

Original Research Article

In vivo-study of antiviral effect of *Gossypium hirsutum* extract on Newcastle Disease Virus.

ABSTRACT

The aim of the study was to evaluate the antiviral effect of *Gossypium hirsutum* extract on Newcastle disease virus. The experimental design involved the use of 59 birds (broiler). The phase involved pre-infection, infection and post-infection. Clinical signs of dyspnea, gasping, greenish diarrhea and paralysis were observed. There was mortality from day 4 in both treated and untreated groups. Gross pathological lesion of both treated and untreated groups were similar and this includes catarrhal haemorrhagic lesion, muscular haemorrhages and congestion of the kidneys, duodenum and small intestine. Also, there was congestion of lungs, liver and necrotic granules in the gland. At histology in the treated group the proventriculus, duodenum, and trachea were normal. Rectal temperature of infected and un-infected birds ranged from 41.75 $^{\circ}\text{C}$ to 42.35 $^{\circ}\text{C}$, while that of treated birds did not vary significantly with treatment regimen ($p>0.05$) and ranged from 40.5 $^{\circ}\text{C}$ on day one post infection to 42.40 $^{\circ}\text{C}$ on day 3. It was observed that both treated and untreated group was eventually overwhelmed by the NDV despite the relatively low effect of the extract. There was no significant antiviral effect of *G. hirsutum* extract on Newcastle disease virus, since all the birds died.

Keywords; *Gossypium hirsutum*, extract, birds, Newcastle disease virus, in vivo-study.

INTRODUCTION

Newcastle Disease is a highly contagious viral disease that affects poultry of all ages (McMullin, 2004). Although the first outbreaks recognized as Newcastle Disease occurred in Indonesia in 1926, it has been suggested that a major outbreak in Scotland in 1986 was due to Newcastle disease virus (Sadiq et al., 2001). The first documented outbreak of Newcastle Disease in Nigeria occurred between December 1952 and February 1953 in Ibadan (Okwor and Eze, 2010). The disease has remained a notable problem in the country (Oladele et al., 2002) and has become endemic in Nigeria in both local and commercial poultry, with annual epidemics recorded in

highly susceptible flocks with pockets of outbreaks occurring in between the annual epidemic periods (Saidu and Abdu, 2008)

Newcastle disease (ND) is a zoonosis of economic importance, it is endemic in Nigeria and constitutes the greatest threat to the development of poultry industry in the country (Okwor and Eze, 2010; Ogundipe, 1998; Hills *et al.*, 1953). The reservoirs are found among wild and local birds (Echeonwu *et al.*, 1993; Ezeifeka *et al.*, 1992). These and other factors leads to the disease occurring in all year round and peaks in the harmattan and dry seasons (Okwor and Eze, 2011). Outbreaks are usually associated with mortality which could be upto 100% and decrease in egg production (OIE, 2009). Possible therapy is presently lacking and control has been by vaccination (Alexander, 2003). Existing vaccines do not seem to effectively protect birds against highly virulent strains of ND virus resulting in reports of vaccination failures and occurrence of post-vaccinal outbreaks (Okwor and Eze, 2010; Nwanta *et al.*, 2008;Saidu and Abdu, 2005; Ezeifeka and Onunkwo, 2005). Sequel to the above problems, the search for additional means of controlling ND in Nigeria becomes very pertinent in order to save the poultry industry from this scourge.

Besides drugs developed from medicinal chemistry, plant natural products constitute major sources of innovative therapeutic agents for various conditions including infectious disease (Cos *et al.*, 2006; Clardy and Walsh, 2004). Only a minute portion of plant diversity has been explored in this regard and ample opportunities in sourcing antimicrobial drugs from plant products selected on the basis of ethno-medicinal use (Fashola *et al.*, 2011; Verporte *et al.*, 2005).

An apparently feasible approach to the control of ND in Nigeria therefore is the use of easily accessible antiviral drugs. This approach could be achieved through the search for ND antiviral

remedies in plant natural products selected on the basis of ethno-medicinal use. This study therefore examines the antiviral effect of *G. hirsutum* extract on ND virus.

Concentration of the extracts

Gossypium hirsutum was concentrated with undiluted and 10 fold dilution of the extract in sterile distilled water.

MATERIALS AND METHODS

Experimental Design

One phase experimental design with fifty nine (59) birds was used for the study.

