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TOXICITY AND ANTI-COCCIDIAL EFFICACY OF *Azadirachta indica* AQUEOUS LEAF EXTRACT IN BROILER CHICKENS EXPERIMENTALLY INFECTED WITH MIXED *Eimeria tenella* and *Eimeria maxima* SPORULATED OOCYSTS

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ABSTRACT

Toxicity and anticoccidial efficacy of Azadirachta indica aqueous leaf extract were studied with four-week old broiler chickens. The acute and sub-acute toxicity test was performed by administering varied doses of the extract once and for 21 days to the birds. Following toxicity studies, only mild signs of depression were observed amongst the birds that received highest dose of Azadirachta indica aqueous leaf extract. For the in vivo AILE anticoccidial effects, use was made of 25 birds arbitrarily allocated into five groups (I – V) of five birds each. Group I birds were the uninfected control group while groups II – V birds were infected with 200,000 mixed Eimeria tenella and Eimeria maxima sporulated oocysts. On the detection of oocysts in all the infected birds, group II and III birds were treated with 200mg/kg of Azadirachta indica aqueous leaf extract once and daily for five days respectively. Group IV was treated with a commercial anticoccidial, Embazine forte® (30 g/50 litres of water) while group V was the infected-untreated group. The body weight, oocyst outputs, packed cell volume, red blood cell count, total leucocytes count, hemoglobin concentration, total protein, serum albumin of the birds were monitored. Mortalities were not recorded following toxicity studies, however, mild signs of depression were observed especially amongst the chickens that received the highest dose of Azadirachta indica aqueous leaf extract. Clinical signs such as bloody diarrhea, drooping, ruffled feathers, etc. and lesions of coccidiosis including ballooned and bloody intestines were observed in the infected birds. Reduction and/or cessation of oocyst outputs, reduction of percentage mortality, disappearance of clinical signs and lesions, and significantly improved body weight and hematological indices were recorded in Azadirachta indica aqueous leaf extract treated groups compared to the infected-untreated group V. It was therefore concluded that Azadirachta indica aqueous leaf extract might possess anticoccidial activity against mixed Eimeria infections of broiler chickens with good margin of safety.

Keywords: Toxicity, Anticoccidial effect, *Azadirachta indica*, Eimeria species, Broiler chickens

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INTRODUCTION

Poultry constitutes a valuable source of animal protein, especially in rural communities of most African countries [1]. However, diseases including coccidiosis constitute a major health problem and limitation to poultry production worldwide [2]. Coccidiosis is endemic in Nigeria and has been documented as the most consistently reported health problem in poultry [3]. Nine species of *Eimeria* are known to infect chickens and mixed infections are usually common [4] especially in Southeastern Nigeria.

Control of coccidiosis is mainly by hygiene and chemotherapy involving anticoccidial drugs [5]. Many anticoccidial drugs have evolved and have been used worldwide. However, their continued use and misuse have resulted in the development of drug resistant strains [2], presence of anticoccidial residues in poultry products, prolonged withdrawal periods and increased cost of poultry production. The emergence of drug resistant strains of *Eimeria* and the escalating cost of drug development have greatly reduced the commercial incentive to develop new anticoccidial drugs [2]. Thus, there is an urgent need for alternative, cost-effective and affordable remedies for the control of these infections, especially under the small-scale system of poultry production in the rural communities.

Amongst the available control options currently under investigation world-wide, use of botanicals is probably the most promising and affordable in sustainable coccidiosis control. Several plants have been claimed traditionally to have medicinal value for the treatment of various ailments in both man and animals in Nigeria [6]. However, the efficacy and safety of these natural products remain doubtful as only a few have been properly identified and documented [5, 6].

The neem (*Azadirachta indica A. juss*) is a tree of Indian origin with many useful compounds. It is popularly called '*Dogonyaro*' in Nigeria. Almost all the parts including the leaf, bark, flower, fruit, twig, gum, seed, oil, and root have been shown to have medicinal values in both man and animals [7]. Studies have demonstrated the insecticidal, antibacterial, antiviral, antifungal, anthelmintic and antiprotozoal effects of the plant [8]. Few studies are available on the anticoccidial effects of leaf extract of the plant [9, 10]. However, such studies concentrated on the efficacy of the leaf extract on *Eimeria tenella vis-à-vis* oocyst count, feed conversion ratio neglecting the haematological and toxicity aspects, and mixed *Eimeria* infections, which is very common especially in Southeastern Nigeria. Thus, this study aims to assess the toxicity and anticoccidial efficacy of aqueous leaf extract of *Azadirachta indica* in broilers experimentally infected with mixed *Eimeria tenella* and *Eimeria maxima*.

