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Haematological Profile and Oxidative Stress Indices of Broiler Chickens Infected with *Eimeria tenella* and Treated with the Methanolic Extract of *Vernonia amygdalina* Leaf and Its Fractions

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Abstract:
Eimeria tenella infection in broiler chickens is associated with haemorrhagic typhlitis and anemia. The study aimed to evaluate the haematological parameters and oxidative stress profile of caecal coccidiosis in broiler chickens infected with E. tenella following the administration of the methanolic leaf extract of Vernonia amygdalina and its fractions. To evaluate the parameters, 210 broiler chickens were divided into seven groups with two replicates (n=15). The experimental groups were negative control (NC), positive control (PC), standard amprolium control (SC), crude methanol extract (ME), hexane fraction (HF), butanol fraction (BF), and aqueous residue fraction (AF). All birds were infected with 10^5 sporulated oocysts of E. tenella except those in the NC group and treated accordingly. The haematological parameters and oxidative stress markers were assessed at 7 and 14 days post-infection as evaluation criteria for the evaluation of the anticoccidial efficacy of treatments. The packed cell volume (PCV) of the NC group showed a significant decrease at 7 days post-infection but markedly improved at 14 days post-infection. There was a non-significant difference in the antioxidant values of the birds in all groups ($p > 0.05$), as no definite trend in the value measured parameters were recorded. It was concluded that treating infected birds with V. amygdalina effectively decreased blood loss and improved oxidative defence during caecal coccidiosis.

Keywords: *Eimeria tenella, haematological profile, antioxidant stress indicators, Vernonia amygdalina, methanolic extract*

1. Introduction

Avian coccidiosis, caused by seven species of parasitic apicomplexan protozoa of genus *Eimeria*, is one of the most economically important diseases of the poultry industry (Chapman *et al*., 2013). Coccidiosis stands out as the parasitic disease with the greatest economic impact on the poultry industry worldwide (Chapman, 2014). *E. tenella* is the most pathogenic *Eimeria* species that parasitizes growing chickens, causing considerable financial loss to the poultry industry (Shirley *et al*., 2007). This species inhabits the caeca causing haemorrhage and inflammation known as caecal coccidiosis. It can be recognized by the accumulation of blood in caecal lumen and by bloody droppings (McDougald & Fitz-Coy, 2013).

Control of coccidiosis is traditionally based on administering anticoccidial drugs and, to a lesser extent, by vaccination. The extensive and abusive use of drug has led to the emergence of drug-resistant strains of *Eimeria* worldwide (Abbas *et al*., 2011).

Currently, there is interest in using natural products, including plant extracts, to reduce problems caused by coccidiosis (Del Cacho *et al*., 2010; Burt *et al*., 2013; Gotep *et al*., 2016; Saranya *et al*., 2020). Vernonia amygdalina is a well-known plant with medicinal and culinary uses (Yeap *et al*., 2010). The positive effects of *V. amygdalina* against avian coccidiosis have been demonstrated by their inclusion as feed additives in broiler feed which lowers oocysts production and improve weight gain in birds (Mohammed & Zakariya'u, 2012; Oyagbemi & Adejinmi, 2012; Osho *et al.*, 2014; Banjoko *et al*., 2018).

Based on previous reports of the beneficial effects of *V. amygdalina*, the current investigation carried out to evaluate the efficacy of the methanolic extract of *V. amygdalina* leaf and its fractions on the basis of haematological profile and antioxidant properties in broiler chickens experimentally infected with *E. tenella*.

2. Materials and Methods

2.1. Plant Collection, Identification, and Processing

Fresh disease-free leaves of *V. amygdalina* were collected at the flowering stage as one batch from a private garden in Jos town. The sample was identified and authenticated at the Department of Biological Sciences, Ahmadu Bello University, Zaria, and a voucher sample numbered 7183 was deposited at the herbarium of the department for future reference. The leaves were washed with tap water and air dried on galvanized-wire screens under the shade with occasional shifting until a constant weight was obtained. A sample of ten kilograms (10 kg) of leaves was then powdered with a mechanical grinder and stored in an airtight plastic container until used.

