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Effects of Neem (*Azadirachta indica*) Leaf Aqueous Extracts on Haematological Parameters of Cockerels Experimentally Infected with Infectious Bursal Disease Virus

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

The study aimed to identify the effects of aqueous extract of Neem (*Azadirachta indica*) leaf on some haematological parameters of cockerels vaccinated and infected with infectious bursal disease virus (IBDV). Four hundred and eighty (480) day old cockerels were purchased for the study and allocated to 8 groups. The birds were grouped as vaccinated/ unvaccinated, challenged/

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unchallenged and neem leaf treated/ untreated groups. The IBD vaccines (intermediate plus strain) were given at 14 and 28 days of age while the experimental infection using very virulent IBD virus (vvIBDV) was inoculated at 35 days of age and the extracts were given from day old to 6 weeks old. Blood samples were collected at 1-week post each IBD vaccination, and vvIBD virus challenge for haematology and the results were analysed and recorded. The results obtained showed heterophilia and lymphopenia in the vaccinated groups while very virulent IBDV strain results in panleucopenia, lymphopenia and heterophilia. However, increased lymphocytes and total white blood cell counts (TWBC) was observed following Neem leaf aqueous extracts administration with resultant decrease in heterophils. These results indicates that neem leaf aqueous extracts enhanced the immune status of the birds by increasing the TWBC and lymphocyte counts.

Keywords: Haematology; very virulent infectious bursal disease virus; neem leaf aqueous extracts; vaccine; cockerels.

1. INTRODUCTION

Infectious bursal disease (IBD) is an infectious, acute, mild or subclinical viral disease of young chickens characterised by trembling, incoordination, inflammation followed by necrosis and atrophy of the bursa of Fabricius (BF) resulting in immunosuppression following the extensive destruction of lymphocytes [1]. Infectious Bursal Disease Virus (IBDV) is a small, non-enveloped virus, belonging to the genus *Avibirnavirus* of the family Birnaviridae*,* which is characterised by a bisegmented double-stranded RNA genome which are packaged into single-shelled, non-enveloped virions [2]. Infectious bursal disease virus is classified into two *serotypes (Serotype 1 and Serotype 2) by* virus neutralisation test [3]. These two serotypes are antigenically distinct. Serotype 1 viruses are pathogenic to chickens and differ in their virulence [4]. They cause lesions in the BF by depleting the B- lymphocytes [5]. Whereas, serotype two viruses are avirulent to chickens and isolated mainly from turkeys and chickens [6].

Four pathotypes of Serotype 1 viruses have been described based on virus virulence as mild, classic, variant and very virulent IBDVs [7]. Until 1985, the IBDV field strain (classical strains) isolated was of relatively low virulence, causing only 1-2% of specific mortality, and vaccination satisfactorily controlled outbreaks. However, since 1985 antigenic and pathogenic variant strains of IBDV, distinct from these classical strains, with increased specific mortality have been described in different parts of the world [4]. In the USA, new strains responsible for up to 5% of specific mortality were described [8]. These virus isolates were designated antigenic-variant viruses since they were antigenically different from the classical strains isolated before 1985. They infected broiler chickens possessing relatively high levels of MA and were highly immunosuppressive [9].

Later on, after 1988, in Europe, and subsequently in Japan, new classes of pathotypic variants with high mortality rates (50-60% in laying hens and 25-30% in broilers) were observed [10]. These isolates caused up to 100% mortality in specific-pathogen-free (SPF) chickens and were therefore designated very virulent IBDV (vvIBDV) strains [11]. They rapidly spread all over Asia, Africa and to other significant parts of the world [4]. It is still a problem in many parts of the world till date [12].

In the present scenario, interest is renewed in herbal medicines due to its fewer side effects and is safer. Interest and demand are increasing to obtain more drugs from plant sources to alleviate the ailments of humankind. The use of different parts of several medicinal plants to cure specific ailments has been in vogue form. The green medicine has been popular since ancient times being safe and multiple benefits. Neem is a rich source of different compounds having medicinal properties, so drug development programme should be started utilising the biological and therapeutic properties of neem. In modern era, emphasis should be on control of diseases of human, animals and environment using non-toxic herbal products. By making quantum of research on biological and medicinal properties of neem, some of the herbal products have been prepared but still there is lot of scope in this field for better utilisation of this wonder plant [13]. Thus, the need to know the effects of this remarkable plant and the IBDV on some haematological parameters that are believed to play a vital role in fighting against infections of

chickens, since no data available to that effects in and around the area of the study.

