The effects of *Azadirachta indica* (Neem) leaf extract on white blood cell count and the immune response of chickens vaccinated with Newcastle disease vaccine

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**Abstract**

Plant-based medicine is gaining importance in veterinary medicine worldwide. Various parts of the neem plant (*Azadirachta indica*) are used by a high proportion of livestock farmers to treat a variety of animal ailments. Neem is known to enhance the immune system in humans. This study sought to evaluate the immune stimulatory effects of aqueous Neem Leaf Extracts (NLE) in chickens vaccinated against Newcastle Disease (ND), a disease, which is responsible for immense losses in unvaccinated rural chicken populations in Africa. Neem Leaf Extract was prepared by soaking pounded mature green neem leaves in boiling water overnight in a ratio of 1:4 (that is 1 part of leaves in 4 parts of water). The resulting fluid extract was strained and passed through a rotary evaporator to obtain a 20% concentrate. Day old cockerels were placed in three experimental groups of 34 birds each, which were given 0%, 5% or 10% NLE, orally, three times a week for 10 weeks. The dosage employed was 100 mg NLE per kg of bird weight. All birds were vaccinated with ND-I-2 vaccine at 2 weeks and La Sota vaccine at 6 weeks. At weekly intervals, 20 birds were selected from each group and bled for haematological and serological studies. Results showed that birds that received 5% and 10% NLE showed significantly higher total WBC counts (2.51 and 2.07 x 10^9 Cells/L respectively) than birds that were not given NLE (1.72 x 10^9 Cells/L) (P<0.05). Mean lymphocyte percentage counts were also significantly higher between week 3 and week 9 (56.13-58.88) in birds given 5% NLE and 54.75-58.5% in birds given 10% NLE compared to 39.12% - 47.62% in control birds. Between 7 and 10 weeks of age, the Geometric Mean Titre of birds vaccinated with LaSota ND vaccine at 6 weeks showed significant increases in birds given 5% and 10% NLE respectively compared to untreated control birds. It is concluded that, aqueous neem leaf extract can improve the immune response in chickens to ND vaccination through enhanced lymphocytogenesis.

**Keywords:** neem, immune response, Newcastle disease

**Introduction**

The need for enhanced immunity is very relevant in any livestock and poultry industry. An animal with a good immune system is considered generally able to overcome many pathogenic infections to a large extent. This is even more evident in some viral infections where control is usually based on vaccination and the ability of the body to recruit cells that can help combat the invading virus. One of such virus is the Newcastle disease virus, which is responsible for a highly contagious disease (Newcastle disease), of poultry, especially chickens, causing devastating losses in the indigenous poultry industry. The
disease is primarily controlled by vaccination but the rural resource poor farmers are unable to patronise vaccination due to the high cost of vaccines used, unavailability of cold-chain systems required by these vaccines in rural areas, small flock sizes as well as multi-aged birds (Samuel, 1987; Okitoi et al., 2000; Gueye, 2002). The scavenging chicken, which forms about 80% of the total poultry industry in Africa (Sonaiya, 1993, 1998; Brankhaert and Gueye, 2000; Amakye-Anim, 2000) are thus left to survive with minimal health management. There is the need therefore to find ways by which these birds could overcome such infections, one of which is the enhancement of the immune system.

The use of botanicals in medicine is gradually gaining importance as a result of some limitations with modern medicine including vaccines. Azadirachta indica, Neem, is one plant that is commonly used by most people to cure various ill conditions. One very common use of neem is its popularity as an insecticide. It has been found out that the major compounds in neem act as feed inhibitors in insects, thus disrupt their lifecycle (National Research Council 1992). Simple aqueous extracts of Neem leaves have also been reported to be the standard treatment for external parasitic infestations such as mange, lice and ticks (Fajimi and Taiwo, 2004) in most villages with far less side effects. Other discovered potential of neem include its use as an antimicrobial against fungi (Selvester, 1999) and viruses (Badam et al., 1999; Helmy et al., 2007). It is also known to have immunostimulatory effects in both man and animals (Ray et al., 1996; Sadekar et al., 1998; Njiru and Kofi-Tsekpo, 1999). It is in view of the latter that the study was carried out to evaluate the immunopotential of neem leaf extract in chickens against the Newcastle Disease (ND) virus. The study aimed to assess the effect of Neem leaf extract on leucocytosis, lymphocytosis and seroconversion in chickens vaccinated with ND vaccine and administered with the extract.

