

# **Original Research Article**

## **Invivo-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 99birds (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 0.12OC to 42.35 0.44OC, while that of treated birds did not vary significantly with treatment regimen ( $p>0.05$ ) and ranged from 40.5 0.5OC on day one post infection to 42.40 0.07OC 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

Keywords; *Gossypium hirsutum* extract, birds, Newcastle disease virus, invivo-study, pre-infection, infection and post infection.

### **INTRODUCTION**

Newcastle Disease is a highly contagious zoonotic viral disease affecting 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 large 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 and around Ibadan (Okwor and Eze, 2010). The disease since this time 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), a zoonosis of economic importance is endemic in Nigeria and constitutes the greatest threat to the development of poultry industry in the country (Okwor and Eze, 2010; Ogunidipe, 1998; Hills *et al.*, 1953). Reservoirs exist commonly among wild and local birds (Echeonwu *et al.*, 1993; Ezeifeke *et al.*, 1992). These and other factors exacerbate the disease resulting in all year round prevalence with peaks in the harmattan and dry seasons (Okwor and Eze, 2011). Outbreaks are usually associated with upto 100% mortality 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 (Okworand Eze, 2010; Nwanta *et al.*, 2008;Saidu and Abdu, 2005; Ezeifeke 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 *Tetrapleura tetraptera* pods 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 PLANTS**

The pod of *Tetrapleura tetraptera* 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 (27<sup>0</sup>C) for 24hrs and filled with what man No 1 filter paper. The residue was discarded and filtrate made

into tenfold and 100fold dilutions of the aqueous extract of *Tetrapleura tetraptera*.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 and of titer 108.2 MLD50/ml. Nine to eleven day old pre-incubated embryonated chicken eggs were obtained from Guffons veterinary centre Hatchery, Owerri, Imo State and 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, inoculated eggs were incubated for four days at 37<sup>0</sup>C in an egg incubator chilled in the refrigerators overnight and the allantoic fluid was harvested as virus. 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 carriedout 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 9-11days embryonated chicken eggs (0.2ml) per egg) by the allantoic route. Inoculated eggs were incubated for five days at 37<sup>0</sup>C in an egg incubator and monitored for egg mortality.

Toxicity of extract was estimated as percentage egg mortality.

## **Experimental birds**

A set of experimental birds were used for the study. The set comprised 100 broiler (Anank), obtained from hatchery stock of bird's distribution in Aba, Abia State. The stock of bird were

brought at day old, brooded and raised to four weeks (broilers) without Newcastle` Disease vaccination. Birds were administered with antibiotics, vitamin and coccidiocidal drugs, during brooding and vaccinated against infectious Bursal disease. The birds were raised in veterinary experimental farm of the university.

## Experimental Design

Experimental design was in one phase and ninety nine (99) birds were used for the study.

Using *Gossypium hirsutum* extract, six birds in each sub-group were treated with 10% aqueous extract and six with 0.1% dilution.

**Table 1: Experimental Design of in-vivo Antiviral study phase 1**

| Plant | Treatment<br>regiment            | Treatment<br>concentration | Test birds | Virus<br>control | Extract<br>control | Diluent<br>control |
|-------|----------------------------------|----------------------------|------------|------------------|--------------------|--------------------|
| Gh    | Pre<br>infection                 | 1.0                        | 6          | 12               | 5                  | 5                  |
|       | At<br>infection                  | 1.0                        | 6          |                  |                    |                    |
|       |                                  | 1.0                        | 6          |                  |                    |                    |
|       | At onset of<br>clinical<br>signs | 1.0                        | 6          |                  |                    |                    |
|       |                                  | 1.0                        | 6          |                  |                    |                    |

## *Gosypium hirsutum*

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

## Results

### Profile of clinical signs

Birds treated with *Gossypium hirsutum* extract from one day preinfection showed signs of dyspnea, gasping and greenish diarrhea from the third day post-infection, followed by paralysis on the fourth day. Birds treated from 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 2).

Mortality started on the 4<sup>th</sup> day in all experimental birds both treated and untreated irrespective of treatment regimen adopted. However whereas mortality lasted for only two days (4<sup>th</sup> and 5<sup>th</sup>) in the untreated group, it extended till the 6<sup>th</sup> 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 3).