2. MATERIALS AND METHODS

2.1 Study Area

The study was conducted in Sokoto, in the Poultry pen of the Faculty of Veterinary Medicine, Usmanu Danfodiyo University, Sokoto. Sokoto State is geographically located to the North Western part of Nigeria between the longitudes 4° 8'E and 6° 54'E, and latitudes 12° and 13° 58N [14]. The state falls within two vegetation zones: the Sudan Savannah and Northern Guinea Savannah. The climate is characterised by altering dry and wet seasons with slight cold and dry period of harmattan usually accompanied by dust-laden winds and fogs which start from October and last through February. The duration and intensity of annual rainfall ranges from 60-160 days and 635-1000 mm (occurring between May to October) respectively. The mean monthly temperature is generally 20-46°C; relative humidity ranges from 12-17% with the highest occurring in August [14].

2.2 Experimental Birds

Four hundred and eighty (480) day old cockerels were purchased from a commercial hatchery (Farm support^{R}) in Ibadan were used for the study. The experimental birds were raised for 8 weeks.

2.3 Housing and Feeding

The birds were managed on deep litter system in cleaned formalin- potassium fumigated pens. One 200 watt electric bulb was fixed to provide warmth to the birds for each of the groups with additional source of heat around the pens. The pen temperature was maintained at 33 to 35°C for 1 to 3 weeks old and 28 to 29°C for the remaining 3 to 8 weeks old. They were fed on commercial feed; chick mash (Animal care^{R}) for the 8 weeks but the treated water was given from day old to 6 weeks. The feed and water were provided *ad libitum*.

2.4 Biosecurity Measures

Strict biosecurity measures were taken for all the groups. Moreover, the challenged groups were separated into a different pen entirely, employing 2 well trained poultry attendants to routinely feed the birds, disinfect themselves, watering and feeding utensils as well as the environment in order to prevent the spread of microorganisms into or outside the poultry pen. Each of the attendants was responsible for particular groups of birds (unchallenged: groups A to D and challenged: groups E to H). Groups A to D were raised in the main Faculty of Veterinary Medicine poultry pen while groups E to H within a separate pen. Complete personal protective wears were used during vaccination and experimental infection which were incinerated immediately after used. At the entrance of each pen, footbath was provided containing 2% formalin and shoes were provided and hanged just at the interior side of the pens door for use specifically within the pens. At the end of the experiment, the whole pens were fumigated with formalin- potassium composition while the litter was removed and incinerated.

2.5 Preparation and Extraction of Neem (*Azadirachta indica*) Leaf Aqueous Extracts

Mature green neem leaves were used for the experiment. The leaves were obtained from Shehu Kangiwa Square and a botanist from UDUS herbarium professionally identified the Neem and labelled it (UDUH/ANS/0004). The leaves were rinsed in distilled water, air dried and pulverized. The extract was prepared following the procedure reported by [15]. The concentration of the aqueous neem leaf extract used was 3.0 mg/ml which was administered through drinking water for 6 weeks. The choice of the concentration was based on the earlier preliminary work done on the safety margin of the extract.

2.6 Vaccine

A live IBD (intermediate plus strain) vaccine containing $\geq 10^{3}$ EID_{50}/ml was used for the study which was sourced from an Agro- Veterinary Company in Kaduna, Nigeria. The vaccination was carried out at 2 and 4 weeks of age via oral route.

2.7 Challenge IBD Virus

At day 35 the birds in groups E, F, G and H were challenged orally with 0.1 ml of a live vvIBD virus containing $10^{9.76}$ CID/ml.