2.2. Extraction and Fractionation of V. amygdalina Leaves

A 2 kg portion of the powder of the leaves of *V. amygdalina* obtained was subjected to extraction with 10 L of absolute methanol in Soxlet apparatus at 70^{o}C (Youn *et al*., 2003) to obtain a methanolic extract. The extract was dried into powder using a rotary evaporator at a temperature of 40^{o}C and stored in an air-tight amber-coloured glass bottle under refrigeration until used (Momoh *et al*., 2010).

2.3. Experimental Birds and Management

A batch of 300 broiler chicks of both sexes was purchased at 1 day of age and raised as a group in a brooder pen. Clean tap water and commercial feeds free of anticoccidial drugs were provided *ad libitum*. Chickens were vaccinated against the infectious bursal disease and Newcastle disease.

2.4. Eimeria tenella Inoculum

A local strain of E. *tenella* previously subjected to molecular identification was propagated in 5 broiler chicks at 2 weeks of age to propagate and harvest the working oocysts as described (Toah *et al*., 2021)

2.5. Experimental Design

The experiment was carried out in a randomized and completely block design. Two hundred and ten (210) broilers with similar body weights were selected for the study, as recommended by Holdworths *et al*. (2004). The birds were assigned to 7 experimental groups designated from A-G, represented by two replicates of 15 birds per cage, and allowed to acclimatize for 7 days.

Each bird was inoculated with 10^5 sporulated oocysts at 21 d of age as prescribed (Holdsworth *et al*., 2004) except group A which served as a negative control. Birds were treated for 7 days, starting from 1 d prior to infection through 5 d post-infection, according to the following schedule:

- Group A: Uninfected and normal saline (negative control) per os
- Group B: Infected and normal saline (positive control) per os
- Group C: Infected, medicated with 125 mg/kg of amprolium for 7 consecutive days per os
- Group D: Infected, medicated with 1000 mg/kg of methanolic extract for 7 consecutive days per os
- Group E: Infected, medicated with 500 mg/kg of hexane fraction for 7 consecutive days per os
- Group F: Infected, medicated with 500 mg/kg of butanol fraction for 7 consecutive days per os
- Group G: Infected, medicated with 500 mg/kg of aqueous residue fraction for 7 consecutive days

2.6. Collection of Blood Samples

Two chickens from each replicate within all groups were randomly selected on post-infection days 7 and 14 for blood sample collection. The blood samples were collected from the cutaneous ulnar vein in a 5 mL syringe and divided into two parts. The first portion of the blood was dispensed into a 5 mL tube coated with EDTA for haematological parameters. The second portion was collected in a 5mL container free of anticoagulant, and the supernatant serum was stored at -20^{o}C for subsequent antioxidant assay.

2.7. Determination of Haematological Parameters

The erythrocytes and the leucocytes were counted with a Neubauer haemacytometer using the standard method (Natt & Herrick, 1952). The packed cell volume (PCV) was determined by microhaematocrit centrifugation technique and a

PCV reader according to the method described by Coles (1986), and differential blood count was estimated according to the method described by Drabkin (1949).

2.8. Determination of Oxidative Stress Markers

2.8.1. Assessment of Lipid Peroxidation

Lipid peroxidation was determined by measuring the levels of malondialdehyde (MDA) produced during lipid peroxidation according to the method described by Varshney and Kale (1990). This method is based on the reaction between 2-thiobarbituric acid (TBA) and malondialdehyde (MDA): an end product of lipid peroxide during peroxidation. On heating in acidic pH, the product is a pink complex that absorbs maximally at 532 nm and is extractable into organic solvents such as butanol. Malondialdehyde is often used to calibrate this test; thus, the results are expressed as the amount of free MDA produced.

Briefly, an aliquot of 400 µL of the serum was mixed with 1.6 mL of tris-KCl buffer, to which 500 µL of 30% TCA was added. Then 500 µL of 0.75% TBA was added and placed in a water bath for 45 min at 80°C. This was then cooled in ice and centrifuged at 3000 g for 5 min. The clear supernatant was collected, and absorbance was measured against a reference blank of distilled water at 532 nm. Lipid peroxidation expressed as MDA formed/mg protein or gram tissue was computed with a molar extinction coefficient of 1.56 x 105 M^{-1} cm^{-1}.