### **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, petechial haemorrhages in the proventriculus, muscular haemorrhages and congestion of the kidneys.

However, lesions were more widely distributed in the untreated groups than in the treated groups (Table 2). At histopathology in the treated group the proventriculus, duodenum and trachea were normal. 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 (Plate 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. The lungs and kidney were congested and there was myocarditis with

mononuclear infiltrations of muscle fiber. Spleen was necrotic with depletion of mature lymphocytes, for uninfected and untreated groups, all organs and tissues examine were normal. Histopathology showed erosion of the trachea, duodenum, small intestine with manisfestation of erosive enteritis. The liver, lungs and kidney were congested and proventriculus had necrotic glands in the glands (Plate 1 and 2). There was intravascular haemolysis and presence of haemosiderin granules.

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 ( $41.25 \pm 0.124$ - $41.35 \pm 0.06^{\circ}\text{C}$ ) than in untreated birds ( $41.75 \pm 0.12$  and  $42.20 \pm 0.20$  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 one of the birds in both group survived infection.

Table 2: Profile of clinical signs in chicks infected with Newcastle disease virus and treated under various regimen with aqueous extract of *Gossypium hirsutum*

| Treatment Regiment | Clinical Sign | Onset duration of clinical signs on various days post infection |   |   |   |   |   |   |
|--------------------|---------------|-----------------------------------------------------------------|---|---|---|---|---|---|
|                    |               | 1                                                               | 2 | 3 | 4 | 5 | 6 | 7 |
| Pre infection      | Dysnoea       |                                                                 |   | + | + |   |   |   |
|                    | Diarrhea      |                                                                 |   | + | + |   |   |   |
|                    | Paralysis     |                                                                 |   | - | + |   |   |   |

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

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157 **Table 3: Distribution of Newcastle disease virus haemagglutinating antigen in various**  
158 **organs of infected chicks treated with aqueous extracts of *Gossypium hirsutum*.**

| Treatment        | Virus haemasggiutinating 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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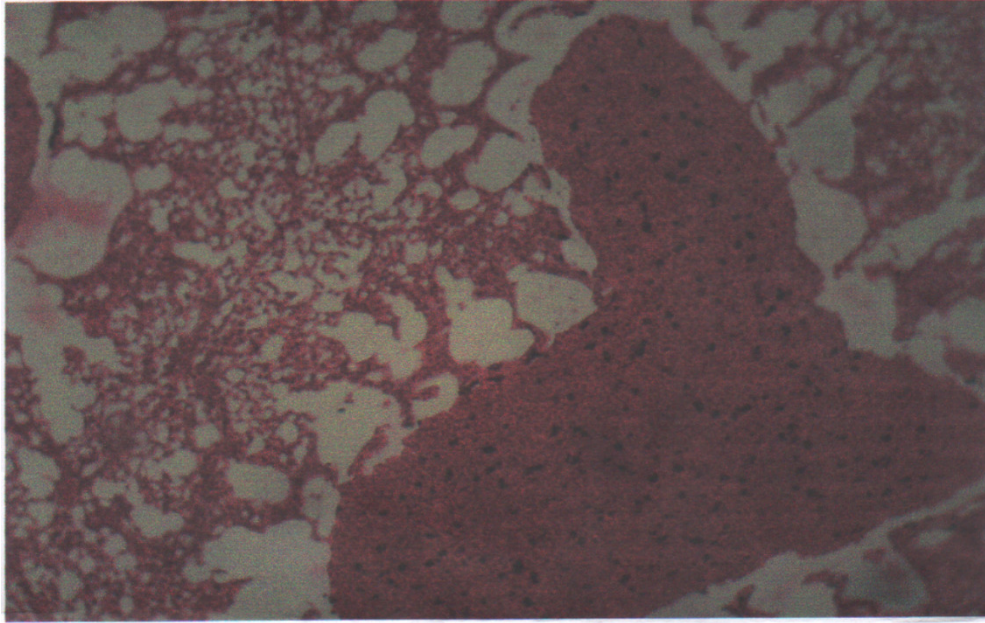
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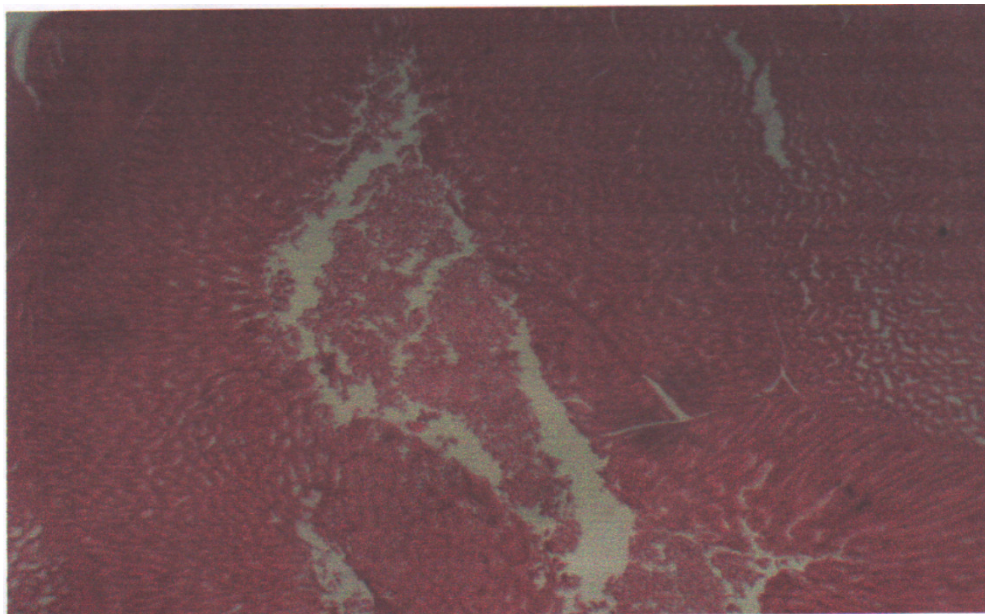
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171 **Plate 1: Congested lung**