Materials and methods

Experimental animals

A total of 85 broiler birds were procured at day old from a hatchery at Ibadan for this study. The birds were routinely vaccinated against Newcastle disease and infectious bursal disease. The birds were housed in a deep litter system at the Laboratory animal house of the Department of Veterinary Parasitology and Entomology, University of Nigeria, Nsukka. Standard commercial feed (Vital feed®, UACN, Jos, Nigeria) were used throughout the experiment and water was provided *ad libitium*.

Preparation of *Azadirachta indica* extracts

The leaves of *Azadirachta indica* used in this study were collected from trees at the Botanical Garden of the University of Nigeria, Nsukka. Voucher specimen of the leaves was deposited following its confirmation in the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka. The leaves were dried, ground into powder and soxhlet-extracted with water for 8 hours at 60^0C. The soluble extract was later concentrated in water bath maintained overnight at 60^oC after which the concentrated extract was collected, weighed and stored at 4^0C until when required.

Infective Material
Eimeria oocysts were obtained from naturally infected broiler chickens, identified as a mixture of *Eimeria tenella* (80%) and *E. maxima* (20%), and sporulated using 2.5% potassium dichromate following standard protocols [11].

To determine the infective dose, 25 broiler chickens aged 4 weeks old were randomly assigned to five groups (I – V) of five birds each. Birds in groups II – V were infected with 4 - 25 x 10^4oocysts per bird, respectively. Those in group I served as the uninfected control. Following infection, the birds were daily examined for the presence of oocysts in faeces and for clinical signs of coccidiosis such as bloody diarrhea, drooping, ruffled feathers, etc. The infective dose was chosen as 20 x 10^4 based on the severity of clinical signs and number of oocysts found in the faeces.

Acute and Chronic Toxicity Tests
Twenty-five broiler chicks aged four weeks old, randomly assigned into five groups (I - V) of five birds each were used to determine the acute toxicity of the aqueous leaf extract of *Azadirachta indica*. Birds in group I (control) were orally given only distilled water equivalent to the highest volume of the extract. The birds in groups II - V were treated orally with graded doses (200, 400, 800, 1200 and 1600 mg/kg, respectively) of 0.25 mg/ml *Azadirachta indica* aqueous leaf extract (AIALE). The birds were observed for 24 hours for signs of toxicity and death.

Twenty-five broiler birds aged four weeks old were also used for the chronic toxicity test. They were randomly assigned into six groups (I - V) of five birds each. Group I birds served as control and were treated orally with distilled water. The birds in groups II - V were treated orally with graded doses (200, 600, 1200, 1600 and 2000 mg/kg, respectively) of the AIALE daily for 21days. The birds were monitored for signs of toxicity and mortality.

***In vivo* Anti-coccidial Effects of AIALE**
Twenty five broiler birds were randomly allocated into 5 groups (I – V) of five birds each. Birds in group I served as the uninfected control while birds in groups II - V were orally infected with 20 x 10^4 sporulated oocysts [mixed *Eimeria tenella* (80%) and *E. maxima* (20%)]. Thereafter, the birds were observed daily for the development of clinical signs of coccidiosis, changes in body weight and the presence of oocysts in faeces. Following detection of oocysts in faeces of all infected birds on day 4 post infection, group II birds were treated orally once with 200 mg/kg of AIALE determined from toxicity tests while group III birds were treated for five days using the same route and dose of AIALE. Group IV birds were treated orally with Embazine forte® (30 g/50 litres of water) for 3 days followed by 2 days of plain water and another 3 days of Embazine forte® medication. Birds in group V were not treated. Total leucocytes counts (TLC), red blood cell counts (RBC) and packed cell volume (PCV) of the birds were assessed every other day while hemoglobin concentration (HbC), total protein (TP), serum globulin concentration (SGC) and serum albumin (SA) of the birds were assessed every four days. Post mortem examinations of the dead and humanely sacrificed birds were also carried out.