Materials and Methods

Aqueous extraction of Neem Leaves

Mature green Neem leaves were harvested from Neem plant and air-dried for two hours. Air-dried leaves were pounded in a wooden mortar and pestle until soft to touch and transferred into a clean plastic bowl. Pounded Neem leaves were then weighed and boiling water added in a ratio of 1:4 (that is 1 part of pounded leaves in 4 parts of boiling water). For instance, to twenty grams (20 g) of pounded leaves, eighty milliliters (80 ml) of boiling water was added. Leaves were allowed to steep overnight. Suspension was strained through a 1mm sterile cheese cloth and the residue discarded. The filtrate was then transferred into a 100 ml round bottom flask and water was evaporated to obtain a 20% extract concentrate using a rotary evaporator. The resulting filtrate containing the extract was allowed to cool and then transferred into tightly corked bottles wrapped in aluminium foil to prevent interactions with direct light and then stored in a 4°C refrigerator until tested.

Experimental Birds

One hundred and two (102) day-old cockerels were purchased for the experiment. Fifty of these birds were bled from the right jugular vein and sera obtained to determine levels of maternally derived antibodies. The birds were tagged on the left wing with numbers using a wing tag applicator and distributed into three experimental groups. Group 1 birds received 5% of NLE (prepared by diluting 5 ml of 20% concentrate with 15 ml of distilled water for a 20 ml final volume of extract), at a dosage of 100 mg/kg bird weight, three times a week. Group 2 birds...
received 10% of NLE (prepared by diluting 10 ml of 20% concentrate with 10 ml of distilled water for a final volume of 20 ml), administered as in Group 1 birds and Group 3 birds served as the control group and received no NLE. These birds were drenched with water. Twenty birds from each group were weighed individually and bled weekly for haematological studies.

All birds were put on chicken starter feed ad lib for the first four weeks and then fed grower mash till the end of the experiment at ten weeks. Feed was provided two times daily. Water containing vitamins and antibiotics was provided ad lib for the first 4 weeks after which no antibiotics were given. All birds were vaccinated in the first week with a first ND vaccine, Newcastle Disease vaccine I-2 (ND I-2) administered ocularly. In the second and fourth weeks, experimental birds were vaccinated against Infectious Bursal Disease (IBD). This was given through drinking water. At six weeks, all experimental birds were vaccinated with a second Newcastle disease vaccine, namely, LaSota administered through drinking water.

**Haematological studies: Determination of White blood cell count**

This was determined as described by Lillie (1977) with a few modifications: A volume of 4 μl of each whole blood sample was diluted in 1999 μl of WBC diluting fluid to make a total volume of 2000 μl, that is, a ratio of 1:500. This was determined by a haemocytometer and observed under a microscope. Cells were then counted using a cell counter and the total white blood cells calculated as:

\[
\text{Total white blood cell} = \frac{X}{4} \times 10 \times d_f
\]

where:

- \(X/4\) = average number of cells counted,
- 10 = depth of counting chamber
- \(d_f\) = dilution factor

**Differential white blood cell count**

A thin blood smear was prepared based on the diagnostic method of Garcia (2001) with slight modifications: Giemsa stain was prepared in distilled water at a ratio of 1:4 instead of the stated buffered saline at a ratio of 1:20. The smear was air dried and examined under a microscope for heterophils, eosinophils, monocytes, lymphocytes and basophils. A 100-cell differential was performed and each white blood cell expressed as a percentage of the whole.

**Serological studies**

**Haemagglutination Inhibition Test**

This was to determine the Geometric mean haemagglutination inhibition titre against the ND I-2 and La Sota vaccines. The beta procedure (constant virus, diluted serum) described by Hitchner et al. (1975) was used. A volume of 25 μl PBS was added to all wells in a 96 V-bottom well plate. To the wells of the first and eleventh columns 25 μl of standard ND antisera was added. A two-fold serial dilution was made across columns 1-10. Stock antigen of 4HA unitage determined from viral suspensions by titrating various dilutions of viral suspension against a constant volume of ND antisera and observing the endpoint at which complete agglutination occurs. The dilution that gave a titre of 4 and complete agglutination is considered the 4HA unitage of antigen. This was added to each of the wells at a volume of 25 μl, with the exception of the 12th well which served as the negative control. Standard Newcastle Disease Virus (25 μl each) was added to wells of the last two rows. The sides of the plates were tapped and incubated at room temperature for 20 mins. To all the wells of the plate, 25 μl of 1% chicken RBC was added and incubated at room temperature for 45 minutes.
The eleventh and twelfth columns served as the positive and negative controls respectively. The plate was observed and read. The highest titre at which the viral suspension could be inhibited, (observed by the settled clump of red blood cells at the bottom of the well), by NDV antisera was recorded as the mean HI titre. Results obtained were subjected to analysis of variance using the Statistical Package for Social Sciences (SPSS) version 16 and means were separated by Duncan’s Multiple Range Test (DMRT).