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179 **Plate 2: Proventriculus with necrotic granules in glands**

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## 182 Discussion

183 The phytochemical analysis revealed the presence of some chemicals known to have some  
 184 antiviral effect including alkaloids, terpenoids, saponins and tannins. These have been reported  
 185 to inhibit the synthesis of viral DNA, other polynucleotides and viral proteins (Jassim and Naji,  
 186 2003). The mechanism of action of the extract against ND virus in this study is not clear.  
 187 However, the phytochemical components including saponins, tannins, terpenoid and alkaloids are  
 188 known to affect the genetic synthesis of viruses (Jassim and Naji, 2003). All the three antiviral  
 189 regimen applied in the study resulted in virus inhibition by *Gossypium hirsutum* extract at levels  
 190 between 20.3 and 80.3 percent. Extracts of *Gossypium hirsutum* are widely used in  
 191 ethnomedicine even in the treatment of viral diseases including HIV and Herpes viruses (Brown,  
 192 2001). There was evidence of reduction of viral load, increase in body weight and CD4  
 193 lymphocyte count when extracts were used in AIDS patient volunteers in Anambra State before  
 194 the advent of antiretroviral therapy (Chukwuezi, 2007). Fashola et al. (2011) also reported  
 195 significant antiviral activity of aqueous extract against Yellow fever virus in vero cells.  
 196 *Gossypium* is a polyphenolic compound found in *Gossypium* species has antifertility, anticancer,  
 197 antioxidant, antitrypanosoma, antiviral and antimicrobial activities (Wang *et al.*, 2009). The onset  
 198 of clinical signs in 2 days post infection (dpi) in untreated birds is in line with previous reports on  
 199 the incubation period of velogenic viscerotropic NDV. Brown et al. (1999) reported that four  
 200 weeks old birds experimentally with viscerotropic velogenic NDV manifested clinical signs in 2  
 201 dpi and died between 4 and 5 dpi.

202 In birds treated with *Gossypium hirsutum* (GH), the profile of clinical signs and incubation  
 203 period were not significantly different in treated and untreated birds except one day delay in  
 204 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

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

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

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

## 236 Conclusion

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

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