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In vivo Studies of Antiviral Effect of *Tetrapleura tetraptera* Extracts on Newcastle Disease Virus

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Authors' contributions

This work was carried out in collaboration between all authors. Author GE designed the work and wrote the protocol. Author PN wrote the first draft of the manuscript. Authors CA, IE and AO managed the analysis of the study. Authors I. Ugoama and I. Ukweni managed the literature review. All authors read and approved the manuscript.

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ABSTRACT

Newcastle disease (ND) is an economically important disease of poultry. Vaccination had been the only way of prevention in Nigeria and indeed many countries of the world. Possible therapy is presently lacking. Drugs developed from medicinal chemistry, plant natural products constitute major sources of innovative therapeutic agents for various infectious diseases. Here we studied the in-vivo antiviral effect of *Tetrapleura tetraptera* (*Tt*) pods on ND virus in Birds. The aim of this study is to test the ability of this plant to prevent death in Birds. The study employed one (1) phase experimental design. Two concentrations of *Tt* extract (1.0%, 0.1%) and control were used for treatment in phase 1. All test birds were treated at pre-exposition prophylaxis, at time of prophylaxis

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after exposure and beginning of clinical signs. Clinical signs of greenish diarrhea, dyspnea, increase in temperature and paralysis were observed. All birds (100%) died, while mortality in treated birds was slightly delayed. There was 20% more lesion in untreated group than in treated groups at post-mortem. There was necrosis of intestinal mucosa and depletion of lymphocytes in the treated group while there was erosion of trachea, duodenum and mononuclear infiltration of muscle fiber in the untreated group. There was 14.3% mortality on the second day post-infection, followed by 71.4% and 14.3% mortality on the 6^{th} and 9^{th} day in treated birds that were infected directly in phase 1. Treatment of ND virus with *T. tetraptera* extracts was not significant.

Keywords: Newcastle disease; Tetrapleura tetraptera extract; birds; pre-exposition prophylaxis prophylaxis after exposure; Newcastle disease virus.

1. INTRODUCTION

Newcastle disease (ND) is a disease of major economic importance of chicken in Nigeria and constitutes the greatest threat to the development of poultry industry in the country [1-3].

Reservoirs exist commonly among wild and local birds [4,5]. These and other factors exacerbate the disease resulting in all year round prevalence with peaks in the harmattan (dried moist weather) and dry seasons [6]. Outbreaks are usually associated with upto 100% mortality and decrease in egg production [7]. Possible therapy is presently lacking and control has been by vaccination [8]. Existing vaccines when poorly administered do not give maximum protection to birds against high virulent strain of ND virus resulting in reports of vaccination failures and occurrence of post-vaccinal outbreaks [1,9,10]. It is what noting that the poultry farms often times are in deplorable conditions of poor hygiene caused by water spillage from the drinking trough, extreme temperatures and intake of moist or mould feed [9]. This condition could contribute to the development of Newcastle disease. 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 [11,12]. *Tetrapleura tetraptera* is a specie in the Fabacea family native to West Africa. The plant is commonly called Prekese or Aridan. Only a minute portion of plant diversity has been explored in this regard and ample opportunities exist in sourcing antimicrobial drugs from plant products selected on the basis of ethno-medicinal use [13,14].

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. This study therefore examines the antiviral effect of extract of *Tetrapleura tetraptera* pods on ND virus to avoid death in Birds.

1.1 Experimental Design

The research work was designed in one phase. In this phase called phase I, a total of fifty eight (58) birds were used for the study. Three groups of test birds (12birds/group) were used. The group was subdivided into two sub-group of six each. Group 1 birds were orally treated in drinking water with extracts from one day before infection with virus (pre-infection treated group).

Group 2 birds were infected with ND virus followed by the commencement of treatment (at-infection treated group). Group 3 birds were infected with ND virus and treated from the onset of clinical signs for 7days. Six birds in one sub-group were treated with 1.0% solution of the extract whereas the six birds in the second sub-group were treated with 0.1% dilution of the extract. Controls includes, virus control (12birds infected with virus but without treatment with extract), extract control (five birds inoculated with only the extract without virus infection) and infected control (five birds inoculated with sterile distilled water only).

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

1.2 Declaration

Permission was obtained from animal welfare unit of the university before the commencement of the research work.

2. MATERIALS AND METHODS

2.1 Experimental Plants

The pod of *Tetrapleura tetraptera* was bought in dried form from medicinal natural products shop at Umuahia central market, Abia State Nigeria between April and June 2012. The plant pod was identified by Professor C.U. Okeke, a plant taxonomist of the Department of Botany, Nnamdi Azikiwe University, Awka.