**Results**

**Immunostimulatory Effect of Neem Leaf Extract: Effect on Total White Blood Cell (WBC) Count**

**Fig. 1.** Effect of Neem Leaf Extract on mean total WBC count (Xx10^9 cells/L)

Key: E - exponent

Significant differences (P<0.05) were observed between counts of birds in the control group. (1.72 x 10E9 cells/L ± 0.069 (s.e) - 2.92 x 10E9 cells/L±0.05 (s.e)) and those administered with 5% (2.31 x 10E9 cells/L± 0.04 – 3.59 x 10E9 cells/L±0.08) and 10% (2.07 x 10E9 cells/L±0.03 – 3.51 x 10E9 cells/L±0.1) NLE respectively. No significant differences were however observed between birds treated with 5% and 10% Neem leaf extract although figures for the latter were lower. The total WBC count increased in all three groups within the first 3 weeks but decreased sharply in the fourth week. The numbers however increased steadily after the fourth week through to the tenth week.

**Effect on Lymphocyte Count**

**Fig. 2.** Effect of Neem Leaf Extract on Mean Percentage Lymphocyte Count

Mean percentage lymphocyte count in the control group differed significantly (P<0.05) from treated groups from the third to the tenth week. While mean percentage counts increased from 39.12% in the third week and reached its peak by the ninth week with a count of 47.62% in the control group, those in the treated groups increased from 56.13%-58.88% (5% Neem) and 54.75-58.5% from the third to the ninth weeks respectively. Generally, Lymphocyte counts were higher in treated groups than in the control group. In all groups percentage lymphocyte count increased steadily from the first to the ninth week but decreased slightly in the 10th week. Percentage lymphocyte in birds administered with 5% neem leaf extract was similar to those administered with 10% Neem leaf extract.

**Effect of Neem Leaf Extract on Heterophil Count**

Percentage heterophil count in birds given 5% or 10% NLE were significantly lower (P<0.05) than those of the control birds. The percentages range from 33-42 in NLE-treated birds and 44-57 in control birds. There were no differences in heterophil percentages whether birds received 5% or 10% NLE.

**Effect of NLE on percentage mean count of eosinophil, monocyte and basophil**

Figures 4-6 shows the effects of NLE on percentage
eosinophil, monocyte and basophil counts. No distinct pattern was observed in all three groups.

**Fig. 3.** Effect of different dosage levels of NLE on percentage mean heterophil count

**Fig. 4.** Effect of NLE on Percentage Monocyte count

**Antibody titres of cockerels given Neem Leaf Extract and vaccinated with ND I-2 vaccine and La Sota vaccine**

The mean antibody response of cockerels administered with NLE to ND vaccine over a ten-week period is shown in Fig 7.

Mean HI Titres at day old were high in all three groups ($\log_2 5$-$7$). At (day 7) there was a decrease to $\log_2 4.18$ in control birds $3.82$ in $5\%$ NLE-treated birds and $4.18$ in $10\%$ NLE treated birds. After vaccination in week 1 (day 7) with I-2 ND vaccine, further decreases in titres were observed after primary vaccination with I-2 in week 1 from $2.5$-$2.7$ to $1.7$-$1.8$ ($\log$ to the base 2) by the fifth week. The mean HI titres continued to drop gradually from an average of $\log_2 4.0$ to $\log_2 1.06$, $\log_2 1.41$, and $\log_2 1.29$ in the control, $5\%$ NLE-treated birds and $10\%$ NLE-treated birds respectively by the $6^{th}$ week (day 42). Seroconversion was low in all three groups and no significant difference was observed. At six weeks, second La Sota vaccine was administered. This caused increases in mean HI titres from the $7^{th}$ week (day 49) with high records in the $8^{th}$ and $9^{th}$ week (day 56-$63$) and slight decreases by the tenth week (day 70). At week 8-10, no significant differences were found in the titres of birds given $5\%$ or $10\%$ NLE. However significant differences ($P<0.05$) were found between the titres of control birds and those given NLE. Titres in $5\%$ NLE-treated birds were slightly higher ($\log_2 1.41$-$2.76$) than those of $10\%$ NLE-treated birds ($\log_2 1.29$-$2.53$). Titres in the control birds increased from $\log_2 1.06$-$1.94$ by the eighth week. Antibody titres in NLE-treated birds were however higher in these weeks than in control birds. The values for NLE treated birds were significantly higher ($P<0.05$) within the peak periods than in the control groups, with values close to protective levels.