Experimental design

Phase one

Fifty nine (59) birds were used for the study. Two groups of birds A and B, numbering sixteen (16) in each group served as infected test groups. Each group was treated with one plant extract after infection. Eight (8) birds in each group were infected with ND virus while the remaining 8 were housed together as in-contact with the infected birds to encourage natural transmission. Twelve (12) other birds (Group C) were also used as positive control (six infected and six left as in-contact) without treatment with extracts. Five(5) others were inoculated with sterile distilled water (diluent) and served as negative control, whereas two other groups of five birds each were inoculated with each extract (extract control)

Experimental birds

A set of experimental birds was used for the study. The set comprised 59 broiler (Anank), obtained from hatchery stock in Aba, Abia State. The stock of bird were brought at day old, brooded and raised to four weeks (broilers) without Newcastle` Disease vaccination. Antibiotics, vitamins and coccidiocidal drugs, were administered during brooding while vaccination against infectious Bursal disease was at 2weeks. Treatment with the *G. hirsutum* extract commence at 5weeks. The birds were raised in veterinary experimental farm of the university

Inoculation of birds.

Each infected birds was inoculated intramuscularly with 0.2ml of ND virus Kudu train containing $10^{7.2}$ EID₅₀/ml. Uninfected birds were similarly inoculated but with 0.2mls of either distilled water or the extracts.

Treatment of infected Birds

All test birds were treated either pre-infection, at time of infection, or at the beginning of clinical signs, post-infection with either the 0.1%, 1.0% or 10% concentration of the plant aqueous extract. Each bird received 12.5ml of extract orally twice a day at 8.00am and 4pm (25ml/day) after the drinking water trough had been removed for one hour.

Management of the experimental birds

Each sub group of birds was kept in a separated cage. Control groups were kept about one kilometer away from the test groups. Birds were fed with sufficient feed and water ad-libitum and observed for clinical signs.

EXPERIMENTAL PLANTS

The leaves of *Gossypium hirsutum* was bought in dried form from medicinal natural products shop at Umuahia central market, Abia State between April and June 2012. The plant pod was identified by Professor C.U. Okeke, a plant taxonomist of the department of botany, NnamdiAzikiwe University, Awka.

PRE-EXTRACTION PREPARATION OF PLANT

The plant materials were rinsed in sterile distilled water, air derived at room temperature for 7-20days, and pulverized using pestle and mortar, ground into fine texture in electric grinder and stored at room temperature in air-tight containers for further processing.

Preliminary phytochemical analysis of *Gossypium hirsutum* was performed on the ground plant samples using the method of Harbone (1998).

EXTRACTION OF PLANT MATERIAL

Fifty grams of powdered plant material was mixed with 500mls of distilled water to obtain a 10% suspension in a conical flask. The suspension was maintained at room temperature (27⁰C) for 24hrs and filled with what man No 1 filter paper. The residue was discarded and filtrate made into 10 fold and 100 fold dilutions of the aqueous extract of *Gossypium hirsutum* in distilled water.

VIRUS PROPAGATION AND QUANTIFICATION

Newcastle virus (kudu strain) was obtained from Dr. Ponman of the National Veterinary research institute vom, Plateau State, Nigeria. It is a velogenic strain with a titer of 108.2 MLD50/ml. Nine to eleven day old (pre-incubated embryonated) chicken eggs were obtained from Guffons Veterinary Hatchery in Owerri, Imo State. The chicken eggs were used immediately on arrival in the laboratory for the study.

Virus stock was passaged four times in 9-11 days old embryonated chicken eggs by inoculation of 0.2ml fraction into the allantoic sac. The inoculated eggs were incubated for four days at 37 °C in an egg incubator chilled in the refrigerators overnight and the allantoic fluid was harvested for virus isolation. Virus in allantoic fluid was quantified by cultivation of tenfold serially diluted stock in embryonated eggs and virus titre estimated as egg infections dose fifty per millimeter of allantoic fluid by the endpoint assay of Reed and Muench (1938). This work was carried out in Veterinary Microbiology Laboratory of Michael Okpara University of Agriculture, Umudike.

Assay of toxicity of extract

Graded concentration of plant extract in sterile phosphate buffered saline were inoculated into 9-11 days embryonated chicken eggs (0.2ml) per egg) by the allantoic route. Inoculated eggs were incubated for five days at 37 °C in an egg incubator and monitored for egg mortality.

Toxicity of extract was estimated as percentage egg mortality.

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Gossypium hirsutum

Phytochemical analysis of methanolic extract showed the presence of alkaloids, tannins, terpenoids, saponin and cardiac glycosides, flavonoids and steroids were absent.

