvenone	1.10	9.67
β-santalene	2.50	10.12
α-cubebene	8.49	17.38
β-cayrophyllene	0.77	19.80
D-limonene	50.06	20.57
α-longipinene	2.75	25.41
Terpinen-4-ol	2.04	24.33
α-pinene	1.71	27.06
γ-terpinene	0.94	27.55
γ-eudesmol	1.93	29.10
Capraldehyde	0.16	29.55
Torreyol-α-cadinol	0.07	30.80
β-citrylideneethanol	4.30	30.51
Caryophyllene	5.50	12.81
Phytol	0.10	18.93
4-methyl-2,3-hexadien -1-ol	0.17	33.19
Spathulenol	0.10	29.16
3-methoxy-p-cymene	0.01	34.05
β-Elemene	0.01	34.01
Total	96.76	

R.T: reaction time (minutes)

Table 3: Haematological parameters of broiler chicks fed different level of CASR

Variables	T1	T2	T3	T4	T5	SEM
PCV (%)	22.98	25.71	26.02	26.88	26.92	1.12
Hb (g/dl)	8.02	8.11	8.40	8.52	8.71	0.40
RBC ($\times 10^{12}$/L)	2.01	2.13	2.41	2.50	2.53	0.12
WBC ($\times 10^{9}$/L)	9.11^{c}	11.29^{b}	11.50^{b}	12.74^{a}	12.80^{a}	0.17
LYM (%)	58.18^{c}	65.72^{b}	68.93^{b}	70.90^{a}	72.12^{a}	0.63
MON (%)	1.03	1.08	1.11	1.13	1.17	0.27
EOS (%)	0.12	0.17	0.14	0.10	0.18	0.01
BASO (%)	1.33	1.28	1.30	1.45	1.42	0.02
HET (%)	30.91^{a}	28.42^{b}	28.00^{b}	27.94^{b}	27.90^{b}	0.80

Means in the same row not sharing same superscript are significantly ($P< 0.05$) different.

PCV: pack cell volume; Hb: haemoglobin; RBC: red blood cell; WBC: white blood cell; LYM: lymphocytes; MON: monocytes; EOS: eosinophils; BASO: basophils; HET: heterophils; Treatment 1 (T1) basal diet + Ciprofloxacin 0.2 mL/litre of water, T2 (basal diet + 10 mL/litre CASR), T3 (basal diet + 20 mL/litre CASR), T4 (basal diet + 30 mL/litre CASR) and T5 (basal diet + 40 mL/litre CASR); SEM: standard error of the mean.

Table 4: Serum biochemical indices of broiler chicks fed different level of CASR

Variables	T1	T2	T3	T4	T5	SEM
T.P (g/dl)	4.29	4.38	4.50	4.53	4.59	0.18
Albumin (g/dl)	2.33	2.38	2.41	2.43	2.50	0.13
Globulin (g/dl)	1.96	2.00	2.09	2.10	2.09	0.10
Cholesterol (mg/dl)	218.2^{a}	200.4^{a}	192.8^{b}	188.3^{b}	170.1^{c}	3.92
Urea (mg/dl)	4.10^{a}	3.84^{b}	3.55^{b}	3.40^{b}	3.32^{b}	0.26
Creatinine (mg/dl)	2.41	2.21	2.03	2.10	2.02	0.20

Means in the same row not sharing same superscript are significantly ($P< 0.05$) different.
T.P: total protein; Treatment 1 (T1) basal diet + Ciprofloxacin 0.2 mL/litre of water, T2 (basal diet + 10 mL/litre CASB), T3 (basal diet + 20 mL/litre CASB), T4 (basal diet + 30 mL/litre CASB) and T5 (basal diet + 40 mL/litre CASB); SEM: standard error of the mean.

