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In vivo Antiviral Assay of methanolic extract of *Diospyros mespiliformis* on Newcastle disease virus (NDV)

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ABSTRACT

The *in vivo* antiviral assay of methanolic extract of *Diospyros mespiliformis* on newcastle disease virus (NDV) was performed using chicken. The chickens were brought and kept in well ventilated and sterilized bamboo cages that were covered with mosquito netting, and fed *ad libitum* on locally available commercial feeds and water. Following inoculation, clinical signs and mortality rates were monitored daily from different groups. Body weight and blood samples obtained from chickens from different groups on days 0, 4, 5, 6, 7, 10, and 16 (expected termination day of the experiment). Following the infection of the chickens with NDV in the *in vivo* experiments, the onset of clinical symptoms were rapid from day- 2 PI in Gp2 (infected and untreated) and by day- 6 PI, mortality which was first observed in day- 4PI has reached 80% in all groups. The decrease in body weights in group 2 (infected and untreated) of experiment 1 on day-4 post infection from 2.05kg to 1.10kg was significant. However, in birds that were treated with *Diospyros mespiliformis* extract following inoculation with ND in groups 1and 3 of experiment 1, there was body weights decrease of 1.87kg and 1.74kg from 2.0kg and 20.1kg respectively. Results from this research showed that development of clinical signs from groups of birds that were treated with *Diospyros mespiliformis* extract were delayed as opposed to those not treated. The delay in clinical signs and mortality among the *D. mespiliformis* treated groups as compared with the untreated groups in both experiments is suggestive of the protective role of the extract in the infected but treated chickens.
Keywords: *Diospyros mespiliformis,* antiviral, NDV, chickens and infection.

INTRODUCTION

Newcastle disease virus (NDV), formerly called exotic Newcastle disease [1], [2], [3], is a contagious viral avian disease affecting many domestic and wild bird species; it is transmissible to humans [4], [5]. Though there are rare cases where the disease gives a mild fever and/or conjunctivitis. Its effects are most notable in domestic poultry due to their high susceptibility and the potential for severe impacts of an epizootic on the poultry industries [6, 7, 8]. It is endemic to many countries [9], [10], [11], [12].

Exposure of humans to infected birds (for example in poultry processing plants) can cause mild conjunctivitis and influenza-like symptoms, but the Newcastle disease virus (NDV) otherwise poses no hazard to human healths [13], [14], [15], [16]. No treatment for NDV is known, but the use of prophylactic vaccines [17] and sanitary measures reduces the likelihood of outbreaks.

The causal agent, Newcastle disease virus (NDV), is a variant of avian orthoavulavirus 1, a negative-sense, single-stranded RNA virus. NDV belongs to the subfamily Avulavirinae, which infect birds [18], [19]. Transmission occurs by exposure to faecal and other excretions from infected birds, and through contact with contaminated food, water, equipment, and clothing. NDV is spread primarily through direct contact between healthy birds and the bodily discharges of infected birds. The disease is transmitted through infected birds droppings and secretions from the nose, mouth, and eyes. NDV spreads rapidly among birds kept in confinement, such as commercially raised chickens [20].

High concentrations of the NDV are found in birds bodily discharges; therefore, the disease can be spread easily by mechanical means. Virus-bearing material can be picked up on shoes and clothing and carried from an infected flock to a healthy one. NDV can survive for several weeks in a warm and humid environment on birds feathers, manure, and other materials. It can survive indefinitely in frozen material. However, the virus is destroyed rapidly by dehydration and by the ultraviolet rays in sunlight. Smuggled pet birds, especially Amazon parrots from Latin America, pose a great risk of introducing NDV into the Unites States of America [21]. Amazon parrots are carriers of the disease, but do not show symptoms, and are capable of shedding NDV for more than 400 days.

Diospyros mespiliformis is an evergreen tree that reaches up to 20 m in height, or up to 45 m in forests. It is characterized by a wide spreading and dense canopy and dark grey bark [22]. It is commonly found in tropical Africa, south of the Sahara. West African Ebony has a wide range of medicinal uses. Different parts of the plants can be made into variation and used in the treatment of a range of conditions like fever, pneumonia, dysentery, syphilis, leprosy, yaws, menorrhoea, diarrhoea, headaches, arthritis, gingivitis, toothache, cuts and wounds, otitis, stomach pains, sores and ulcers.