Results

Profile of clinical signs

Birds treated with *Gossypium hirsutum* extract at preinfection showed signs of dyspnea, gasping and greenish diarrhea from the third day after treatment, followed by paralysis on the

fourth day. Birds treated at time of infection had whitish diarrhea on the third day. Untreated birds and those treated from onset on signs showed greenish diarrhea and respiratory sign from day 2 but whereas the untreated birds had paralysis from day 3, those treated did not show nervous signs (Table 1).

Mortality started on the 4th day in all experimental birds of both treated and untreated groups irrespective of treatment regimen adopted. However mortality lasted for only two days (4th and 5th) in the untreated group, it extended till the 6th day in all the treated groups. Neither the treated nor the untreated groups survived the infection as all the infected birds were dead by the sixth day (Table 2).

Gross Pathology

Gross pathological lesions in both treated and untreated birds were similar. There were catarrhal haemorrhagic lesions in the trachea, congestion of the lungs and kidney, petechial haemorrhages in the proventriculus and muscular haemorrhages.

However, lesions were more widely distributed in the untreated groups than in the treated groups (Table 1). The proventriculus, duodenum and trachea were normal at histology in the treatment group. The lungs had bronchiopneumonia and intestinal pneumonia. The meninges were inflamed and there was necrosis of intestinal mucosa and splenic lymphocytes leading to severe depletion of splenic mature lymphocytes (Figure 1 and 2). There was pericarditis and myocarditis with mononuclear infiltration of muscle fibers while in the infected but untreated group, there were erosion of the trachea, duodenum and small intestine with necrosis of intestinal mucosa and proventricular glands. There was congestion of the lungs and kidney, as well as myocarditis with mononuclear infiltrations of muscle fiber. There was necrosis of the spleen with depletion of

mature lymphocytes, for uninfected and untreated groups, all organs and tissues examine were normal.

There were erosion of the trachea, duodenum and small intestine with manifestation of erosive enteritis at histopathology. The liver, lungs and kidney were congested and proventriculus had necrotic glands in the glands (Figure 1 and 2). There was intravascular haemolysis and presence of haemosiderin granules.

Rectal temperature was taken with digital clinical thermometer, the rectal temperature of uninfected birds (Negative controls) in the first three days post-infection ranged from $40.17 \pm 0.12^{\circ}\text{C}$ to $40.41 \pm 0.24^{\circ}\text{C}$. Those of infected and untreated birds (Positive controls) ranged from $41.75 \pm 0.12^{\circ}\text{C}$ to $42.35 \pm 0.44^{\circ}\text{C}$. Rectal temperature of treated birds did not vary significantly with treatment regimen ($P < 0.05$) and ranged from $40.5 \pm 0.50^{\circ}\text{C}$ on day 1 post infection to $42.40 \pm 0.07^{\circ}\text{C}$ on day 3.

Rectal temperature was generally lower in all groups of treated birds on day1($40.5 \pm 0.50^{\circ}\text{C}$ - $41.35 \pm 0.02^{\circ}\text{C}$ and day 2 (41.25 ± 0.124 - $41.35 \pm 0.06^{\circ}\text{C}$) than in untreated birds (41.75 ± 0.12 and 42.20 ± 0.20 respectively).

In the directly infected untreated group, 33.3% mortality occurred on days 2, 4 and 6 p.i, whereas in the indirectly infected groups 33.3% and 66.7% of the birds died respectively on days 8 and 9 p.i none of the birds in both group survived infection.

Table 1: Profile of clinical signs in chicks infected with Newcastle disease virus and treated under various regimen with aqueous extract of *Gossypium hirsutum*

Treatment Regimen	Clinical Sign	Onset duration of clinical signs on various days post infection						
		1	2	3	4	5	6	7
Pre infection	Dysnoea			+	+			

At infection	Diarrhea		+	+	
	Paralysis		-	+	
	Dyspnoea	-	+	+	+
At disease onset	Diarrhea	+	+	+	+
	Paralysis	-	-	-	-
	Dyspnoea	+	+	+	+
Untreated	Diarrhea	+	+	+	+
	Paralysis	-	-	-	-
	Dyspnoea	-	+	+	
	Diarrhea	+	+	+	
	Paralysis	-	+	+	

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180 **Table 2:** Distribution of Newcastle disease virus haemagglutinating antigen in various
181 organs of infected chicks treated with aqueous extracts of *Gossypium hirsutum*.