2.2 Pre-extraction Preparation of Plant

The plant materials were rinsed in sterile distilled water, air-dried at room temperature for 7-20 days, 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.

2.3 Extraction of Plant Material

Fifty grams of powdered plant material was mixed with 500 mls of distilled water to obtain a 10% suspension in a conical flask. The suspension was maintained at room temperature (27°C) for 24 hrs and filtered with Whatman No 1 filter paper. The residue was discarded and filtrate made into tenfold and hundred fold dilution of the aqueous extract of *Tetrapleura tetraptere* in distilled water.

2.4 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 $10^{8.2}$ EID50/ml. Nine to eleven day old pre-inocubated 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 0.2 ml fraction into the allantoic sac. Inoculated eggs were incubated for four days at 37°C in an egg incubator chilled in the refrigerator 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 titer estimated as egg infectiou dose fifty (EID50/ mL) per millimeter of allantoic fluid by the endpoint assay [15]. This work was carried out in Veterinary Microbiology Laboratory of Michael Okpara University of Agriculture, Umudike.

2.5 Haemagglutination Test

Harvested chick organs were washed in strile phosphate buffered saline (PBS, pH7.2), ground in pestle and mortal containing some sterile sand, diluted with equel volume of PBS, clarified by centrifugation at 3500 rpm for 5 minutes and passed through 0.45 μl Millipore filter. Two fold serial dilution of 50 μl filtrate were made with PBS in wells of V-bottom microtitre plate. Fifty microlitres of 2% chick red blood cells prepared in PBS was added to each well followed by 50 μl of PBS. The plate was tapped to mix the content, incubated at room temperature for 30 minutes and observed for haemagglutination.

2.6 Experimental Birds

One set of experimental birds were used for the study. The bird was 100 Anak, Broilers. Both stock of birds distribution were from Abia State. The stocks of birds 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.

2.7 Treatment and Management of the Experiment Birds

All test birds were treated either pre-exposition prophylaxis, at time of infection or at the beginning of clinical signs post-infection with either the 0.1%, or 1.0% concentration of the plant aqueous extract. Each bird received 12.5 ml of extract orally twice a day at 8.00 am and 4.00 p, (25 ml/day) after the drinking water trough had been removed for one hour.

2.8 Post-infection Observation of Birds

The following indices were monitored post-infection; rectal temperature, clinical signs, morbidity, mortality, pathology and assay of virus in organs of birds. The pathology and assay of virus in organs were conducted at death or by sacrificing the birds (control) and samples of trachea, lungs, duodenum, proventriculus, kidney and brain were aseptically collected during gross pathological examination. A portion of each

organ sample was stored in 10% formalin and forwarded for histopathological examination while the remaining portions was ground with sterile sand in sterile pestle and mortar and separated by centrifugation at 3000 rpm for five minutes. Two fold dilutions of the supernatant were assayed for virus quantification by the haemagglutination assay and identification with specific antiserum by the haemagglutination inhibition test [16]. The specific antisera used was obtained from National Veterinary Research Institute, Vom.

3. RESULTS

3.1 Profile of Clinical Signs

Birds treated with aqueous extract of *T. tetraptera* starting from a day pre-infection or at the time of infection manifested clinical signs from the third day post-infection as greenish diarrhea which persisted till death on the fifth day. Birds which were treated from the time of onset of clinical signs manifested clinical signs of both greenish diarrhea and paralysis as early as the second day post-infection. On the other hand, birds which were not treated at all had greenish diarrhea on day two followed by both dyspnea with gasping and paralysis on the third day post infection (Table 1). The rectal temperature of uninfected birds (negative control) in the first three days post-infection ranged from 40.17±0.12°C to 40.41± 0.24°C. Those of infected and untreated birds (positive control) ranged from 41.75 ± 0.12°C to 42.35°C± 0.44°C. Except for birds treated at the onset of clinical signs, the increase in rectal temperature of treated birds were less on day 2 in pre-infection treated birds (41.25± 0.13)-41.35 ± 0.05) than in untreated birds (42.20 ± 0.02°C)-42.35±0.05. On the 3rd day post infection increase in rectal temperature was similar in both treated and untreated groups of birds.