The plant is widely used in traditional medicine in parts of Africa, and a number of medically active constituents have been isolated. The principle constituent appears to be plumbagin, which has been shown to have antibiotic, antihaemorrhagic and fungistatic properties. It is found in the root-bark to a concentration of 0.9% and but a trace in the leaves. Tannin, saponin and a substance probably identical to scopolamine are also present [23].

Experimental birds Sources

Chickens (broilers and cockreals) 2 months and 2 weeks of age respectively were purchased from a local poultry industry practitioners in Nnewi and Nnobi both in Nnewi North and Idemili North Local Government Area in Anambra State, Nigeria. None of the chicken sources were reported to have been vaccinated against NDv. Two replica experiments were carried out. In the first experiment, 75 chickens were used. The chicks were wing – tagged body weights taken and determined. They were also screened for antibodies against NDV using haemagglitination inhibition test (HI) [1]

Reconstitution of Extract

Phosphate buffered saline (PBS) was used to reconstitute the methanolic plant extract of *Diospyros mespiliformis* used in the *In vivo* Antiviral Assay on Newcastle disease virus (NDV) using live chickens. By carefully weighing out 400mg of the extract using an automatic electric weighting balance and dissolving it in 1liter (1000ml) of water to gire a concentration of 400mg/kg.

Pre-inoculation, Monitoring and Grouping

The chickens were brought and kept in well ventilated and sterilized bamboo cages that were covered with mosquito netting, and fed ad libitum on locally available commercial feeds and water. During this period of monitoring and reering, the chicks were stabilized by the administration of antibiotics (OTC$^{(R)}$), anticoccidials (Amprotium 200$^{(R)}$) and antihelmintics (Kukuzole$^{(R)}$) for a period of one month to reduce and eliminate bacteria, Coccidians and helminth endoparasites infections respectively. The doses were administered two times daily orally.

Prior to the infection of the chickens, they were screened for antibodies against Newcastle disease (ND), body weights determined wing-taged and then were randomly assorted into five experimental groups. The chicks were randomly sorted into five experimental groups namely: Group1 (pre-treated and Infected), Group2 (Infected and Untreated), Group3 (Infected and treated), Group4 (Uninfected and Untreated) and Group5 (Extract Treatment only). Fifteen (15) chicks were used in each of the group.

In the second experiment, 37 chickens (acquired and stabilised as above) were used. After screening for antibodies against Newcastle disease virus, they were divided into different groups namely: Group1 (Pre-treated and Infected), Group2 (Infected without Treatment), Group3 (Infected and treated) and Group4 (Uninfected and Untreated) and Group5 (Treatment control). Group1-3 consisted of 9 chickens each while Groups 4 -5

consisted of 5 chickens each. The detailed experimental protocol is shown in figure.1.

Figure **1**. *Invivo* Experimental Protocol (NDV)

CHICKENS (2months and 2 weeks)

↓

Period of Stabilization with antibiotics, anticoccidials and antihelmintics for 1 month. Prior to infection of chickens, screened for antibodies against ND, body weights determined,wing-taged and then randomly assorted into five experimental groups.

↓

Grouped randomly into 5

Group1	Group2	Group3	Group4	Group5
Gp1 Pre-treated and infected (n=15) DM treated orally x2 for 7days before infection and 7 days PI 400mg/kg.	Gp2 Infected and untreated (n=15) No treatment after infection	Gp3 Infected and treated (n=15) 0-7day PI	Gp4 Uninfected and untreated n=15 No Infection, no treatment	Gp5 Treated and uninfected n=15 treatment control 7 days

↓

Following inoculation, Clinical sign and mortality rates were monitored daily from different groups. Body weights and blood samples obtained from chickens from different groups on days 0,4,5,6,7,10, and 16. Pathological changes and Histopathology determined.

Haemagglutination Inhibition (HI) test

Antibody response to the Haemagglutinin protein in the Newcastle disease virus envelope can be measured by the HI test as described by Allan and Gough for serological testing [1]. By performing two-fold serial dilutions on the serum prior to testing, the concentration of the serum antibodies can be expressed as an HI titre to the log base 2. Using a micropipette, 25ul of fresh PBS was dispensed into each well of a plastic V-bottomed micro titre plate. Twenty-five micro liter (25ul) of well shaked serum sample was added into the first well and the last (control) well of a row of a micro well plate. A serial two-fold dilution (along each row) of 25ul volume was carried out until the second last well from the end. From the second last well, 25ul volume of dilution was discarded. The last well was used as the serum control and was not diluted. Twenty-five micro liter (25ul) of 2HA virus antigen (Haemagglutinin 2HA units) was added in each of the well excluding the control well in the last column. The content of the micro titre plate was mixed properly by flapping gently, covered with a lid, and allowed to stand for 30minutes at room temperature. Twenty-five micro liters (25ul) of 1 percent v/v washed chicken red blood cells was added to each well including the control well in the last column. The content of the micro titre plate was gently tapped by the sides to mix the reagents which was covered with a lid