Determination of the Various Parameters
Modified McMaster technique was employed in determining the oocysts counts per gram of faeces using saturated solution of sodium chloride as the floating medium [12]. The body weights of the birds were assessed using a triple beam balance. Packed cell volume and HbC were determined using the microhaematocrit method and the cyanomethaemoglobin method, respectively; while RBC and TLC were estimated using the improved Neubauer technique [13]. Serum albumin was determined using the bromocresol green method [14], whereas TP was estimated using direct Biuret method [15].

Statistical Analysis
The data were summarized as means ± standard error of the mean and variations in means were analyzed using one way analysis of variance (ANOVA). Variant means were separated using Duncan multiple range test. The level of significance was considered at $p < 0.05$.

RESULTS

Toxicity Tests
There were no signs of toxicity in the groups of birds that received 200 – 800 mg/kg doses of *Azadirachta indica* extract following acute toxicity tests. The birds that received 1600 mg/kg dose of the extract exhibited mild signs of depression. However, no mortality was recorded. Slight enlargement of the liver was the only gross lesion observed in 1600 mg/kg dose group following humane sacrifice. Following chronic toxicity test, no mortality or signs of toxicity was observed amongst the groups except for the group of birds that received highest dose of the extract (2000 mg/kg). Enlargement of the liver was the only gross lesion observed in the 2000 mg/kg dose group.

Anticoccidial Effects
Clinical Signs
Oocysts appeared in the faeces of all the birds in the infected groups on day 4 post infection. Ruffled feathers, drooping, depression and bloody diarrhoea with whitish streaks, frank blood in faeces and death were some of the clinical signs observed. Gradually, these signs disappeared in the treated groups whereas it continued in the infected and untreated group E. Mortality (100 %) was recorded in the infected and untreated group V by day 12 post infection (PI) whereas the uninfected and untreated groups (negative control group) had the lowest (0%) percentage mortality. The groups of birds that were infected and treated with AIALE for 5 days and Embazine forte® had a low percentage mortality rate of 20% (Table 1).

Body Weight
The body weights of the treated infected groups of birds significantly ($p < 0.05$) improved when compared to the infected and untreated group from day 9 PI (Figure 1). However, the body weights of the treated groups were significantly ($p < 0.05$) lower compared to the uninfected control group I.

Table 1. Percentage mortality of broiler chickens experimentally infected with mixed sporulated *Eimeria tenella* and *Eimeria maxima* oocysts and treated with aqueous leaf extract of A*zadirachta indica.*

Group/Treatment	No. in group	No. (%) dead
Group I (Uninfected + Untreated)	5	0 (0)
Group II (Infection + *Azadirachta indica* × 1 day)	5	2 (40)
Group III (Infection + *Azadirachta indica* × 5 days)	5	1 (20)
Group IV (Infection + Embazine forte®)	5	1 (20)
Group V (Infected + Untreated)	5	5 (100)

Oocyst Output/Counts
Oocyst outputs rose rapidly in the infected groups attaining peak output on day 8 PI (Figure 2) except Embazine forte® treated group IV. Although treatments with AILE and Embazine forte® significantly ($p < 0.05$) suppressed the oocyst output, the oocyst output of the extract treated groups were significantly ($p < 0.05$) higher than that of the Embazine forte® treated group. Oocysts were completely cleared on days 12, 18 and 16 PI in groups treated with Embazine forte®, AIALE once and AIALE for five days respectively (Figure 2).