2.8.2. Determination of Catalase Activity

Catalase activity was determined according to the method of Claiborne (1985). This method is based on the loss of absorbance observed at 240 nm as catalase splits hydrogen peroxide. Although hydrogen peroxide has no maximum absorbance at this wavelength, its absorbance correlates well enough with concentration to allow its use for a quantitative assay. An extinction coefficient of 0.0041 mM^{-1} cm^{-1} (Noble & Gibson, 1970) was used. Briefly, hydrogen peroxide (2.95 mL of 19 mM solution) was pipetted into a 1 cm quartz cuvette, and 50 µL of the sample was added. This was done to reduce the dilution of the samples (done according to the other protocols whereby H2O2 was prepared separately in distilled water (100 mL), and the buffer was also prepared separately. The mixture was rapidly inverted to mix and placed in a spectrophotometer. Change in absorbance was read at 240 nm every minute for 5 min.

2.8.3. Determination of Superoxide Dismutase (SOD) Activity

The level of superoxide dismutase (SOD) activity was determined by the method of Misra and Fridovich (1972). The ability of superoxide dismutase to inhibit the autoxidation of epinephrine at pH 10.2 makes this reaction a basis for a simple assay for this dismutase. Superoxide ($O_2^{\bullet-}$) radical generated by the xanthine oxidase reaction caused the oxidation of epinephrine to adrenochrome and the yield of adrenochrome produced per $O_2^{\bullet-}$ introduced increased with increasing pH (Valerino & McCormack, 1971) and increasing concentration of epinephrine. These results led to the proposal that autoxidation of epinephrine proceeds by at least two distinct pathways, only one of which is a free radical chain reaction involving superoxide ($O_2^{\bullet-}$) radical and hence inhabitable by superoxide dismutase.

In the procedure, 0.2 mL of the sample was diluted in 0.8 mL of distilled water to make a 1-in-5 dilution. An aliquot of 0.2 mL of the diluted sample was added to 2.5 mL of 0.05 M carbonate buffer (pH 10.2) to equilibrate in the spectrophotometer. The reaction started by adding 0.3 mL of freshly prepared 0.3 mM adrenaline to the mixture, which was quickly mixed by inversion. The reference cuvette contained 2.5 mL buffer, 0.3 mL of substrate (adrenaline), and 0.2 mL of water. The increase in absorbance at 480 nm was monitored every 30 seconds for 150s.

2.9. Data Analysis

The mean values were compared by one-way analysis of variance (ANOVA) followed by Tukey's test. Variations in the data were expressed as the pooled standard error (SE). The statistical analysis was performed with SPSS version 20. The differences between groups were considered significant if $p < 0.05$.

3. Results

3.1. Haematological Profile

The most remarkable finding in the haematology was the significant decrease in the PCV of untreated chickens. At 7 days post-infection, the PCV of untreated chickens showed a significant decrease below the normal range ($p < 0.05$), but that of the treated chickens remained within the normal values (Table 1). The RBC and WBC were generally below the normal range, though no significant differences were observed between treatments ($p > 0.05$).

At 14 days post-infection, a drastic improvement in the PCV of the untreated birds, tending towards the normal values, was observed. The RBC numbers of the treated groups returned to normal levels. The proportion of lymphocytes (LYM) and heterophils (HET) did not show a definitive trend, but a high percentage of eosinophils (EOS) was recorded (Table 2).

3.2. Oxidative Stress Status

The status of oxidative stress as measured by the oxidative stress markers in experimental birds at 7 days post-infection is presented in table 3. There seem to be inconsistencies in the values of the markers evaluated. The general trend is that the value remained the same or mildly altered during the infection. Similar values were obtained in experimental chickens at 14 days post-infection in all groups, irrespective of treatment (Table 4).