and allowed to stand at room temperature for 45minutes. The wells were then observed for haemagglitination inhibition indicating the presence of antibodies. The setting pattern of each serum sample was observed. The red cells will settle as a button where antibodies were present but in the well where antibodies were absent, the cells will agglutinate .the end point of the titration is the well that shows complete haemagglitination inhibition. The HI titre is the highest dilution of serum causing complete inhibition of 2HA of antigen. The agglutination was assessed by tilting the plates with only those wells where the RBCs stream at the same rate as the control well should be considered to show inhibition. The antibody level for each serum sample was recorded and was expressed as a log base 2.

Controls

Positive control: Serum sample with HI titre of 2^{10} and showed inhibition of viral haemagglitination activity when tested by the HI lest.

Negative control: Serum sample with HI titre of 2^{1} and showed no inhibition of viral haemagglitination activity when tested by the HI test.

Inoculation/Infection and Treatment regimen of Various Groups

The various chickens of Groups1, -groups2 and groups3 of experiments 1 and 2 were infected with 1ml of NDV stain characterized as velogenic viscerotropic strain with the virus containing an effective infective dose of EID_{50} $10^{8.6}$ /ml. The infectious titre was obtained by first inoculating the virus into embryonated chicken eggs hence multiplication as described by O.I.E. (1996) [16].

NDV Infection: Inoculation was done as ocular and nasal drops, with a drop on each location on one side of the face, while the remainder of the inoculum was given orally (Yongolo, 1996). Observations for signs of Newcastle disease and mortality was made twice daily for the entire period of the study.

The treatment schedule was effected as indicated below:

Group1-Pre-treated and Infected (n = 15). Treated orally with reconstituted extract of *Diospyros mespiliformis* at a concentration of 400mg/kg two times daily. Morning and evening – for 7 days before infection followed for another 7 days post Infection (PI).

Group2 -Infected and untreated (n = 15). No treatment was administered after infection.

Group3- Infected and treated (n = 15). Infected and Treatment began immediately from day 0 to 7 PI.

Group4-Uninfected and Untreated (n = 15). No infection and no treatment.

Group5-Treatment control (n = 15). Treatment given but no infection for 7 days.

In this study, the same experimental protocol used in 1 was also used in 2. **Figure. 1.** Experiment 2 was carried in order to verify the findings of experiment 1.

Post-Inoculation monitoring.

Following inoculation, clinical signs and mortality rates were monitored daily from different groups. Body weight and blood samples obtained from chickens from different groups on days 0, 4, 5, 6, 7, 10, and 16 (expected termination day of the experiment). The serum obtained to be investigated for antibodies against NDV using Haemagglutination Inhibition (HI) test. Pathological changes were determined in addition to histopathology.

***In vivo* Activity of *Diospyros mesipiliformis* Against NDV Clinical signs**

The clinical signs were recorded/observed on days-2 post infection where 4 chickens representing 26.7% of total from GP2 (infected and untreated) in both experiments showed clinical signs. The clinical signs observed were those of dropping wings, sneezing and coughing (Table. **1**) No clinical signs were recorded from the treated groups (Gp1 and Gp3) on this day.

On day-3 post infection, about 60% of these untreated chickens in Gp2 came down with more clinical signs like anorexia, nasal discharge, greenish and watery diarrhoea and were crouching together in a group. On this day about six birds representing 40% of total (15) from *Diospyros mespiliformis* treated group three (Gp3) and 20% treated group one (Gp1) started developing respiratory distress (anorexia, sneezing, crouching together). By the 4^{th} day P1 several birds started developing more clinical signs and greenish diarrhea was observed in many of the bird in all groups irrespective of whether treatment was given or not. From the 5^{th} day P1, many birds from different groups developed nervous clinical signs

like twisting necks and head with some coming down with paralysis. The trend continued until the seventh day where few remaining birds developed nervous signs. The control groups (Gp4 and Gp5) recorded no clinical signs at the end of the experiment (Table. **1 and 2**).