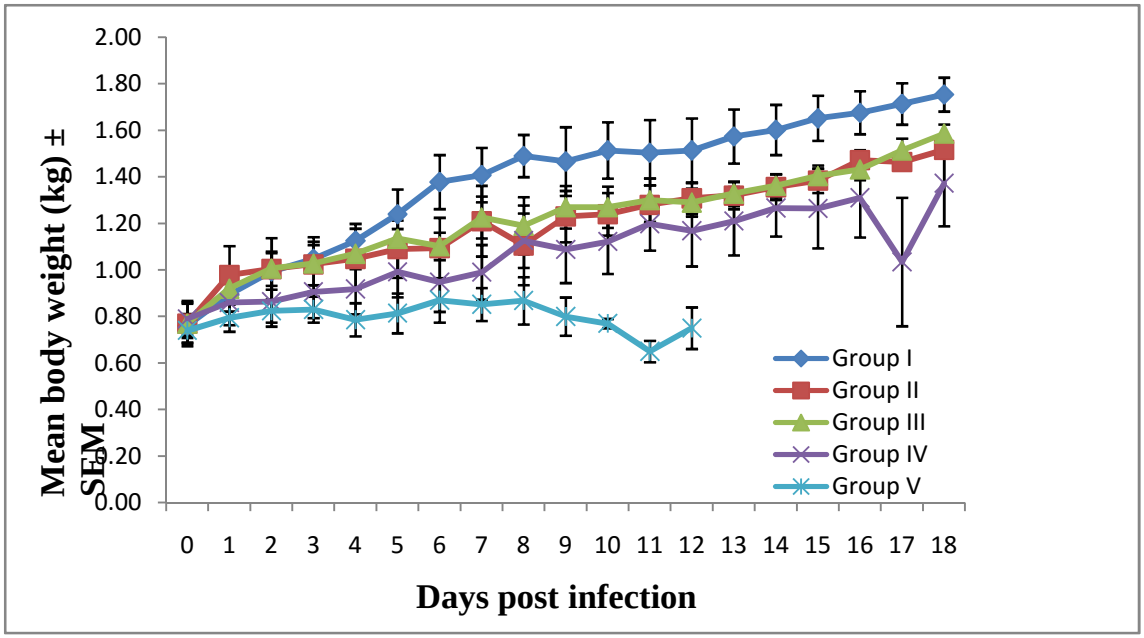

Figure 1: Mean body weight of birds infected with mixed *Eimeria species* and treated with aqueous leaf extract of A*zadirachta indica.*
KEY: Group I = Uninfected + Untreated; Group II = Infected with mixed *Eimeria spp.* + AILE × 1 day on day 4 PI; Group III = Infected with mixed *Eimeria spp.* + AILE × 5 days on days 4, 5, 6, 7 and 8 PI; Group IV = Infected with mixed *Eimeria spp.* + Embazine Forte® on days 4, 5, 6, 9, 10 and 11 PI; Group V = Infected with mixed *Eimeria spp.* + Untreated.

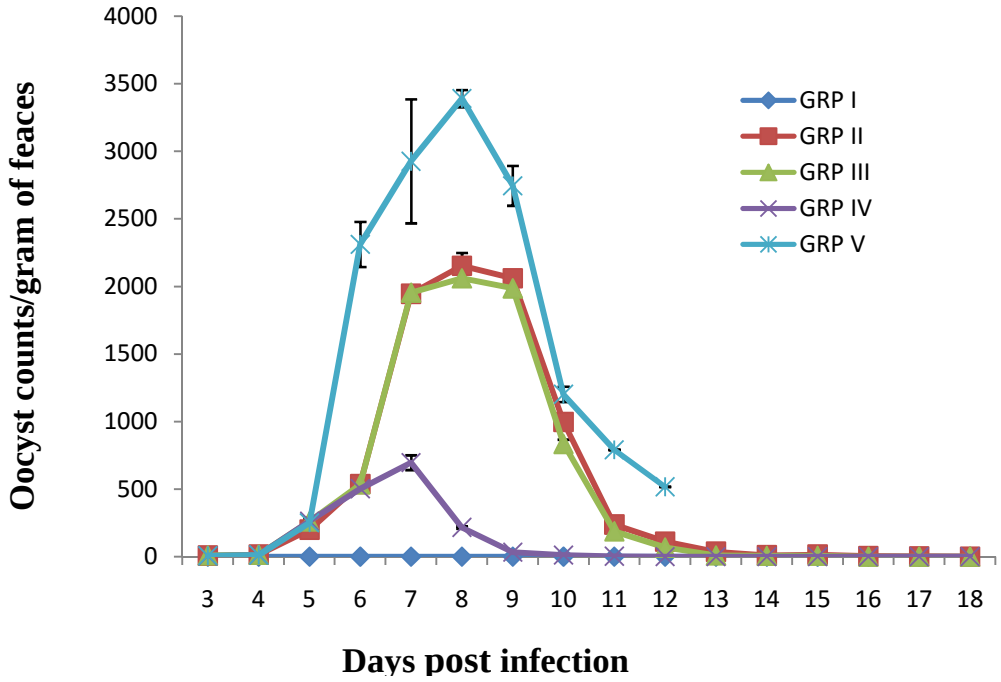

Figure 2: Mean Oocyst output of birds infected with mixed *Eimeria species* and treated with aqueous leaf extract of A*zadirachta indica.* KEY: same as in Figure 1.