2.8 Experimental Design

2.8.1 Grouping

The 480 birds were assigned randomly into eight groups (A to H) with 60 birds per group. Group A was the negative control group and therefore, neither receive the aqueous extracts nor were they vaccinated against IBD. Group B was the positive IBD- vaccinated control group, thus, did not receive the aqueous extracts but was vaccinated against IBD. Group C, D, G and H were treated with 3.0 mg/ml of Neem leaf aqueous extracts in drinking water from day old to 6 weeks. Group C, F and G were vaccinated against IBD while D, E and H were not vaccinated against the IBD. Birds in group E, F, G and H were challenged with vvIBD virus while those in group A, B, C and D remained unchallenged.

2.8.2 Vaccine and vaccination

Birds from groups B, C, F and G were vaccinated against IBD at 14 and 28 days of age with IBD intermediate plus vaccine containing $\geq 10^3$ EID_{50}/ml, 0.2 ml/bird via oral route while groups A, D, E and H remained IBD-unvaccinated.

2.8.3 Experimental infection

Birds from group E, F, G and H were experimentally challenged orally with 0.2 ml of vvIBD virus at 35 day of age (5 week old) while A, B, C and D were not challenged.

2.9 Blood Collection

At week post each IBD vaccination and vvIBD virus challenge, blood samples from five birds in each group were collected randomly for haematology. Using 21 gauge needle and 2 ml syringe blood was collected directly from the heart which was immediately transferred into an EDTA bottle.

2.10 Haematology

Total leukocytes count and differential leukocytes count were determined as described by [16] from the blood samples collected immediately after the collection.

2.10.1 Total leucocyte counts

The blood was drawn up to 0.5 mark on the WBC blood cell diluting pipette. Glacial acetic acid fluid was further drawn into the pipette to bring the fluid up to the mark 101 above the bulb. The pipette was then shocked to mix the blood and the diluting fluid. The WBC was then counted using an improved neubeurhaemocytometer as described by [16].

2.10.2 Differential leukocyte counts

A drop of blood was placed on one end of the glass slide. A spreader slide was placed in front of the drop of blood at about 30° and pushed back till it just touched the drop. As soon as the spreader touched the blood, it was pushed forward smoothly and quickly at an angle of about 30°. The blood smears were air-dried for 2 minutes and stained with May- Grunwald-Giemsa for 10 minutes. The smears were then gently rinsed with distilled water and air-dried the stained slides were examined under oil. Immersion objective in a meander method to cover the entire slide. The cells (heterophils, basophils, eosinophils, lymphocytes and monocytes) were counted with the aid of tally counter.

Table 1. Experimental design

Group	IBD vaccination status	Experimental challenge with vvIBDV	Neem extract treatment
A	Unvaccinated	Unchallenged	No Extracts given
B	Vaccinated	Unchallenged	No Extracts given
C	Vaccinated	Unchallenged	3.0mg/ml Extract
D	Unvaccinated	Unchallenged	3.0mg/ml Extract
E	Unvaccinated	Challenged	No Extracts given
F	Vaccinated	Challenged	No Extracts given
G	Vaccinated	Challenged	3.0mg/ml Extract
H	Unvaccinated	Challenged	3.0mg/ml Extract

2.11 Statistical Analysis

The haematological parameters were presented in tables and their standard deviations calculated.

They were further subjected to one way analysis of variance (ANOVA) at 95% confidence interval using GraphPad® instat software.

3. RESULTS

3.1 Result of Haematological Parameters

3.1.1 Effect of neem leaf aqueous extract on haematological parameters of cockerels following vaccination against IBD

The haematological values for groups B and C showed that at 3 weeks of age (one week post primary vaccination; PPV), the basophils (BAS) and monocytes (MON) were not statistically different when compared to group A (negative control group). However, group B (untreated-vaccinated group) had a significantly (P<0.05) lower lymphocyte and total white blood cell counts (TWBC) values with higher heterophils (HET) when compared to group A. Group C (treated-vaccinated group) had a significantly (P<0.05) higher lymphocytes and TWBC when compared to group B. In addition, highest lymphocytes and TWBC values were recorded in group D when compared to other experimental groups (Table 2).

At 5 weeks of age (one week post booster vaccination; PBV), the lymphocyte and TWBC count in group B was lower when compared to that in group A but the difference was not significant. Significantly (P<0.05) higher value of lymphocytes and TWBC counts were evidenced in group C when compared to that in group B. Highest values of lymphocyte and TWBC counts was observed in group D when compared to other groups. Significantly (P<0.05) higher value of heterophil was observed in group B and C when compared to group A (Table 3).