MORTALITY TREND (NDV)

Mortality of the birds in the study were concentrated on days 0-6 as about 80%, 90%, and 87% of chickens in groups 1,2 and 3 respectively of experiment 1 died by day 6 post infection. The trend was similar in experiment 2, where about 55%, 88% and 65% of the experimental birds died by the end of day 6 PI in all the infected groups 1, 2, and 3 respectively (Table. **3 and 4**). The first chicken mortality was observed in day 4 PI in experiment 1, where 2 chickens were lost in both Gp2 and Gp3, (infected and un- treated, and infected and treated) respectively. In experiment 2, two chickens were lost in Gp2 (infected and untreated) whereas only one chicken was lost in Gp3 (infected and treated). Only one chicken was lost in Gp1 (pretreated and infected) of experiment 1 on this 4th day post infection, whereas in experiment 1, Gp1 (pretreated and infected) recorded no death (Table. **3 and 4**)

No statistically significant difference in mortality between infected groups ($p>0.05$) was observed on day -4 post infection. As the disease progressed into the acute phase, the mortality rates were significantly different (<0.05), in experiment 1, with highest rates in Gp2 (infected and untreated) which recorded eleven(11) dead on the 5th day PI representing about 73.3%. Gp3 (infected and treated) recorded 53.3% chickens mortality, while Gp1 (pretreated and infected) recorded 46.7% chicken mortality. By the end of day – 6 post infection, additional four (4) chickens representing 26.7% were lost in Gp1 (pretreated and infected) bringing the total birds lost at twelve (12) by the end of 6- day PI. In Gp2 (infected and untreated) additional one chicken died bringing the number of dead birds to fourteen (14) out of the total of fifteen (15). This fourteen lost chickens represented about 93.3% of total. In Gp3 (pretreated and infected) additional three (3) chickens representing 20% got lost by the end of 6-day PI. Altogether, thirteen (13) chickens died in this group by the time the experiment swas terminated on the 16th day (Table**. 3**).

No mortality was recorded among Gp4 (uninfected and untreated) and Gp5 (uninfected and treated) at the end of the experiment.

In experiment 2, almost the same pattern of mortality did occur, however, not with same variation in the trend of mortality. Death were recorded in Gp2 and Gp3 on the 4th day PI (2; 1 deaths respectively) but mortality was first recorded on day -5 PI in Gp1 (pretreated and infected) (22.2%). Gp2 and Gp3 recorded five and three death on this day (55.6%; 33.3% respectively). Mortality on day -6 PI was higher in Gp3 (22.2%) than in Gp2 (11.1%) and Gp1 recorded (33.3%) (Table **6**). By the end of the 7th -day and 8th -day PI, GP1 lost one chicken on each day bringing the total PI number of birds lost to seven (77.7%) of the total (9) by the 16th day when the experiment was terminated. In Gp2, there was 100% mortality by the 16th day when the experiment was terminated. In Gp3 where additional one chicken died by the end of 7-day PI. This brought the total number of chicken lost in this group to seven (77.7%). No death was observed in the control groups Gp4 (uninfected and untreated) and Gp5 (treated and uninfected) at the end of the experiment. Tables **3 and 5** summarized the results.

BODY WEIGHTS

The changes in body weights of experimental birds in various groups studied are shown in table. **3**. It was observed that by the end of 4th day P1 (when almost 80% of chickens from all treated groups were gone), there were decrease in the mean body weights of the infected group s (Gp1, Gp2 and Gp3). In the first experiment, there was decrease in mean body weights at the end of 4th day by 6.5%, 46.3% and 13.4% respectively for groups 1, 2 and 3.

Whereas in the 2nd experiment .the mean body weights decreased by 17.9%,53.8% and 18.5% for G1,Gp2 and Gp3 respectively.

Interestingly, there was increase in mean body weight of 29.5% and 35.3% for untreated groups 4 and 5 as against 53.8% and 55.6% in the 2nd experiment.

There was a significant decrease in the mean body weights of chickens in Gp2 ($p<0.05$) in both experiments. The body weights of the chickens in the uninfected groups (Gp4 and Gp5) increased significantly ($P<0.001$) between days 0 and 4 the 16th day P1, the mean body weight in

group 2 came down by 17.1% in experiment 1 and an increase of 9% and 4.5% in treated groups 1 and 3 of experiment 1, whereas the untreated groups (4 and 5) recorded increased mean body weights of the chickens by 44.7% and 55.1% respectively. In experiment 2 there was increased mean body weights of groups 1, 3, 4 and 5 respectively to 32.1%, 33.3%, 111.6% and 146.2% at the end of 16th day post infection.