Table 2. Mean packed cell volume, total leucocytes and red blood cell counts of broiler chickens experimentally infected with mixed *Eimeria tenella* and *Eimeria maxima*-sporulated oocysts and treated with aqueous leaf extract of A*zadirachta indica.*

Groups/ treatment	Days post infection								
	0	2	4	6	8	10	12	14	16
Packed cell volume (%)									
Group I	35.25±1.75	35.5±1.19	34.5±0.65^{a}	34.25±1.11^{a}	34.75±0.75^{a}	35.5±0.96^{a}	34.5±0.5^{a}	35.3±0.75	35.8±0.48
Group II	35.75±1.25	32.5±0.65	22.25±1.31^{b}	20.75±0.48^{b}	20.75±1.11^{b}	24.5±0.65^{b}	31.25±1.11^{a}	34±0.71	34±0.58
Group III	35.75±1.03	32.25±1.25	22.25±1.25^{b}	21.5±0.87^{b}	21±1.08^{b}	27.75±1.75^{b}	32±1.22^{a}	34.3±0.48	34.25±0.85
Group IV	35±0.71	32±1.08	23.75±1.44^{b}	21.5±1.19^{b}	28.5±1.32^{c}	32.25±1.75^{a}	34.25±1.78^{a}	35.8±0.48	35.5±0.5
Group V	35.5±0.87	31.75±1.55	22.25±1.18^{b}	14.5±1.19^{c}	14.5±0.87^{d}	13.3±0.33^{c}	12.67±0.33^{b}		
Total leucocytes count (×10^{3}/µl)									
Group I	5.83±0.2	6.09±0.1^{a}	6.8±0.44^{a}	6.54±0.75^{a}	5.96±0.8^{a}	5.79±1.53^{a}	6.33±2.3^{a}	6.3±0.65^{a}	7.4±0.49^{a}
Group II	5.9±0.24	10.69±0.12^{b}	14.54±0.12^{b}	14.59±0.17^{b}	14.34±0.25^{b}	11.41±0.86^{b}	8.7±0.87^{b}	9.3±0.41^{b}	7.6±0.45^{a}
Group III	5.86±0.2	10.1±0.47^{b}	14.7±0.18^{b}	14.59±0.19^{b}	14.14±0.07^{b}	10.98±0.1^{b}	8.93±0.12^{b}	9.1±0.18^{b}	7.35±0.13^{a}
Group IV	5.15±0.15	10.48±0.22^{b}	13.75±0.28^{c}	14.97±0.19^{b}	9.1±0.17^{c}	8.43±0.17^{ab}	6.15±0.11^{a}	6.1±0.11^{a}	7.430.87^{a}
Group V	5.36±0.06	10.58±0.06^{b}	14.73±0.23^{b}	15±0.34^{b}	13.93±0.23^{b}	15.19±0.11^{c}	4.75±0.21^{c}		
Red blood cell count (×10^{6}/µl)									
Group I	2.56±0.013	2.76±0.119^{a}	2.59±0.017^{a}	2.65±0.023^{a}	2.62±0.05^{a}	2.54±0.011^{a}	2.31±0.244^{a}	2.6±0.027^{a}	2.55±0.0
Group II	2.54±0.049	2.24±0.025^{b}	2.22±0.014^{b}	2.15±0.058^{b}	2.14±0.023^{b}	2.28±0.044^{b}	2.46±0.011^{a}	2.4±0.026^{b}	2.51±0.0
Group III	2.560.036	2.3±0.053^{b}	2.19±0.018^{b}	2.09±0.027^{bc}	2.11±0.018^{b}	2.36±0.039^{bc}	2.47±0.012^{a}	2.4±0.014^{b}	2.61±0.053
Group IV	2.59±0.039	2.22±0.043^{b}	2.2±0.010^{b}	2.08±0.042^{bc}	2.37±0.078^{c}	2.4±0.018^{c}	2.52±0.009^{a}	2.5±0.005^{a}	2.51±0.032
Group V	2.81±0.255	2.13±0.068^{b}	2.135±0.022^{c}	2.01±0.019^{c}	1.47±0.081^{d}	1.42±0.003^{d}	1.39±0^{b}		

^{abc} Figures in a row with different superscripts for each hematological parameter are significantly differences ($p < 0.05$).