3.1.2 Effect of neem leaf aqueous extract on haematological parameters of cockerels challenged and unchallenged 6 weeks old cockerel chicks

Significantly lower lymphocytes and TWBC counts were identified in group E and F when compared to those in group A. Statistically, significantly (P<0.05) higher values of lymphocytes and TWBC counts were found in group G and H when compared to those in group E and F while group G on the other hand, had significantly (P<0.05) higher values of lymphocyte and TWBC counts when compared to group H. Significantly (P<0.05) higher values of heterophils were observed in all the challenged groups (E, F, G and H) as compared to group A. Similarly, significantly (P<0.05) higher values of heterophils were observed in group E and F as compared to group G and H while group H had a significantly higher value of heterophil as compared to group G. All the challenged groups had significantly (P<0.05) lower monocytes counts when compared to the negative control group (group A). Group G had statistically unsignificant (P>0.05) higher values of monocytes when compared to the other challenged groups (Table 4).

Table 2. Effect of neem leaf aqueous extract on haematological parameters of vaccinated and unvaccinated 3 weeks old cockerels

Haematological parameters									
				3 Weeks Old					
GRP	Ext	Vac	Ch	LYMP	HET	EOS	BAS	MON	TWBC
A	-	-	-	87.8±1.39^{b}	10.4±1.47^{b}	0.79±0.38^{a}	0	0.9±0.36^{a}	16.51±2.37^{b}
B	-	+	-	82.50±1.41^{a}	14.99±1.09^{a}	0.89±0.69^{a}	0	1.61±0.7^{a}	13.22±1.93^{a}
C	+	+	-	85.50±1.50^{b}	13.0±1.42^{a}	0.4±0.28^{a}	0	1.1±0.3^{a}	20.7±3.0^{c}
D	+	-	-	89.8±0.26^{c}	8.10±0.9^{c}	0.9±0.1^{a}	0	1.2±0.3^{a}	21.0±3.1^{c}

$^{a-c}$ Values within column with no common superscripts differ significantly at P<0.05
KEY: GRP: Group, LYMP: Lymphocyte,MON: Monocyte, Ext: Extract, HET: Heterophil, TWBC: Total White Blood Cells, Vac: Vaccination, EOS: Eosinophil Ch: Challenge, BAS: Basophil

Table 3. Effect of neem leaf aqueous extract on haematological parameters of vaccinated and unvaccinated 5 weeks old cockerels

Haematological parameters									
				5 Weeks Old					
GRP	**Ext.**	**Vac.**	**Cha.**	**LYMP**	**HET**	**EOS**	**BAS**	**MON**	**TWBC**
A	-	-	-	83.20±2.1[a]	10.8±0.89[a]	4.8±0.52[a]	0	1.10±0.49[a]	13.90±1.23[a]
B	-	+	-	81.23±1.19[c]	12.97±1.19[b]	3.62±0.57[a]	0	2.12±0.51[b]	13.08±0.89[a]
C	+	+	-	83.99±1.0[a]	12.21±0.9[b]	1.8±0.34[b]	0	2.0±0.9[b]	15.3±3.2[b]
D	+	-	-	87.2±1.2[b]	8.59±0.7[a]	2.9±0.25[b]	0	1.31±0.8[a]	15.9±2.9[b]

[a-b] *Values within columns with no common superscripts differ significantly at P<0.05*

KEY: GRP: Group, LYMP: Lymphocyte, MON: Monocyte,Ext: Extract, HET: Heterophil, TWBC: Total White Blood Cells, Vac: Vaccination, EOS: Eosinophil, Ch: Challenge, BAS: Basophil

Table 4. Effect of neem leaf aqueous extract on haematological parameters of challenged and unchallenged 6 weeks old cockerels