ANTIBODY LEVELS (NDV)

Antibody against ND was first detected on day-4, PI. From then onwards, there was a progressive and systematic increase in antibody levels in the infected groups in both experiments during the 16days periods of observation. Antibody levels increased to 10log2 in all infected groups, however, there was no significant difference ($p>0.05$) between the groups.

In experiment 1, the antibody levels in the infected groups were first detected in day 4 PI without any significant difference between Gp2 and Gp3. By days -6 PI, the antibody levels were the same in Gp1,Gp2 and Gp3 after which there was a parallel increase in Gp1 and Gp2. Gp3 had slightly higher titre although it was statistically not significant.

In experiment 2, antibody levels were first detected on day -6 PI, from the surviving chickens in the infected groups. After this there was a parallel increase in antibody titre in both Gp1 and Gp3 with no significant difference up till the last estimation on day -16 when the experiment was terminated.

In the control groups, Gp4 and Gp5 (uninfected and untreated and treated and uninfected respectively), the mean antibody levels of all the chickens were estimated as titers of 2^1

Table 1: Clinical signs in bird after Infection with NDV and treatment with Extracts of *Diospyros mespiliformis*

Days Post infection	GPI (pretreated and infected) n =15	GP2 (Infected and Untreated) n = 15	GP3 (Infected and Treated) n = 15	GP4 (Uninfected and Untreated) n = 10	GP5 (Treated and Uninfected) n = 10
2nd day	No clinical signs	4chickens (26.7%) showed dropping wings, sneezing coughing	No clinical signs	No clinical signs	**No clinical signs**
3rd day	3chickens (20%) started showing respiratory distress.	9chickens (60%) came down with anorexia, nasal discharge, crouching together and watery diarrhea	6chickens (40%) showed signs of respiratory distress	No clinical signs	**No clinical signs**
4th day	Several chickens developed greenish diarrhoea with one (6.7%) death	More chickens developed observable clinical signs: watery diarrhea, crouching together, recorded 2 (13.3%) death	More clinical signs observed greenish diarrhoea. 2 (13.3%) deaths recorded	No signs	**No signs**
5t day	7 (46.7%) deaths, swelling of the tissues around the eyes and in the neck	11 deaths. Twisting neck and heads and general (complete) paralysis, 7 (73.3%) deaths	8 (53.3%) deaths	No signs	**No signs**
6th day	4 (26.7%) deaths	1 (6.7%) dead	3 (20.0%) deaths	No signs	**No signs**
7th day	**1death (6.7%) nervous signs**	**Nervous signs: depression, muscular tremors,**	**Nervous signs: dropping wings, twisting of neck.**	**No clinical signs**	**No clinical signs**

Table 2. Scoring of the clinical signs in birds after Infection with NDV and Treatment with the Extract of *Diospyros Mespiliformis*

P1D Day Post Infection	G1(n=15)	G2(n=15)	G3(N=15)	G4(n=10)	G5(n=10)
2	-----	(4) +	-----	-----	-----
3	(3) +	(4) +(9) ++	(6) +	-----	-----
4	(6) ++ (4) +	(11) ++ (4) ++	(8) ++ (3)+++	-----	-----
5	(9) ++ (3) +	(2) ++ (11) +++	(12) ++ (1) ++	-----	----
6	(5) ++ (2) +	(2) ++ (1) +++	(4) ++ (1) +	-----	-----
7	(1) ++ (2) +	(1) +	(2) +	-----	-----
10	(2) +	(1) +	(2) +	-----	-----
13	(2) +	(1) +	(2) +	-----	-----
16	(2) -	(1) +	(2) -	-----	-----

Scoring procedures:
-, no clinical symptoms; +, drooping wings, sneezing; ++, anorexia, respiratory distress, crouching together, greenish watery diarrhoea; +++, watery diarrhoea, crouching together, anorexia, respiratory distress, paralysis. Scoring procedures according to Christensen et al (1996).

