www.iaajournals.org
ISSN: 2636-7246

In vivo Antiviral Assay of methanolic extract of *Diospyros mespiliformis* on Fowl Pox virus (FPV)

Onwuatuegwu J. T. C., Ebo D.U. and Chinweude C. V.

Department of Microbiology Tansian University, Umunya Anambra State, Nigeria.
joeonwuatuegwu@gmail.com.

ABSTRACT

The *in vivo* antiviral assay of methanolic extract of *Diospyros mespiliformis* on Fowl Pox virus (FPV) 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). Infection of the birds was done as previously enumerated in Fowl Pox virus experimentation Inoculation was done as ocular drops, with a drop on each location on one side of the face, while the remainder of the inoculum was given orally. The results of *in vivo experiments of the current* study have shown an indication than crude *D. mespiliformis* extracts could play a significant role in the control and management of fowl pox diseases in ethnoveterinary practice.
Keywords: *Diospyros mespiliformis,* antiviral, FPV, chickens and infection.

INTRODUCTION

Fowl pox is a relatively slow-spreading viral infection that affects bird species [1], [2], [3], [4]. It occurs in both wet and dry forms. The wet form is characterised by plaques in the mouth and upper respiratory tract [5], [6], [7], [8]. The dry form is characterised by wart-like skin lesions that progress to thick scabs. The disease may occur in any age of bird, at any time [9], [10], [11]. Mortality is usually not significant unless the respiratory involvement is severe [12], [13], [14]. Fowl pox can cause depression, reduced appetite and poor growth or egg production [15], [16], [17], [18].

Fowl pox is caused by an avian DNA pox virus [19]. There are five or six closely related viruses that primarily affect different species of birds but there is some cross-infection. Infection occurs through skin abrasions or bites, through the respiratory route and possibly through ingestion of infective scabs [20]. It can be transmitted by birds, mosquitoes or fomites (inanimate objects such as equipment) [21]. The virus is highly resistant in dried scabs and under certain conditions may survive for months. Mosquitoes can harbour infective virus for a month or more after feeding on affected birds and can subsequently infect other birds. Recovered birds do not remain carriers. A flock may be affected for several months as fowl pox spreads slowly [22].

There is no treatment for fowl pox and prevention is through vaccination of replacement birds. Where preventative vaccination is used, all replacement chickens are vaccinated when the birds are six to ten weeks of age and one application of fowl pox vaccine results in permanent immunity [23]. Vaccination of broilers is not usually required unless the mosquito population is high or infections have occurred previously. Chicks may be vaccinated as young as one day of age. During outbreaks, unaffected flocks and individuals may be vaccinated to help limit the spread [5]. If there is evidence of secondary bacterial infection, broad-spectrum antibiotics may help reduce morbidity and mortalities. As mosquitoes are known reservoirs, mosquito control procedures may be of some benefit in

limiting spread in poultry confined in houses [7].

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 bird Sources

Chickens (broilers and cockreals) 2months and 2weeks 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 Fowl Pox virus (FPV). 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 FPV 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 Fowl pox virus (FPV) 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.

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 haemagglutination 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 haemagglutination 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 haemagglutination activity when tested by the HI lest.

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

Grouping of experimental birds

Group1 - Infected and treated (n=21) made up of 10 older birds of 3months and 11young birds of 3weeks

Group2 - Infected and untreated (n=21) made up of Ten 3months old and eleven 3 weeks old.

Group3 - Pre-treated and infected (n=21) comprised Ten 3months old and eleven 3 weeks old.

Group4 - Uninfected and untreated (n=10) housed five 3months old and five 3weeks old.

Infection of birds with FPV

Infection of the birds was done as previously enumerated in Fowl Pox virus experimentation Inoculation was done as ocular drops, with a drop on each location on one side of the face, while the remainder of the inoculum was given orally [23].

Treatment was administered orally with reconstituted etract of *Dispyros mespiliformis* at a concentration of 400mg/kg two times daily- morning and evening for 7 days as indicated below:

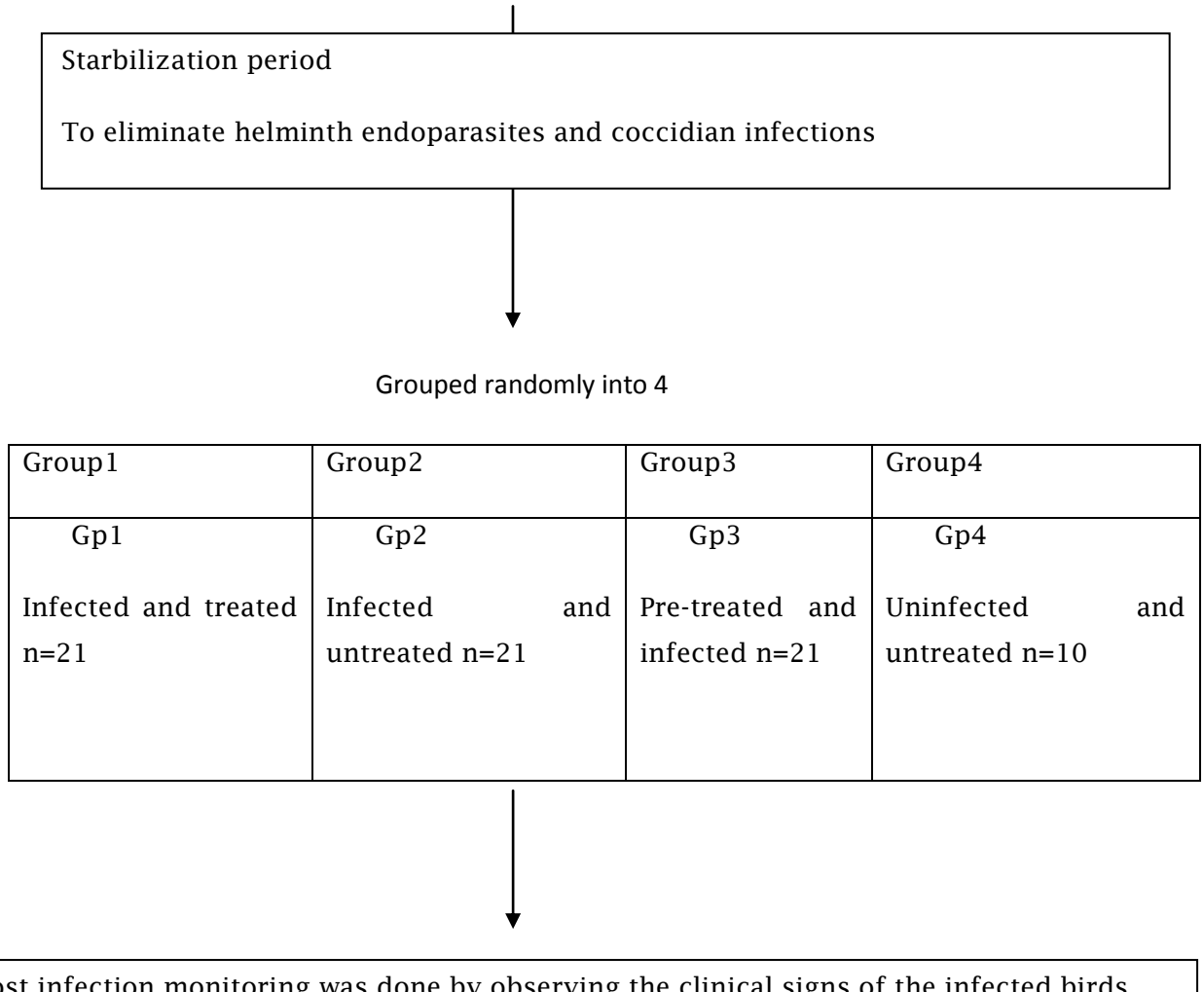
Group 1 – Infection with the virus (FPV) was done and immediately treatment was administered from day 0 to 14 PI.

Group 2 – Birds were infected with the virus and no Treatment with the extract was administered.

Group 3 – Treated orally with the reconstituted extract concentration as earlier described for 7 days before infection with the virus, followed for another 7 days post infection.

Group 4 – No Treatment was administered for 14 days without infection of the virus. Figure 1: Overleaf Summarized the Test.

Figure 1: *In vivo* Experimental Protocol (FPV)



Post-Infection monitoring

The post-Infection monitoring was done by observing the clinical signs of the infected birds from different groups after 7 day PI. Body weights and mortality rates were taken from different groups periodically. Pathology and Histopathology examinations of clinical and dead birds were analyzed. Samples were obtained for autopsy and Tissues were fixed in 10% buffer neutral formalin. Paraffin embedded section was stained with Haematoxylin and Eosine (H&E) according to the procedure of Luna (1968) [13].