Hematology

There was a significant ($p < 0.05$) decline in the PCV, RBC and HbC of the infected groups compared to the uninfected control group I (Tables 2 and 3). Following treatment, significant ($p < 0.05$) improvement was observed in the treated groups reaching normal levels on day 12 PI compared to the infected untreated group V. However, the PCV values of the extract treated group was significantly lower ($p < 0.05$) than the Embazine forte® treated group IV on days 8 – 10 PI. The RBC and HbC values of the extract and Embazine forte® treated group did not differ significantly ($p > 0.05$). The TLC values of the infected groups increased significantly following infection compared to the uninfected control group (Table 2). However, following treatment, the TLC returned to its pre-infection level.

The total protein levels of the AILE treated groups, though comparable with that of Embazine forte® treated group, were significantly ($p < 0.05$) lower than the uninfected control group from day 8 (Table 3). However, the TP level of the infected untreated group V was significantly ($p < 0.05$) lower than that of the treated groups from day 8 PI. Serum albumin values of the infected untreated group V were comparable with the treated groups. However, they were lower ($p < 0.05$) than those of group I birds (Table 3). Also, the values of the AILE treated groups were lower ($p < 0.05$) than the uninfected group on day 16 PI.

Table 3. Mean hemoglobin concentration, total protein and serum albumin levels of broiler chickens experimentally infected with mixed sporulated oocysts of *Eimeria* species and treated with aqueous leaf extract of A*zadirachta indica.*

Groups/treatment	Days post infection				
	0	4	8	12	16
Haemoglobin concentration (g/dl)					
Group I	10±0.64	7.3±0.2 8.3	5±0.43^{a}	7.18±0.24^{a}	9.13±0.18
Group II	10.13±0.39	6.38±0.79	7.33±0.56^{a}	6.5±0.29^{a}	8.67±0.43
Group III	10.35±0.33	6.5±0.38	7.43±0.39^{a}	6.5±0.38^{a}	8.68±0.18
Group IV	10.13±0.7	6.48±0.35	7.55±0.14^{a}	6.73±0.4^{a}	8.8±0.29
Group V	10.13±0.48	6.03±0.13	5.28±0.43^{b}	4.1±0^{b}	
Total protein (g/dl)					
Group I	3.3±0.13	3.13±0.27	2.75±0.1^{a}	3.03±0.06^{a}	3.05±0.05^{a}
Group II	3.25±0.1	2.75±0.1	2.18±0.15^{bc}	2.38±0.23^{b}	2.45±0.15^{b}
Group III	3.35±0.17	2.65±0.1	2.45±0.1^{ab}	2.33±0.27^{b}	2.55±0.17^{b}
Group IV	3.23±0.15	2.6±0.38	2.55±0.15^{a}	2.78±0.11^{ab}	2.75±0.1^{ab}
Group V	3.25±0.18	2.63±0.36	2±0.07^{c}	1.8±0^{c}	
Serum albumin (g/dl)					
Group I	0.78±0.09	0.93±0.03	1.23±0.16^{a}	0.83±0.03^{a}	1.2±0.14^{a}
Group II	0.78±0.06	0.83±0.11	0.95±0.06^{ab}	0.7±0.04^{ab}	0.95±0.03^{b}
Group III	0.8±0.04	0.83±0.05	0.95±0.06^{ab}	0.73±0.05^{ab}	0.95±0.03^{b}
Group IV	0.8±0.04	0.85±0.03	0.95±0.03^{ab}	0.77±0.07^{ab}	1±0.04^{a}
Group V	0.83±0.11	0.8±0	0.78±0.05^{b}	0.59±0^{b}	

^{abc}Figures in a row with different superscripts for each hematological parameter are significantly differences ($p < 0.05$)

Gross Lesions

Pale carcass, atrophied bursa of Fabricus, congested breast and thigh muscles, coagulated blood on the caeca, ballooned and bloody intestines and pale kidneys were amongst the gross lesions observed in the

infected groups of birds. However, these lesions were mild in the groups that received Embazine forte® and AIALE for five days.