3.2 Mortality Pattern

All infected birds both treated and untreated died of the virus. However, onset of mortality was delayed by one day in those treated pre-infection and at time of infection than in untreated groups. Mortality started on day 4 in the untreated birds and those treated at onset of clinical signs, but started on the fifth day in the other treated groups. Whereas all the untreated birds died within two days (4th -5th day), the treated birds lasted till the sixth day (Table 2).

3.3 Pathology

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

Table 1. Profile of clinical signs in chicken infected with Newcastle disease virus and treated for 7 days under various regimen with aqueous extract of *Tetrapleura tetraptera*

Treatment regiment	Clinical sign	Onset and duration of clinical signs on various days post infection						
		1	2	3	4	5	6	7
Preinfection	Dyspnea		NA	-	-	-		
	Diarrhea		NA	+	+	+		
	Paralysis		NA	-	-	-		
At infection	Dyspnea		NA	-	-	-		
	Diarrhea		NA	+	+	+		
	Paralysis		NA	NA	NA	NA		
At Disease Onset	Dyspnea		-	-	-	-		
	Diarrhea		+	+	+	NA		
	Paralysis		+	+	+	NA		
Untreated	Dyspnea		-	+	+	NA		
	Diarrhea		+	+	+	NA		
	Paralysis		-	+	+	NA		

NA=not available

Table 2. Mortality pattern of chicken infected with Newcastle disease virus and treated by various regimen with aqueous extracts of *Tetrapleura tetraptera*

Treatment regimen	Extract concentration (%)	Mortality rate (%) on various days past-infection					
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Preinfection	1.0					50.0	50.0
	0.1					66.7	33.3
At infection	1.0					50.0	50.0
	0.1					100.0	
At disease onset	1.0				0.0	100.0	
	0.1				16.7	66.7	16.7
Untreated					33.3	66.7	

Table 3. Distribution of Newcastle disease virus haemaglutinin antigen in various pooled organs of infected birds treated with aqueous extracts of *Tetrapleura tetraptera*

Treatment regimen	Virus haemaglutinin antigen titer in various organs					
	Trachea	Lungs	Duodenum	Proventriculus	Kidney	Brain
Preinfection	16	32	256	128	256	256
At infection	2	64	1024	1024	512	512
At disease onset	512	512	512	128	128	128
No treatment	128	64	1024	512	1024	1024

However, lesions were more widely distributed in the untreated groups than in the treated groups (Table 3). At histopathology, in the treated group the proventriculus, duodenum and trachea were normal. The lungs had bronchiopneumonia and interstitial 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 were pericarditis and myocarditis with mononuclear infiltration of cardiac 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 cardiac muscle fiber. Spleen was necrotic with depletion of mature lymphocytes. In the uninfected and untreated groups, all organs and tissues examined were normal.