***In vivo* Activity of *Diospyros mespiliformis* against FPV Clinical signs**

The birds from different groups were checked for pox lesions, one week after

exposure to the virus. Observed pox lesions were considered evidence of infection. Clinical examination of the birds showed individuals with nodular lesions, on the head, including the eyelids, beak, the legs including the digits. At the close of 7 day post infection, 17 chickens from Gp2 (infected and untreated) representing 80.95% developed active lesions on the head and legs. In Gp1 (infected and treated), 5 birds representing 23.8% had light pox lesion. Some of the affected birds came down with cutaneous lesions around the head and featherless regions of the leg. In Gp3 (pre-treated and infected), 3 birds came down with observable clinical signs representing about 14.3% of total (Table 2). With passing days and weeks, more clinical signs developed especially in Gp2 birds (conjunctivitis with a clear discharge from the eyes accompanied by ocular congestion and weakness) while also there was tremendous recovery and receding of clinical signs especially from the treated groups (Gp1 and Gp3). The recovery of the clinically down birds started from the second week where pox lesion that developed on the skin later dried and peeled off. By 28-day post infection, there was virtually no noticeable signs from affected birds in all the groups. No recorded and observed clinical signs occurred in Gp4 (untreated and uninfected) during the entire period.

Mortality Rates (FPV)

The trend in mortality of infected groups showed that Gp2 (infected and untreated) recorded the first observed death by the 21st –day PI, where 7 deaths were recorded (2 adults and 5 young birds) representing 33.3% of total. In group one Gp1 (infected and treated), there were two recorded deaths representing 9.5% of the total birds or 12.5% of those showing clinical signs. There was no recorded death among those with clinical signs in Gp3 (pre-treated and infected). All the birds in this group survived the challenge. In Gp4 (uninfected and untreated), all the birds survived the entire duration of the experiment with no death recorded (Table 4).

Body Weight Monitoring (FPV)

There were no-significant increase in average body weights as observed from the infected and treated birds in all the groups as at the end of 3 weeks PI. However, there was a significant increase in weight ($p < 0.05$) in the uninfected group at the end of the experiment, whereas there was mean weight decrease of 11.1% in group 2.

Table 1: Variation in Average Body weight (kg) of Birds Infected with Fowl Pox virus (FPV) and Treated with Extracts of *Diopspyros 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)**

Table 2: Observed Clinical Signs in Birds after infection with Fowl Pox Virus (FPV) and treatment with extract of *Diospyros mesiliformis*.

Body part	Gp1 infected and treated	Ggp2 infected and untreated	Gp3 pre-treated and infected
Legs and feet	N= 3 Adult – 1 Young – 2	N = 10 Adult – 4 Young – 6	N =3 Young ones only
Head and beak	N = 2 Young ones only	N =5 Adult – 2 Young – 3	0
Eye lids	0	N = 2 Old – 1 Young – 1	0
Total	5 21	17 21	3 21

Table 3: Body Distribution of Fowl Pox lesions in Birds infected with Fowl pox virus and treated with 400mg/ml Extracts of *Diospyros mespiliformis*.

Groups	Days post Infection	Number of deaths	Percentage (%) mortality	Number of birds survived	morbidity	% morbidity
GP1Infected and Treated	7th day 21st day 28th day	0 2 0	0% 12.5% 0%	21 19 19	5 3 0	23.8% 14.3% 0.0%
GP2 Infected and Untreated	7th day 21st day 28th day	0 7 0	0% 33.3% 0%	21 14 14	17 10 0	80.95% 47.6% 0.0%
GP3 pre-Treated and infected	7th day 21st day 28th day	0 0 0	0% 0% 0%	21 21 21	3 3 0	14.3% 14.3% 0.0%
GP4 uninfected and Untreated	7th day 21st day 28th day	0 0 0	0% 0% 0%	10 10 10	0 0 0	0.0% 0.0% 0.0%

Table 4: Trends of morbidity and mortality rates of the chickens infected with FPV and treated with extracts of *Diospyros mespiliformis.*

Age (Months)	Days Post Infection	Group 1 Infected and treated		Group 2 Infected and Untreated		Group 3 Pretreated and Infected		Group 4 Uninfected and Untreated	
		Average weight	% Increase % Decrease	Average weight	% Increase % Decrease	Average weight	% Increase % Decrease	Average Weight	**% Increase % Decrease**
	0	2.11		2.04		1.97		1.92	
	7	2.0	5.2% ↓	1.78	12.7% ↓	=	=	2.14	**11.5%** ↑
3 Mouths	21	2.15	1.9% ↑	1.84	9.8% ↓	=	=	2.45	**27.6%** ↑
3 weeks	0	0.27		0.27		0.27		0.26	
	7	0.25	7.4% ↓	0.24	11.1% ↓	0.28	3.7% ↑	0.33	**26.9%** ↑
	21	**0.30**	**11.1%** ↑	**0.24**	**11.1%** ↓	**0.31**	**14.8%** ↑	**0.65**	**150.0%** ↑

Table 5: Data on mean body weights (kg) and standard deviation during and after the experiment 1

Days	GP1 pre-infected and treated		GP2 Infected and Untreated		GP3 Infected and Treated		GP4 Uninfected and Untreated		GP5 Treated and Uninfected	
	MW	SD	MW	SD	MW	SD	MW	SD	**MW**	**SD**
0	2.0	± 0.18	2.05	± 0.21	2.01	± 0.23	1.90	± 0.10	1.87	± 0.08
4	1.87	± 0.15	1.10	± 0.15	1.74	± 0.24	2.46	± 0.11	2.53	± 0.08
16	2.18	± 0.075	1.7		2.1	± 0.15	2.75	± 1.13	2.90	± 0.10

Key: MW = Mean weight in Kg, SD = Standard deviation.