Haematological Parameters									
Group	**Treatments**	**PCV (%)**	**RBC (10^{12}/L)**	**WBC (10^9/L)**	**LYM (%)**	**HET (%)**	**MON (%)**	**EOS (%)**	**BAS (%)**
A	Negative control	29.50^{a}	1.85^{ab}	2.33^{a}	58.75^{c}	43.75^{a}	0.00	1.75^{b}	0.00
B	Positive control	21.75^{b}	1.58^{c}	4.38^{a}	79.50^{a}	16.25^{c}	0.00	5.50^{a}	0.00
C	Standard control	27.25^{bc}	1.88^{a}	3.15^{bc}	79.50^{a}	17.50^{c}	0.00	3.00^{ab}	0.00
D	Methanol extract	25.00^{c}	1.73^{abc}	3.25^{bc}	79.50^{a}	16.75^{c}	0.00	3.75^{ab}	0.00
E	Hexane fraction	27.50^{ab}	1.68^{bc}	3.38^{b}	79.75^{a}	17.75^{c}	0.00	2.50^{ab}	0.00
F	Butanol fraction	27.25^{bc}	1.63^{c}	3.25^{bc}	77.50^{a}	18.75^{c}	0.00	3.75^{ab}	0.00
G	Aqueous fraction	29.75^{a}	1.88^{a}	2.98^{c}	67.00^{b}	31.00^{b}	0.00	2.00^{b}	0.00
SE±		0.523	0.040	0.071	1.032	1.467	0.000	0.962	0.000

Table 1: Mean Values of Haematological Parameters at 7 Days Post-Infection in Experimental Chickens
Mean Values Bearing Different Letters within the Same Column Differ Significantly at P < 0.05

Haematological Parameters									
Group	**Treatment**	**PCV (%)**	**RBC (10^{12}/L)**	**WBC (10^9/L)**	**LYM (%)**	**HET (%)**	**MON (%)**	**EOS (%)**	**BAS (%)**
A	Negative control	28.75^{a}	2.23^{a}	2.20^{bc}	58.75^{c}	36.00	1.00^{a}	3.50	0.75^{a}
B	Positive control	23.50^{b}	1.88^{c}	3.40^{a}	72.75^{a}	21.50^{c}	0.50^{ab}	4.00	0.75^{a}
C	Standard control	29.75^{a}	2.00^{bc}	1.55^{d}	59.75^{c}	35.75^{a}	0.25^{ab}	4.00	0.00
D	Methanol extract	30.00^{a}	2.05^{abc}	2.13^{bcd}	66.00^{b}	29.75^{b}	0.00^{b}	4.25	0.00
E	Hexane fraction	25.00^{ab}	2.08^{abc}	2.55^{b}	69.50^{ab}	25.75^{bc}	0.00^{b}	4.75	0.00
F	Butanol fraction	26.25^{ab}	1.90^{c}	3.50^{a}	73.50^{a}	22.00^{c}	0.00^{b}	4.25	0.00
G	Aqueous fraction	30.00^{a}	2.15^{ab}	1.85^{cd}	68.00^{ab}	29.75^{b}	0.00^{b}	2.00	0.00
SE±		1.109	0.047	0.141	1.196	1.195	0.211	0.648	0.204

Table 2: Mean Values of Haematological Parameters at 14 Days Post-Infection in Experimental Chickens
Mean Values Bearing Different Letters within the Same Column Differ Significantly at P < 0.05

Group	**Treatment**	**MDA (mmol/mg protein)**	**CAT (H_2O_2/min/mg protein)**	**SOD (µmole SOD/min/mg protein)**
A	Negative control	0.58^{b}	75.95^{c}	20.25^{b}
B	Positive control	1.22^{ab}	258.49^{b}	40.58^{ab}
C	Standard control	0.60^{b}	219.16^{bc}	34.11^{ab}
D	Methanol extract	0.94^{ab}	232.49^{bc}	57.63^{a}
E	Hexane fraction	0.86^{ab}	150.35^{bc}	46.33^{ab}
F	Butanol fraction	1.10^{ab}	207.77^{bc}	48.38^{ab}
G	Aqueous fraction	1.27^{a}	226.33^{a}	56.95^{a}
SE±		0.146	38.415	7.029

Table 3: Mean Values of Oxidative Stress Markers at 7 Days Post-Infection in Experimental Chickens
Mean Values Bearing Different Letters within the Same Column Differ Significantly at P < 0.05