Table 3. Trends in mortality rates of the chicken infected with NDV and treated with 400mg/kg extracts of *Diospyros mespiliformis*

Groups	Days (post Infection)	Number of deaths	Percentage (%) mortality	Number survived
GP1 Pretreated and Infected	4th day	1	6.7%	14
	5th day	7	46.7%	7
	6th day	4	26.7%	3
	7th day	1	6.7%	2
	16th day	0	0.0%	2
GP2 Infected and Untreated	4th day	2	13.3%	13
	5th day	11	73.3%	2
	6th day	1	6.7%	1
	16th day	0	0.0%	1
GP3 Infected and Treated	4th day	2	13.3%	13
	5th day	8	53.3%	5
	6th day	3	20.0%	2
	16th day	0	0.0%	2
GP4 Uninfected and Untreated	4th day	0	0.0%	15
	5th day	0	0.0%	15
	6th day	0	0.0%	15
	16th day	0	0.0%	15
GP5 Treated and Uninfected	**4th day**	**0**	**0.0%**	15
	5th day	**0**	**0.0%**	15
	6th day	**0**	**0.0%**	15
	16th day	**0**	**0.0%**	15

Table 4: Variation in Average Body weight (kg) of birds infected with Newcastle disease virus and Treated with 400mg/kg Extracts of *Diospyros mespiliformis.*

Days Pest Infection	Group 1 Pre Treated and Infected		Group 2 Infected and Untreated		Group3 Infected and Treated		Group 4 Uninfected and Untreated		Group 5 Treated and Uninfected	
	Average weight	% Increase % Decrease	Average weight	% Increase % Decrease	Average weight	% Increase % Decrease	Average weight	% Increase % Decrease	Average Weight	% Increase % Decrease
0	2.0	=	2.05	=	2.01	=	1.90	=	1.87	=
4	1.87	6.5% ↓	1.10	46.3% ↓	1.74	13.4% ↓	2.46	29.5% ↑	2.53	**35.3%** ↑
16	**2.18**	**9.0%** ↑	**1.7**	**17.1%** ↓	**2.1**	**4.5%** ↑	**2.75**	**44.7%** ↑	**2.90**	55.1% ↑

Table 5. Mean Antibody titres (Log2 reciprocal of dilution factor) of chicken serum infected with NDV after treatment with 400mg/kg extract of *Diospyros mespiliformis*

4th day	**4.0 (14 chicks)**	**5.0 (13 chicks)**	**6.0 (13 chicks)**	**1.0 (15 chicks)**	**1.0 (15 chicks)**
5th day	6.0 (7 chicks)	7.0 (3 chicks)	7.0 (5 chicks)	1.0 (15 chicks)	**1.0 (15 chicks)**
6th day	8.0 (3 chicks)	8.0 (1 chicks)	8.0 (2 chicks)	1.0 (15 chicks)	**1.0 (15 chicks)**
10th day	8.0 (2 chicks)	8.0 (1 chicks)	10.0 (2 chicks)	1.0 (15 chicks)	**1.0 (15 chicks)**
16th day	**10.0 (2 chicks)**	**10.0 (1 chicks)**	**12.0 (2 chicks)**	**1.0 (15 chicks)**	1.0 (15 chicks)

Table 6. Trends in mortality rates of the cockrel infected with NDV and treated with 400mg/kg extracts of *Diospyros mespiliformis*

Groups	Days (post Infection)	Number of deaths	Percentage (%) mortality	Number survived
GP1 Pretreated and Infected	4th day	0	==	**9**
	5th day	2	22.2%	**7**
	6th day	3	33.3%	**4**
	7th day	1	11.1%	**3**
	8th day	1	11.1%	**2**
	16th day	0	0.0%	**2**
GP2 Infected and Untreated	4th day	2	22.2%	**7**
	5th day	5	55.6%	**2**
	6th day	1	11.1%	**1**
	7th day	1	11.1%	**0**
	16th day	==	==	**0**
GP3 Infected and Treated	4th day	1	11.1%	**8**
	5th day	3	33.3%	**5**
	6th day	2	22.2%	**3**
	7th day	1	11.1%	**2**
	16th day	0		**2**
GP4 Uninfected and Untreated	4th day	0	0.0%	**15**
	16th day	0	0.0%	**15**
GP5 Treated and Uninfected	4th day 16th day	0	0.0%	**15**
		0	0.0%	**15**

Invivo: 2nd experiment using Cockrel.

Table 7: Mean Antibody titres (Log2 reciprocal of dilution factor) of cockrel serum infected with NDV after treatment with extract of *Diospyros mespiliformis*.