Table 6: Data on mean body weights (kg) and standard deviation before , during and after the Experiment 2.

Days	Gp1 (pre-treated and infected)	Gp2 (infected and untreated)	Gp3 (infected and treated)	Gp4 (uninfected and untreated)	Gp5 (treated and uninfected)
	MW SD	MW SD	MW SD	MW SD	MW SD
0	0.28 ± 0.02	0.26 ± 0.01	0.27 ± 0.02	0.26 ± 0.01	0.27 ± 0.02
4	0.23 ± 0.02	0.12 ± 0.01	0.22 ± 0.01	0.40 ± 0.01	0.42 ± 0.02
5	0.20 ± 0.01	0.12 ± 0.01	0.19 ± 0.01		
6	0.22 ± 0.02	0.13	0.24 ± 0.01		
7	0.25 ± 0.03		0.27 ± 0.01		
8	0.23 ± 0.07		0.28 ± 0.07		
16	0.37 ± 0.12		0.36 ± 0.07	0.55 ± 0.02	0.65 ± 0.02

Key: MW = mean weight in kg; SD = standard deviation.

Table 7: Data on Body weights and standard deviations before, during and after infection of chickens with FPV and treatment with extract of *Diospyros mespiliformis*

3 months old chickens

Days	Gp1 (infected and treated)	Gp2 (infected and untreated)	Gp3 pre (treated and infected)	Gp4 (uninfected and untreated)
	MW SD	MW SD	MW SD	MW SD
0	2.11 ± 0.14	2.04 ± 0.20	1.97 ± 0.14	1.92 ± 0.12
7	2.00 ==	1.78 ± 0.10	== ==	2.14 ± 0.14
21	2.15 ==	1.84 ± 0.07	== ==	2.45

3 weeks old chickens

Days	Gp1 (infected and treated)	Gp2 (infected and untreated)	Gp3 pre (treated and infected)	Gp4 (uninfected and untreated)
	MW SD	MW SD	MW SD	MW SD
0	0.27 ± 0.02	0.27 ± 0.02	0.27 ± 0.02	0.26 ± 0.01
7	0.25 ± 0.01	0.24 ± 0.02	0.28 ± 0.01	0.33 ± 0.04
21	0.30 ==	0.24 ± 0.03	0.31 ± 0.03	0.65

Keys: MW =mean weight in kg, SD = standard deviation.

DISCUSSION

The mortality rates recorded showed that the extract of *D. mespiliformis* offered remedy to the birds as only 2 birds in Gp1 succumbed to the challenge of FPV infection. This is in contrast to 7 deaths recorded in GP2 (infected and untreated). These seven deaths represent 45.5% young and 20% adult. Gp3 (pre- treated and infected) birds recorded no death as all the 3 clinically affected birds survived the challenge at the end of the experiment. Gp4 (uninfected and untreated) recorded no clinical signs and no death at the end of 21- day PI. In another epornitic of FPV that occurred in rosy- faced birds, there was an increased risk of mortality in young birds (150/400, 37.5%)

compared with adults (breeders) (5/160, 3.1%) [18]. Morbidity and mortality in Gp2 at the end of 21- days PI was 80.9% which concurs with the report of Gonzalez-Hein and others (2008) [11] that mortality and morbidity rates in birds with FPV may reach 75% of the population at-risk. In this study, the conventional laboratory procedures for FPV confirmed the diagnosis of FPV and established the inhibitory and protective properties of *D. mespiliformis* in traditional and ethno-veterinary practices.

CONCLUSION

The results from this research showed that methanolic extract of *D.mespiliformis* used 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* extracts could play a significant role in the control and management of fowl pox diseases in ethnoveterinary practice. Thus role which could be protective or curative and this needs to be verified further by using the relevant indicators. Further investigations are necessary in order to draw solid conclusion. The bioactive compounds from the leaves need to be isolated and screened for their pharmaceutical and biotechnological applications in order to cure chronic and infectious diseases. The development of more potent antiviral agents for human race may be enhanced, perhaps, by harvesting these plant constituents and harnessing their potencies.

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