DISCUSSION

Cessation of oocyst outputs, disappearance of clinical signs and lesions and an improvement of body weight are the common indices for evaluating anticoccidial efficacy of botanicals [16]. In the present study, AIALE induced dramatic cessation of oocyst outputs, disappearance of clinical signs and lesions and significantly improved the haematological indices and body weight of the treated birds. These anticoccidial effects of AIALE could be attributed to the ability of the extract to interfere with the multiplication of the parasites and mitigate the damages in the intestinal cells induced by the parasites. The findings of the present study are analogous to the findings of Nweze and Obiwulu [17] and Gotep*et al.*[18] using *Ageratum conyzoides* extract and combined aqueous extracts of *Azadiracta indica* and *Khaya senegalensis* respectively. Biu *et al.*[9] reported 100 % survival rates, cessation of oocyst production and an improvement in the body weight of birds following administration of 800 mg/kg *Azadiracta indica* extract. However, 200 mg/kg of the extract was utilized in the present study. Five days AIALE treatment produced better anticoccidial effect than the single treatment in the present study though the difference is not significant, perhaps due to the sustained and increased concentration of the extract in the birds.

The haematological indices (RBC, WBC, PCV, HbC), TP and SA of the infected birds were significantly improved following treatment with AIALE. This could be ascribed to the extract's haemopoietic and anticoccidial effects. National Research Council [7] reported an increase in red blood cells, white blood cells and lymphocyte counts following oral intake of neem; thus, enhancing cellular immune response and antibody production. Ola-Fadunsin and Ademola [19], Dar *et al.* [20] and Gotep *et al.*[18] also observed improvement in the hematological indices of the infected birds treated with extracts of *Moringa oleifera*, garlic and combination of *Azadirachta indica* and *Khaya senegalensis* respectively.

The antioxidant properties of *Azadirachta indica* have been reported [8] and could also be attributed to the anticoccidial effects recorded by AIALE in the present study. Neem has been reported to decrease oxidative damage and *Eimeria* infection severity [21] by preventing increased nitric oxide and malondialdehyde production and glutathione loss induced by *Eimeria* parasites, thereby decreasing the rate of lipid peroxidation [22].

The anticoccidial effects of AIALE observed in the study could also be due to the bio-active molecules contained in the extract acting additively or synergistically at single or multiple target sites. Though the phytochemical analysis of AIALE was not performed in the present study, AIALE has been shown to contain alkaloids, terpenoids, azadirachtin, flavonoids, glycosides, tannins, saponins, limonoids, quercetin, coumarin [23]. Azadirachtin, a major phytochemical component of AIALE is reported to be responsible for both the antioxidant and antiprotozoal effects of the extract [21]. Limonoids in AIALE is thought to compromise parasites' nutrient utilization [8] while saponins induce parasite death via altering parasite membrane integrity and loss of homoeostasis [24].

The toxicity results revealed that none of the animals in the different dose groups died during toxicity test, though mild signs of toxicity were observed in the groups that received the highest doses (2000mg/kg), thus indicating the safety of AIALE. Water suspensions of herbal extracts have been reported by Hashemi *et al.* [25] to be non-toxic in birds when administered orally. Biu *et al.*[26] also recorded no mortality with leaf extract of *Azadirachta indica* at doses of up to 3200mg/kg, although there were dose-dependent gross and histopathological changes. The liver is the major site for biotransformation of drugs and active principles of extracts [27]. Thus, damages (gross lesions) observed in the liver could have occurred following biotransformation of the extract.

In conclusion, this study highlighted the safety and *in vivo* anticoccidial efficacy of AIALE against mixed *Eimeria* species infections in broiler birds. Consequently, the leaf extract of *Azadirachta indica* may be useful as phytomedicine for the treatment of avian coccidiosis.

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