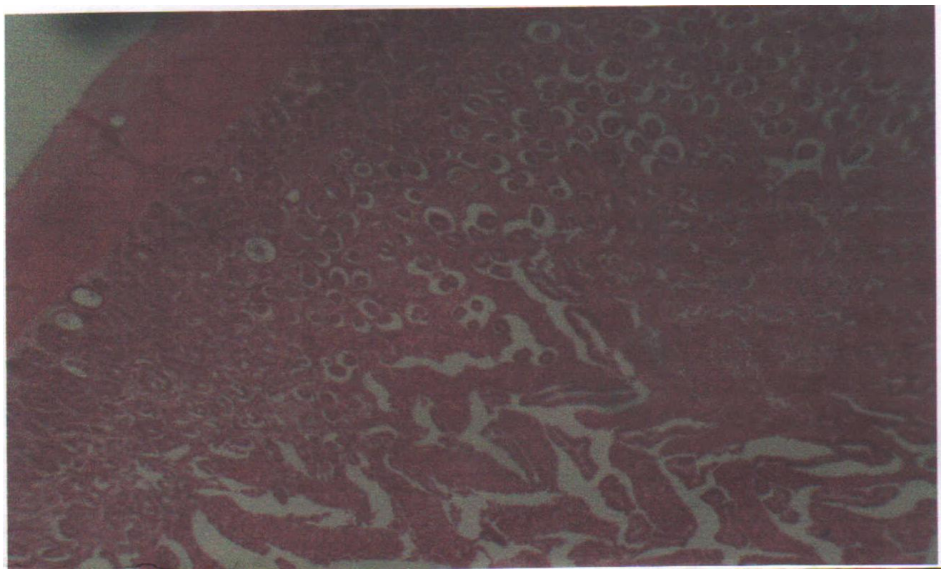

Plate 1. Chicken intestine shows necrotic enteritis x40

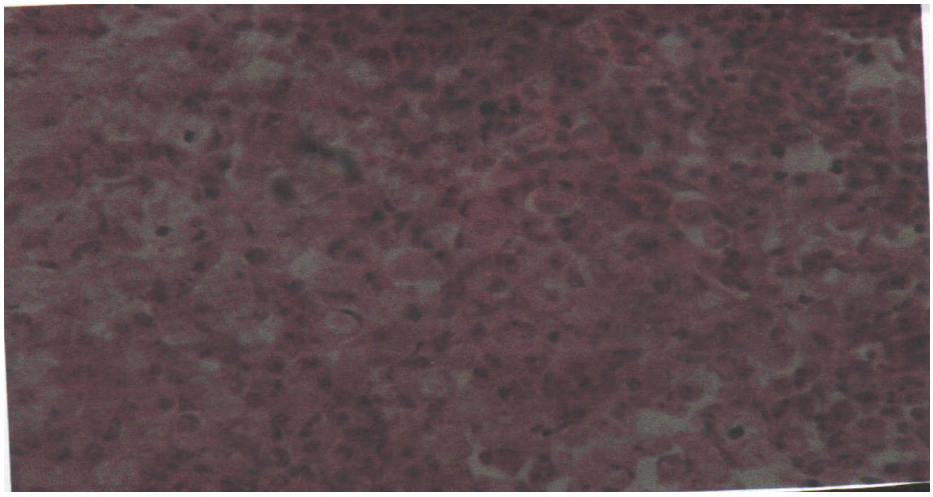

Plate 2. Spleen of chicken shows lymphocytic depletion x40

4. DISCUSSION

Aqueous extract of *T. tetraptera* is used for the treatment of Newcastle disease in the poultry producing areas of Anambra State and there are reports of positive results. The onset of clinical signs in 2days post infection (dpi) in untreated birds is in line with previous reports on the incubation period of velogenic viscerotropic NDV. [17] reported that 4 weeks old birds experimentally infected with viscerotropic velogenic NDV manifested clinical signs in 2dpi and died between 4 and 5 dpi. Birds treated with *T. tetraptera* delayed disease progression till 6dpi whereas untreated birds not only manifested signs one day earlier but also died one day earlier. Clinical signs were also more pronounced in untreated birds than treated ones. There were suggestive effects of *T. tetraptera* extract on the pathogenesis of NDV although the effect was not completely protective. Clinical signs observed were more of velogenic viscerotropic NDV which usually gives rise to the most acute ND [18]. Use of velogenic neurotropic strain would have produced a more protracted illness with a longer incubation period of 5-10dpi [17]. The longer morbidity period would have produced more appreciable effect of the extract on the velogenic neurotropic NDV infection which would have supported previous reports on the antiviral effect of *T. tetraptera* [19]. It is therefore possible that local poultry farmers in Anambra state derived some remedy from the use of aqueous *T. tetraptera* extracts in treating ND especially when their birds are infected with less virulent strains of the virus.

Because the extract lacked complete efficacy against NDV, similar gross lesions were observed in both treated and untreated birds with widespread organ involvement resulting from the high virulence of the velogenic viscerotropic virus used [17,20]. However, the relatively low effect of the extracts on the virus activity was manifested by the distribution of lesions being relatively wider in the untreated birds. Pathological lesions observed were typical of velogenic viscerotropic NDV [17].

Since the birds, both treated and untreated were 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. [20] 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 Fabricous in immune birds challenged with velogenic NDV.

During incubation period (2-3dpi) in treated group, rectal temperatures were relatively lower compared to the untreated group and this is in agreement [21,22]. Who opined that pyrexia often associated with viraemia in viral pathogenesis may be due to the effect of extracts.

This activity probably could not be sustained due to high virus virulence leading to fatal disease in both treated and untreated groups. One percent and ten percent suspensions of crude extracts of

T. tetraptera had no significant efficacy against Newcastle disease in chicks, although pyrexia was lowered and both disease progression and mortality rate were slightly delayed. Though, here was delay mortality and slight decrease in temperature in treated birds, the overall antiviral effect of *T. tetraptera* extracts on ND virus as not significant.

5. CONCLUSION

Though there were suggestive effects of *T. tetraptera* extracts on the pathogenesis of ND virus, none of the three different test groups of treatment showed positive result. Invivo-studies of *T. tetraptera* extracts has no effect on Newcastle disease virus.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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