Results and Discussion

Chemical composition of experimental diet

Table 1 reveals the chemical composition of experimental diet. The diet formulated meets the nutrient requirement of broiler chicks according to NRC (1994). Nutrition plays a vital role in the overall health and performance of an animal (Etim *et al*., 2014) [54]. For instance, Proteins play a central role in biological processes. They catalyze reactions in the body transport molecules such as oxygen, keep the body healthy as part of the immune system and transmit messages from cell to cell (Ojewuyi *et al*., 2014). The ash content of a feed is an index used to determine the amount of minerals present in a particular sample, which are important in many biochemical reactions functioning as co-enzyme and aid physiological functioning of major metabolic processes in the body (Alagbe *et al*., 2020; Shittu and Alagbe, 2020) [1, 2, 3, 4, 41]. Ether extracts are very good sources of energy and helps in the transport of fat-soluble vitamins, insulate and protect internal tissues, and contribute to important cell processes (Pamela *et al*., 2005; Agubosi *et al*., 2021) [22, 17, 20]. Fibre in diet aids effective digestion and also prevents the risk of cardiovascular diseases in animals (Fasola *et al*., 2011; Alagbe, 2019) [4, 5].

GC-MS compositions of *Citrus aurantium* stem bark

Phytochemicals or secondary are biologically active compounds, found in plants in varying amounts with therapeutic properties capable of protecting animals against diseases (Omale and Okafor, 2008; Oluwafemi *et al*., 2021) [21]. Secondary metabolites are normally formed in the plant from basic metabolic pathways, such as glycolysis, the Krebs cycle or the shikimate pathway, using very specific enzymes, and degraded by less specific ~-glucosidase enzymes, esterases and hydrolases (Wink, 1999) [30]. An increased use of medicinal plants in animal nutrition is the result of their positive properties including bactericidal, fungicidal, antiviral, antioxidant capacity, growth-promoting efficacy, immune stimulating effects, stimulation of the secretion of digestive enzymes and absorption of nutrients (Cross *et al.*, 2007). For instance, D-limonene is the most abundant bioactive compound (50.06%) is a monoterpene known to posses antimicrobial, anti-cancer and antioxidant properties (Chow *et al*., 2002; Singh *et al*., 2021) [23]. They are also capable of scavenging free radicals thus preventing the degeneration of vital organs in the body of animals (Adewale *et al*., 2021; Alagbe and Ushie, 2022) [42]. Variation in the composition of D-limonene and other compounds in *Citrus aurantium* stem bark could be attributed to differences in variety or specie, age of plant, preparation technique used in extraction as well as geographical location (Oluwafemi *et al*., 2020). Other bioactive compounds above 2% evaluated in this study such as: α-cubebene (8.49%), β-citrylideneethanol (4.30%), myrcene (3.44%), α-longipinene (2.75%), β-santalene (2.50%), terpinen-4-ol (2.04%) have shown to show potent antimicrobial, antioxidant, hypolipidemic, antibacterial and anti-fungal activities (Alagbe and Ushie, 2022; Salem *et al*., 2019 [28]; Radan *et al*., 2018). γ-eudesmol, α-pinene, Cis-4-thujanol, β-fenchol, γ-terpinen, carvenone, β-cayrophyllene, capraldehyde, torreyol-α-cadinol have strong antibacterial activities against *E.coli* and *Staphyllococcus spp* (Medina *et al*., 2017; Sanei *et al*., 2016) [27, 26, 29].

Haematological and serum biochemical indices of broiler chickens fed *Citrus aurantium* stem bark (CASR)