Haematological parameters									
				6 Weeks Old					
GRP	**Ext.**	**Vac.**	**Cha.**	**LYMP**	**HET**	**EOS**	**BAS**	**MON**	**TWBC**
A	-	-	-	83.80±1.67[a]	10.8±0.99[a]	3.7±0.52[a]	0	1.7±0.49[a]	14.40±1.66[a]
B	-	+	-	79.5±1.03[b]	15.40±1.1[b]	3.2±0.74[a]	0	1.9±0.43[a]	13.55±2.21[a]
C	+	+	-	84.67±1.34[a]	10.56±1.43[a]	2.22±0.98[b]	0	2.56±0.56[b]	26.77±2.31[b]
D	+	-	-	86.90±3.36[a]	9.20±2.05[a]	2.05±0.53[b]	0	2.0±0.45[a]	28.8±1.14[b]
E	-	-	+	71.5±1.86[c]	23.8±1.45[c]	3.8±0.83[a]	0	0.9±0.21[c]	9.85±1.07[c]
F	-	+	+	73.5±2.64[c]	23.3±2.69[c]	3.1±0.79[a]	0	0.4±0.42[c]	10.26±1.28[c]
G	+	+	+	81.5±2.19[a]	14.6±1.21[b]	1.6±1.1[b]	0	2.3±0.59[b]	24.06±1.28[d]
H	+	-	+	77.4±2.23[b]	18.1±2.0[d]	1.8±0.95[b]	0	2.6±0.34[b]	20.91±2.38[e]

[a-e] *Values within columns with no common superscripts differ significantly at P<0.05*

4. DISCUSSION

A week post IBD vaccination (3 weeks of age), the haematological parameters of the vaccinated groups (B and F) revealed a significant reduction in lymphocytes (lymphopenia) and TWBC when compared to the negative control group (group A). However, significant increase in heterophils (heterophilia) was observed in the vaccinated groups. The lymphopenia and decreased TWBC might be associated to the viraemia induced by the IBD vaccine virus as well as the gross/ microscopic lesions present in the BF possibly due to IBD vaccine which can also lead to immunosuppression as earlier reported by [5]. The tissue destruction due to the IBD vaccines administered could be responsible for the heterophilia seen. Conversely, group C and G had a significantly higher lymphocytes and TWBC when compared to group B and F while on the other hand, the group B and F had a higher heterophils when compared to group G and H even though not significant. The increased lymphocytes and TWBC observed in group C and G could be attributed to the immunostimulatory effects of Neem leaves aqueous extract used as earlier reported by [17] and [18], while the decreased heterophils observed in the same group C and G when compared to group B and F could be associated to the minimal tissue damage seen post IBD vaccination when compared to those in group B and F. Hence, more heterophils would be formed in the circulation in order to meet up with the demand to the target. Also, there may be a possibility that, these increased heterophils observed in group B and F was associated to the possible bacterial infection in addition to the vaccinal virus. This was because; throughout the study we did not administer any prophylactic antibiotic to any group. However, Neem leaves extract has been shown to have antibacterial effects, and as such those groups (C and G) that were on the Neem extract may have less heterophils than the others.

At 1 week post vvIBDV challenge (6 weeks of age), significantly lower lymphocytes and TWBC were recorded in group E and F when compared to group G and H and this further signifies that the Neem leaves extract has an immunostimulatory effects as earlier reported by Kwawukume et al. [17]. On the other hand, significantly higher values of lymphocytes and TWBC were observed in group G when compared to group H and this indicates that chicks in group G were more immunocompetent than those in group H and this could be attributed to the acquired immunity had following IBD vaccinations in addition to the extract administered while those in group H had no acquired immunity even though, they were also on the same extract and then both groups became challenged with vvIBDV. The significantly higher values of heterophils observed in the challenged groups (E, F, G and H) when compared to the negative control group was attributed to the viraemia due to the vvIBDV inoculation while the significant decrease in basophils observed in the challenged groups (E, F, G and H) when compared to the negative control group may also be associated to vvIBDV infection.

5. CONCLUSION

In conclusion, the IBD vaccines cause some changes in the haematological parameters (heterophilia and lymphopenia) and the very virulent IBDV affects some haematological parameters, resulting in panleucopenia, lymphopenia and heterophilia. However, increased lymphocytes and TWBC was observed following Neem leaf aqueous extracts administration with a resultant decrease in heterophils.

CONSENT

It is not applicable.

ETHICAL APPROVAL

As per international standard or university standard written ethical permission has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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