Days post infection	Gp1 pre-Treated and Infected	Gp2 Infected and Untreated	Gp3 Infected and Treated	Gp4 Uninfected and Untreated	Gp5 Treated and Uninfected
6th day	3.0 (4 chicks)	2.0 (1 chicks)	3.0 (3 chicks)	1.0 (5 chicks)	**1.0 (5 chicks)**
7th day	5.0 (3 chicks)	==	6.0 (2 chicks)	1.0 (5 chicks)	**1.0 (5 chicks)**
8th day	5.0 (2 chicks)	==	8.0 (2 chicks)	1.0 (5 chicks)	**1.0 (5 chicks)**
12th day	8.0 (2 chicks)	==	8.0 (2 chicks)	1.0 (5 chicks)	**1.0 (5 chicks)**
16th day	**10.0 (2 chicks)**	==	**12.0 (2 chicks)**	**1.0 (5 chicks)**	**1.0 (5 chicks)**

Days post Infection	GP1 Infected and Treated	GP2 Infected and Untreated	GP3 Pre-treated and Infected	GP4 Uninfected and Untreated
	N = 21 Adult chicks 10 Young chicks 11	N = 21 Adult chicks 10 Young chicks 11	N = 21 Adult chicks 10 Young chicks 11	**O = 10 Old chicks 10 Young chicks 5**
7th day	Clinical examination of the birds showed individuals with nodular lesions, on the head including the eyelids beak on the legs including the digits of the 21 birds, five showed clinical signs representing 23.8%. Four young ones and one adult.	Clinical signs showed in 17 (80.95%) birds Eleven young and six adults.	3 (14.3%) birds showed clinical signs. Only the young ones.	**No clinical observation recorded.**
21st day	2 (9.5%) deaths – all young ones	7 (33.3%) deaths –2 adults and 5 young ones.	All those with clinical signs survived	
28th day	**Others with clinical signs survived**	**Others with clinical signs survived**		

DISCUSSION

Following the infection of the chickens with NDV in the *in vivo* experiments, the onset of clinical symptoms were rapid from day- 2 PI in Gp2 (infected and untreated) and by day- 6 PI, mortality which was first observed in day- 4PI has reached 80% in all groups. The strain of the virus used was velogenic viscerotropic and by definition of Office International des Epizooties [16], such a strain has potential to cause up to 100% mortality in natural infection of a flock by day- 3 PI.

By day- 3 PI with NDV, nine of the fifteen chickens representing 60% in Gp2 of experiment 1 showed clinical signs of NDV challenge, including depression, huddling, ruffling of feathers and reluctance to move. By day- 4 after infection, all the infected chickens were down clinically and apart from other noticeable symptoms, had greenish diarrhoea, oedema of the face and various degrees of nervous disorders. The nervous signs included torticolis, paresis of limbs, turning in circles and resting on the beak. There was no clinical sign in Gp1 (pretreated and infected) and Gp3 (infected and treated) on day – 2PI. Clinical signs started manifesting in these groups on day – 3 PI.

Mortality started from day four post infection in all the groups, with two each representing 13.3% in Gp2 and Gp3, with one dead in Gp1 representing 6.7%. By the 8- day PI only one chicken in Gp2 and two chickens in groups 1and 3 were alive (exp. 1). This is in contrast with experiment 2 where all the infected birds in Gp2 died by the end of 7th - day PI while 2 each from groups 1 and 3 survived till the termination of the experiment on day- 16 PI. Result from the experiments did demonstrate that development of clinical signs from groups of birds that were treated with *Diospyros mespiliformis* extract were delayed as opposed to those not treated. The survived bird in group2 of experiment 1 showed severe nervous signs while the other 2 each from group 1 and 3 showed signs of recovery with less nervous signs. Although the remaining chicken in group 2 survived the virus challenge, it remained lame and was sacrificed on day sixteen after infection. The sacrificed birds from treated groups showed similar necropsy picture with the untreated birds that succumbed to infection but the intestinal haemorrhagic lesions were milder. The delay in clinical signs and mortality among the *D. mespiliformis* treated groups as compared with the untreated groups in both experiments is suggestive of the protective role of the extract in the infected but treated chickens. The control birds in both experiments showed no sign of the disease and none of the birds dropped dead throughout the entire period of the experiment. There have been reports of antimicrobial activity of root, leaf and bark of *D. anisandra* [1], *D. peregrina* fruits [7] and *D. melanoxylon* [20]. Antimicrobial activity of *D. mespiliformis* has been reported on it's leaves because traditionally it has the potential to improve nutrition, treatment of wide range of ailments including Measles and Mumps which are in the same virus family (paramyxoviruses) with Newcastle disease virus [21].The leaves and bark of *D. mespiliforms* are also used as antibacterial agent and astrigent in diarrhoea [22]. *D.mespiliformis* also provides support for the receive of pains and fever, as antipyretic, analgesic and anti-inflammatory in experimental mice and rats [23].