Treatment	Virus haemagglutinating antigen (Log base 2) in various pooled organs					
Regimen	Trachea	Lungs	Duodenum	Proventriculus	kidney	Brain
Preinfection	64	16	512	256	128	128
At infection	28	64	1024	1024	128	64
At disease onset	256	128	1024	512	128	128
No treatment	128	64	1024	512	1024	8

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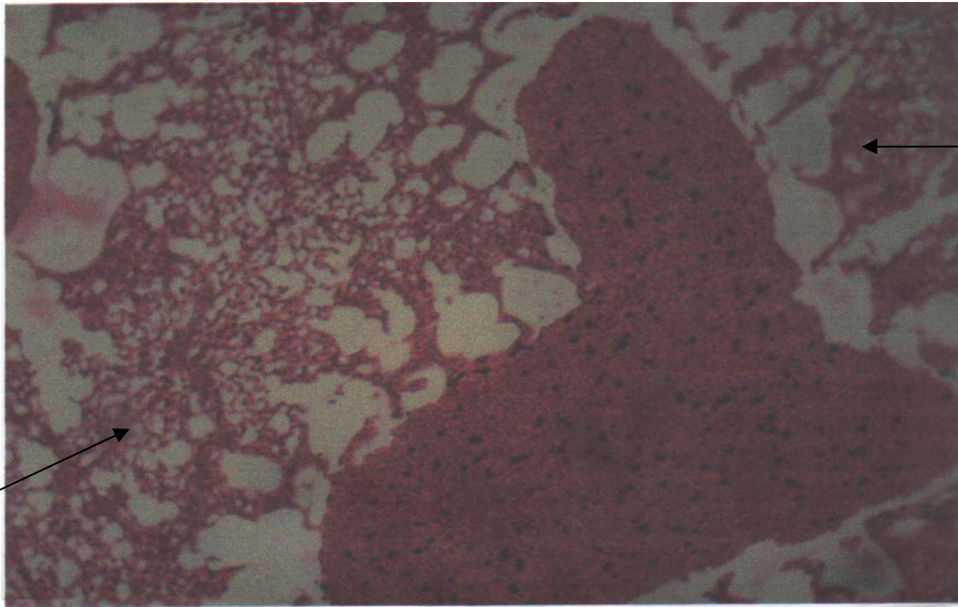


Figure 1: Congested lung x40 at 6days post infection

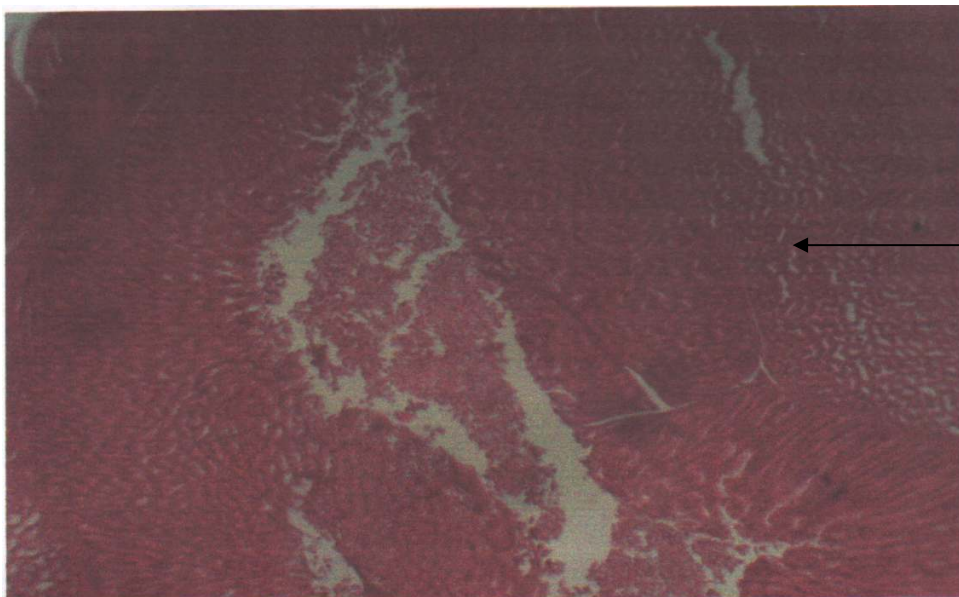


Figure 2: Proventriculus with necrotic granules in glands x40 at 6days post infection