Blood is an important index in evaluating physiological, pathological and nutritional state of birds (Olorede *et al*., 2007) [59]. Pack cell volume (PCV) ranged from 22.98 – 26.92% which is in harmony with values observed by Livingston *et al*. (2020) [9]. PCV values slightly increases as the level of CASR increased though not a significant rate ($P> 0.05$). Lower PCV values in birds are clear indications of anaemia (Aster, 2004) [31]. PCV are also actively involved in the transportation of nutrients and oxygen in the body thus promoting good health (Isaac *et al*., 2013; Orians and Heller, 2003) [33]. Haemoglobin (Hb) and red blood cells play important role in the conveyance of oxygen to tissues of the animal for oxidation of ingested food so as to release energy for the other body functions as well as transport carbon dioxide out of the body of animals (Ugwuene, 2011). RBC values ranged from 2.01 – 2.53 ($\times 10^{12}$/L) while Hb values (8.02 – 8.71 g/dL) fell within the range reported by Talebi *et al*. (2005) [6]; Alagbe, 2019 [5]. RBC and Hb values may be affected by available nutrients in diet of birds as well as the safety of test ingredients used during an experiment (Olafadehan *et al*., 2021). No significant ($P< 0.05$) difference was recorded among the treatments in the above parameters. Haemoglobin is synthesized within developing red blood cells and its synthesis is coordinated with the developmental stages of the erythroid precursors (William, 2003; Iwuji and Hebert, 2011; Agubosi *et al*., 2021) [38, 17, 20].

White blood cells (WBC) are important in boosting the immune system of animals and could be triggered during period of infection, starvation, exercise, unfavorable conditions and stress (Kabir *et al*., 2011) [34]. The WBC values recorded in this experiment ranged from 9.11 – 12.08 ($\times 10^{9}$/L) which is less than the values of 13.02 – 20.00 ($\times 10^{9}$/L) reported by Alagbe (2019) [5]; Adelaja *et al*. (2020). However, it fell within the normal physiological range 9.00 – 20.00 ($\times 10^{9}$/L) earlier reported by Livingston *et al*. (2020) [9]. Lymphocytes, eosinophils, basophils and heterophils values ranged from 58.18 – 72.12%, 0.12 – 0.18%, 1.33 – 1.42% and 27.90 – 30.91% respectively.

WBC and lymphocytes values were significantly ($P< 0.05$) different among the treatments. Lymphocytes been triggered once an animal is sick or any inflammation in the body (Holland, 2013) [35]. Monocytes are actively restricting replication of intracellular microorganisms in animals (Stafford *et al*., 2002) [37]. Basophils and eosinophils can digest bacteria and other foreign bodies (phagocytosis) and also have some roles in allergic reactions (Young and Meadow, 2010) [39]. No significant ($P> 0.05$) differences were observed in the basophils, eosinophils and monocytes values across the treatments.

Serum biochemistry is used to monitor progression of disease before final evaluation such as pathology of arteries and organs (Marinou *et al*., 2010) [36]. Total protein values obtained in this study ranged from 4.29 – 4.59 (g/dL). According to Omokore and Alagbe (2019) [40] total protein could be related to the quality of feed ingredients and safety of test materials. Total protein, albumin and globulin values were highest in T4 and T5 compared to the other treatments ($P>0.05$). Albumin, globulin, cholesterol, urea and creatinine values ranged from 2.33 – 2.50 g/dL, 1.96 – 2.09 g/dL, 170.1 – 218.2 mg/dL, 3.32 – 4.10 mg/dL and 2.02 – 2.41 mg/dL respectively. Cholesterol and urea values decreases as the level of CASR increased across the treatment ($P< 0.05$). The significant decrease in cholesterol levels could be attributed to the presence of bioactive compounds in CASR especially D-limonene which have been reported to exhibit antioxidant, antimicrobial and hypolipidemic activities (Alagbe and Ushie, 2022). Excessive cholesterol level in the blood could affect the activities the heart that can lead to cardiovascular diseases (Musa *et al*., 2020). High creatinine concentration in the blood is a sign of poor protein and amino acid metabolism which can compromise the activities of the kidney (Shittu *et al*., 2021; Alagbe, 2021) [41]. However, all the values were within range recommended by Subhadarsini and Silpa (2020) for healthy birds.

Conclusion

It was concluded that CASR contains several phytochemicals which are natural, less toxic, environmental friendly and can be effectively used to bridge the gap between livestock production and food safety. Feeding broilers at 40 ml/litre does not have any deleterious effect on the health of the animal.

Funding: This research received no external funding.

Conflicts of Interest: The author declares no conflict of interest.

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