In terms of natural disease potentials, the results of this study indicate that the breeds of chickens studied during the entire experiment were all susceptible to Newcastle disease (ND). The clinical signs, mortality rates and necropsy picture presented by the infected birds confirm this. These results agree with the study of Alexander (1997) [2], who reported the lack of breed, age or genetically determined susceptibility to ND. The results of this study also concur with other authors who reported that ND was the number one killer of chickens in Africa [3, 5, 23]. The one chicken in group 2 of experiment 1 that had severe nervous symptoms, survived the virus challenge, up to the sixteen day when the experiment was terminated, which is in agreement with the literature that chickens showing nervous signs have a tendency to recover [23, 5].

The decrease in body weights in group 2 (infected and untreated) of experiment 1 on day-4 post infection from 2.05kg to 1.10kg was significant. However, in birds that were treated with *Diospyros mespiliformis* extract following inoculation with ND in groups 1and 3 of experiment 1, there was body weights decrease of 1.87kg

and 1.74kg from 2.0kg and 20.1kg respectively.

In experiment 2, the mean body weight came down significantly from 0.26kg to 0.12kg on dag-4 PI in group 2 (infected and untreated), whereas the treated groups 1 and 3 had a reduction in body weights of 0.23kg and 0.22kg from 0.28kg and 0.27kg respectively.

The mean body weight of Gp1 (pre- treated and infected) in experiment 1 decreased by 6.5% on the 4- day PI while Groups 2 and 3 (infected and untreated, infected and treated respectively) recorded decreased mean body weights of 46.3% and 13.4%. On the 16 – day PI, the mean body weight of Gp2 decreased by 17.1% whereas there were improved mean body weights in groups 1 and 3 by 9% and 4.5% respectively. Nevertheless, there were increase in mean body weights of uninfected groups 4 and 5 by 44.7% and 55.1% respectively at 16- day PI

The Trend followed the same pattern in experiment 2 where in GP 1 (pre- treated and infected), there was a decrease in mean body weight by 17.9% in day- 4 PI. whereas Group 2 and 3 recorded decreased mean body weights of 53.8% and 18.5% respectively. By 16-day PI, the mean body weight in group 1 increased by 32.1% whereas group 3 recorded increased mean body weight of 33.3%. The uninfected groups 4 and 5 recorded increased mean body weights of 111.5% and 146.2% respectively by 16- day PI when the experiment was terminated. There have been reports of antiviral activity and antipyretic properties of *D. mespiliformis* (leaves and bark) and D. variegate (stem) [3, 5, 14].

Similarly, it has been reported that following oral administration of *D. mespiliformis*, they are absorbed and metabolized, possibly in the liver, to give active antraquinones, which are then secreted into the intestine giving their observed purgative action [9]. In human, *Diospyros mespiliformis* is used in Nigeria as a vermifuge, and a remedy of dysentery [6]. It is also used as dressing for burns, antibacterial agent and astringent in diarrhea [10]. This could be used to explain the healing power played by *D. mespiliformis* in the reduction of lesions among the plant extract treated chickens. The decrease in body weights could be attributed to anorexia developed from day – 2 to day-4 of which is expected in case of Newcastle disease from all groups. However, in the birds that were given *D. Mespiliformis* extract following inoculation, the decrease in body weights was not significant, in both experiments. This could be attributed to the fact that *D. mespiliformis* has been found to have several nutritive components that include vitamins, carbohydrates and essential amino acids that could probably sustain the chicken during the infection [14].

Hence, in this study, antibodies of NDV appeared to be the key factor in the recovery of the chickens. As observed, the birds that survived until day – 16 PI irrespective of whether they were treated or not treated had to a reasonable extent, very high level of antibodies that were measured up to 10log2.

The high titers recorded from both experiments with the surviving chickens could be attributed to what may have essentially played a significant role in the recovery of the chickens. This finding was also made by [21] who observed that high antibody titre against ND contributed to the recovery of the chickens challenged by Newcastle disease virus and not the herbal preparation used to treat the chickens in ethnoveterinary practice.

CONCLUSION

The diverse conventional uses of *D.mespiliformis* correlate well with the present findings. The results gave evidence that aqueous methanolic extract of the plant employed in this study portrayed some level of antiviral activity. The results of *in vivo experiments of the current* study have shown an indication than crude *D.mespiliformis* extract could play a significant role in the control and management of Newcastle diseases in ethnoveterinary practice. Thus role which could be protective or curative needs to be verified further by using the relevant indicators.

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