Discussion

The phytochemical analysis of *Gossypium hirsutum* extracts revealed the presence of some chemicals known to have some antiviral effect including alkaloids, terpenoids, saponins and tannins. These have been reported to inhibit the synthesis of viral DNA, other polynucleotides and viral proteins (Jassim and Naji, 2003). The mechanism of action of the aqueous extract against ND virus in this study is not clear. However, the phytochemical components including saponins, tannins, terpenoid and alkaloids are known to affect the genetic synthesis of viruses (Jassim and Naji, 2003). All the three antiviral regimens applied in the study resulted in virus inhibition by *Gossypium hirsutum* extract at levels between 20.3 and 80.3 percent. Extracts of *Gossypium hirsutum* are widely used in ethnomedicine even in the treatment of viral diseases including HIV and Herpes viruses (Brown, 2001). There was evidence of reduction of viral load, increase in body weight and CD4 lymphocyte count when extracts were used in AIDS patient volunteers in Anambra State before the advent of antiretroviral therapy (Chukwuezi, 2007). Fashola et al. (2011) also reported significant antiviral activity of aqueous extract against Yellow fever virus in vero cells. *Gossypium* is a polyphenolic compound found in *Gossypium* species has antifertility, anticancer, antioxidant, antitrypanosoma, antiviral and antimicrobial activities (Wang et al., 2009). The onset of clinical signs in 2 days post infection (dpi) in untreated birds is in line with previous reports on the incubation period of velogenic viscerotropic NDV. Brown et al. (1999) reported that four weeks old birds experimentally with viscerotropic velogenic NDV manifested clinical signs in 2 dpi and died between 4 and 5 dpi.

In birds treated with *Gossypium hirsutum* (GH), the profile of clinical signs and incubation period were not significantly different in treated and untreated birds except one day delay in disease onset observed in birds treated a day before infection. This correlates with the level of

antiviral effect observed in the invitro antiviral study in embryonated chicken eggs, it is at variance with reports of activity of *Gossypium hirsutum* extracts against other viruses including HIV, herpes viruses and yellow fever virus (Brown,2001; Chukwueze,2006;Fashola,2011). These viruses belong to different families from NDV. There are to the best of our knowledge, no reports of remedy against ND by extracts of GH.

Mortality started same day (4 dpi) in both untreated birds and those treated with GH. Survival period of birds was one day longer in GH treated groups than in the untreated. Although these extracts did not protect the birds from mortality due to NDV, they showed some minor effects on the progression of the disease.

Similar pattern of clinical sign and survival period observed in cockerels was also replicated in broilers they were directly inoculated with the virus. In the GH treated group, both treated and untreated had same onset of signs but survival was increased by one day in those treated. Because the extract lacked complete efficacy against NDV, similar gross lesion was observed in both treated and untreated birds with widespread organ involvement resulting from the high virulence of the velogenic viscerotropic virus used (Brown *et al.*, 1999; Parede and Young, 1990). However, the relatively low effect of the extracts on the virus activity was manifested by the distribution of lesions being relatively wide in the untreated birds. Pathological lesions observed typical of velogenic viscerotropic NDV (Brown *et al.*, 1999).

Since the birds, both treated and untreated was eventually overwhelmed by the NDV despite the relatively low effect of the extract, NDV haemagglutinating antigen was detected in many organs. This was so because the birds lack immunity and the challenge virus was of high virulence. Parede and Young (1990) observed a wider distribution of virus in most organs in

non-immune birds but a restriction of virus distribution to the proventriculus, caecal, tonsils and Bursa of Fabricius in immune birds challenge with velogenic NDV.

During the period of incubation (2-3dpi), rectal temperature was relatively lower in treated birds than in the untreated group. Pyrexia is known to correlate with viraemia in viral pathogenesis (Vaughn *et al.*, 1997). It is possible that the lower rectal temperature observed in the treated birds at the stage of infection resulted from decreased viraemia due to the effect of the extracts.

This activity probably could not be sustained due to high virus virulence leading to fatal disease in both treated and untreated groups. There is usually a direct relationship between pyrexia and viraemia in viral infections (Leitao *et al.*, 2001).

Conclusion

The degree of antiviral activity exhibited by aqueous extract of *Gossypium hirsutum* was very low. One percent and ten percent suspensions respectively of crude extracts of *Gossypium hirsutum* had no significance efficiency against Newcastle disease in chicks, although pyrexia was lowered, both disease progression and mortality rate were slightly delayed

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