Group	**Treatment**	**MDA (mmol/mg protein)**	**CAT (H_2O_2/min/mg protein)**	**SOD (µmole SOD/min/mg protein)**
A	Negative control	1.37	78.24^{b}	23.51^{c}
B	Positive control	1.27	146.73^{a}	118.97^{a}
C	Standard control	1.17	100.83^{ab}	74.67^{b}
D	Methanol extract	1.05	110.85^{ab}	67.67^{b}
E	Hexane fraction	0.81	115.11^{ab}	23.32^{c}
F	Butanol fraction	0.94	116.08^{ab}	22.24^{c}
G	Aqueous fraction	1.03	107.26^{ab}	29.34^{c}
SE±		0.145	10.720	3.100

Table 4: Mean Values of Oxidative Stress Markers at 14 Days Post-Infection in Experimental Chickens
Mean Values Bearing Different Letters within the Same Column Differ Significantly at P < 0.05

4. Discussion

Among the causes of haemorrhagic coccidiosis in chickens, *Eimeria tenella* is well-represented, being used as a model for the study of *Eimeria* spp (Chapman & Shirley, 2003). Infection with haemorrhagic species, including *E. tenella*, *E. necatrix*, and *E. brunetti*, are associated with haemorrhagic diarrhoea, anaemia, and retarded growth. The endogenous stages of *E. tenella* develop deep in infected chickens' caecal mucosa, causing tissue damage, inflammation, and haemorrhagic enteritis (McDounald & Shirley, 1987). In this study, the haematological parameters of broiler chickens experimentally infected with *E. tenella* showed a decrease in PCV, which is reflective of blood loss associated with caecal coccidiosis (Akhtar *et al*., 2015; Hirani *et al*., 2018). The decreased PCV in untreated birds observed in this study is in accordance with other studies (Fukata *et al*., 2007; Adamu *et al*., 2013; Zhang *et al*., 2020; Naseer *et al*., 2022). PCV is used as an index of the severity of haemorrhage resulting from caecal coccidiosis. Improved PCV using plant extracts has been reported in other studies (Dar *et al*., 2014; Ola-Fadunsin & Ademola, 2014; Melkamu *et al*., 2018). A possible explanation for this observation is that dietary administration of *V. amygdalina* leaf extract can inhibit the invasion and establishment of *Eimeria* in the gut tissue, translating to minimal damage and less blood loss (Al-Fifi, 2007; Muthamilselvan *et al*., 2016).

Coccidiosis is associated with oxidative stress caused by free radicals released by damaged tissue during coccidial infection (Fortuoso *et al*., 2019). *E. tenella* has been shown to impair broiler chicken's antioxidant status during infection (Georgieva *et al*., 2006). Traditionally, supplements such as vitamins A, C, and E are used to control oxidative stress during coccidial infection (Jafari *et al*., 2012; Dominguez *et al*., 2015). Natural products are considered viable alternatives to alleviate the damage caused by oxidative stress during coccidiosis (Masood *et al*., 2013; Idris *et al*., 2017; Galli *et al*., 2018; Al-Quraishy *et al*., 2020).

The level of MDA as a measure of the level of the intestinal mucosa was reported to be mitigated by plant-derived antioxidants (Naidoo *et al*., 2008). Antioxidant compounds derived from plants, including alkaloids, flavonoids, saponins, tannins, and phenolics, are known to ameliorate the severity of coccidial infection by mitigating the degree of lipid peroxidation (Zhu *et al*., 2019; Naseer *et al*., 2022). In this study, the abundant antioxidant phytochemicals in the leaf of *V. amygdalina* (Alara *et al*., 2020) might have exerted their anticoccidial activity by protecting the caecal tissue of infected chickens from oxidative damage, thereby reducing the severity of disease in accordance with Oyagbemi and Adejinmi (2012).

Antioxidant enzymes, including SOD and CAT, can prevent antioxidative injury by free radicals during coccidiosis (Alhotan & Adoubas, 2019). In this study, there was no clear trend in the levels of SOD and CAT, probably due to other considerations.

5. Conclusion

This work demonstrated that the methanolic extract of *V. amygdalina* leaf and its fractions can improve some haematological indices and exert protective effects against oxidative stress in broiler chickens during caecal coccidiosis.

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