**Fig. 5.** Effect of NLE on Percentage Eosinophil Count

**Discussion**

In evaluating Neem’s effects on haematological parameters, average total WBC counts ranged between $1.73 \times 10^9$cells/L to $3.59 \times 10^7$cells/L and these are within
the normal range of $1.6 \times 10^{6}$ cells/L to $10.8 \times 10^{9}$ cells/L (Lucas et al., 1961). Neem showed no effect on total WBC count within the first 2 weeks, most likely because of the high maternal antibodies which did not allow for seroconversion (Aning and Brewoo, 1999, 2001). The observed increase from the 5th through to the 10th week in control birds and NLE-treated birds showed that treated birds had higher total white blood cell counts. This is attributable to neem’s ability to boost the macrophage response in the body which stimulates the lymphocytic system and boosts the production of white blood cells (Sadekar et al., 1998). The higher WBC counts in birds that received 5% NLE than 10% NLE may be as a result of probable toxicity of neem at higher concentrations.

**Fig. 6.** Effect of NLE on Percentage Basophil count

![Graph showing effect of NLE on Basophil count](image)

**Fig. 7.** Effect of NLE weekly geometric mean HI titre (log to the base 2) against NDV I-2 and La Sota

Higher lymphocyte counts in treated groups from results, compared to the control group can be attributed to NLE. Results from this study support reports that have shown Neem to significantly give higher lymphocytic proliferative response in treated mice to *in vitro* challenges with spleen cells (Njío and Kofi-Tsekpo, 1999) and rats (Parshad et al., 1994). Generally Neem is thought to cause stimulation or potentiation of the immune system in at least one aspect of the immune response (Van der Nat et al., 1987). Van der Nat et al. (1987) cited by Njío and Kofi-Tsekpo (1999), found that an aqueous *A. indica* extract of the stem bark produced a dose-dependent increase in the production of migration inhibition factor *in vitro*. According to these authors, this will lead to the localization of macrophages and monocytes in vivo which in turn would favour an enhancement of the immune response through the proliferation of lymphocytes. In another study, neem stem bark extract was found to increase both IgM and IgG levels produced by B-lymphocytes but inhibited macrophage migration (Ray et al., 1996). This led the authors to conclude that extracts of Neem enhanced both humoral and cell mediated immunity. Other studies have also shown that chickens with immunosuppressed conditions that were fed powdered dry leaves showed significantly enhanced humoral and cell-mediated immune responses (Sadekar et al., 1998).

Heterophil counts were consistently higher in the control group (44.75-58.25%) than in the treated groups (34.25-40.87% for 5% NLE and 33.38-40.62% for 10% NLE). Numbers were however within range of normal values of 30-75% (Aiello et al., 1998). Heterophils in birds (like neutrophils in mammals) are important during bacterial infections and act as microphages. It is noteworthy that NLE suppressed heterophil levels. Its effect during bacterial infection is worth investigating. Results from the percentage mean counts of eosinophils,

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monocytes and basophils show that NLE has no significant effect in these white blood cells. The numbers were within the expected ranges of 0-5% for monocytes and basophils and 0-4% for eosinophils. Although the differential white blood cell counts exhibited some levels of significance, the basophils, eosinophils and monocytes were within the reported ranges of 3-17, 0-0.5 and 0-5 x 10^3/L respectively for clinically healthy birds (Aiello et al., 1998).

With respect to seroconversion, antibody titres in this study decreased from the 1st week through to the fifth unlike in other reported cases where NDV antibodies were found to increase after treatment with Neem extracts (Sadekar et al., 1998, Ahsan et al., 1999) within the first few weeks. This decrease in Ab titres is likely due to vaccination failure primarily attributable to interference by maternal antibodies in the first week (Tizard, 1977). It is also known that maternal antibody levels undergo decay during the first two weeks of age in birds (Box, 1985). Secondly there is a possibility that vaccine used was not fully viable. With a second ND vaccine administered, slight increases were observed in all three groups with treated birds having significantly higher titres than control birds. These results agree with earlier observations that NDV antibodies were found to increase after treatment with Neem extracts (Sadekar et al., 1998; Ahsan et al., 1999) in chickens.

Conclusion

This study concludes that Neem may be a potential immune system booster in chickens from the proliferation of white blood cells and increased production of lymphocytes as well as increased antibody titres in treated birds. It may therefore be used to help in immunocompromised conditions in chickens such as during Infectious Bursal Disease Virus Infection which is usually found in association with the Newcastle Disease Virus infection. The study recommends that further research in clinical trials should be undertaken to ascertain other factors such as the toxicity of NLE in the liver and other internal organs as well as the determination of the weights of internal organs. Trials should also be undertaken in other groups of chickens such as layers and broilers and local scavenging chickens as well as other breeds in order to get comparable results. It is also recommended that this experiment be repeated to confirm the results reported here.

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