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Antiviral Effect of *Phyllanthus amarus* Leaf Extract against Newcastle Disease Virus in Broilers

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Authors' contributions

This work was carried out in collaboration among all authors. Authors MKO and IAA designed the study, author COF performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors MKO, IAA and TTA managed the analyses of the study. Author COF managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Background and Objective: Newcastle disease (ND) is a viral disease of economic importance in poultry industry worldwide. This study was conducted to investigate the antiviral potential of n-hexane leaf extract from *Phyllanthus amarus (P. amarus*).
Methodology: A hundred and twenty day old broiler chickens were purchased and raised for the experiment. At four weeks, the birds were randomly assigned into 12 groups of 10 birds each. Chickens in groups 1, 2, 3, and 4 were vaccinated while those in 5, 6, 7, and 8 were left unvaccinated. Groups 9 and 10 served as the positive controls while 11 and 12 as the negative controls. All groups except the negative control were infected. To study the prophylactic effect of the

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extract, chickens in groups 1 and 5 received 250 mg/l while those in groups 2 and 6 received 500 mg/l of leaf extract for fourteen days before experimental infection. The chickens in groups 3 and 7 received 250 mg/l while those in groups 4 and 8 received 500 mg/l of leaf extract for fourteen days after infection with ND virus to assess the therapeutic effect of the extract. Clinical signs, bodyweight changes and mortality rates were documented. Antibody titers against the virus were determined and postmortem examination was conducted.

Results: Results revealed reduction in mortality rates following administration of the n-hexane extract after the challenge. Prophylactic administration of the extract was more effective in reducing the mortality rates of birds due to the virus infection compared to the therapeutic administration. Similarly, antibody titers decreased in a dosage dependent pattern in the prophylactic group.

Conclusion: These findings indicate that the n-hexane leaf extract from *P. amarus* has significant antiviral potentials against ND virus in broiler chickens and that prophylactic administration at 500 mg/l might be a safer approach in utilization of the leaf extract against Newcastle disease.

Keywords: Antiviral; newcastle disease virus; Phyllanthus amarus; broiler chickens; medicinal plants.

1. INTRODUCTION

Newcastle disease virus (NDV) is the etiological agent for Newcastle disease (ND), which is a viral disease of birds. The virus belongs to the paramyxovirus (PMV) which is of public health importance and it is significant in poultry as it constitutes one of its major threats [1]. Velogenic strains of Newcastle disease virus (NDV) can cause conjunctivitis in humans, usually when the person has been exposed to the virus consistently in large quantities [2]. Mostly, Laboratory workers, vaccinators, poultry attendants and vaccination crews are affected most often [1]. Humans are among the many species that can be infected by NDV in addition to avian species.

The use of personnel protective equipment and biological safety cabinet has reduced the exposure of laboratory workers. Infection is rarely seen in the workers of a farm; moreover persons handling or consuming poultry products do not appear to be at risk [3]. However, the conjunctivitis usually resolves rapidly, but the virus will be shed in the ocular discharges from 4 to 7 days. In some cases, mild, self-limiting influenza like disease with fever and headache has also been reported in humans [2,4].

The first documented outbreak of Newcastle disease in Nigeria was around Ibadan between Dec. 1952 and Feb. 1953 [5]. The disease has since then become endemic in Nigeria and has remained a dreaded problem in poultry health sector [6]. NDV can infect more than 240 species of birds and it spreads primarily through direct contact between healthy and infected birds. The disease transmits through droppings and secretions from the nose, mouth and eyes of infected birds. The disease spreads by contaminated water, feed and transport. Airborne transmission of the virus is also an important route of transmission for ND especially in flocks with close association [7].

Mechanical transfer of infected faeces could also occur by rodents, insects, dogs, fleas, or scavenging animals [8]. Infection takes place by virus inhalation, ingestion or by contact with conjunctiva. The disease may vary from subclinical with no mortality to severe infection, with 100% mortality [8].

Over the years plants have been known to exert medicinal values and therapeutic functions in humans and animals [9]. The plant *Phyllanthus amarus (P. amarus)* is a leaf flower of Greek origin which belongs to a very large genera in the family of Euphorbiaceae [10]. This *P. amarus* is an upright herbs or shrubs, often with milky acrid juice and commonly found around all tropical regions of Africa, Asia, America, Australia and Europe. It has several medicinal values and claims which include hepato-protective, anti-diabetic, anti-hypertensive, analgesic, anti-inflammatory, and anti-microbial properties [9].

This viral infection currently has no treatment and its outbreak which is rapidly transmitted amongst birds in close groups is fatal, leading to high economic losses. Vaccination is currently a major means of control as there is no treatment.

Vaccines are produced both locally and foreign [11], but could be expensive and also difficult to handle as proper cold storage is required. Therefore there is need to search for alternative, cheaper and readily available means of controlling this dreadful infection. Hence, this study looks into the antiviral effect of plant leaf extract obtainable locally against Newcastle Disease virus.

2. MATERIALS AND METHODS

2.1 Experimental Birds

One Hundred and twenty day-old broiler chickens were procured from a commercial breeder farm based on the experimental design. The chickens were brooded and raised in a pen constructed in an isolated location on the Veterinary Experimental Unit of the Teaching and Research Farm of Federal University Technology Akure (FUTA). Antibiotics, vitamin and glucose were administered accordingly. Feed and drinking water were provided *ad-libitum.*

2.2 Experimental Design

The birds were randomly divided into two groups at 2 weeks of age and blood samples were collected for baseline experimental assay. NDV vaccination was done for one group at 3 weeks of age while the other group was left unvaccinated. These vaccinated and unvaccinated groups were further divided into prophylactics and therapeutic groups. Weight was recorded weekly. The prophylactic group was subdivided into two groups; one of which was administered 250 mg/l and the other 500 mg/l for fourteen days before challenge with the virus while the therapeutic group was also given the 250 mg/l and 500 mg/l of the extract but immediately after challenge with the virus. The positive control was inoculated with the challenge virus but not administered with extract while the negative controls received neither the extract nor the challenge virus. The experimental chickens were challenged with wild NDV (Kudu strain) at 0.2 ml/100EID_{50} via the intraocular route and placed under clinical observation. Blood sample was randomly collected from the chicken and serum samples were harvested to determine the viral antibody titer. This was done according to OIE manual [4]. Post mortem examination was also carried out on dead and sacrificed birds.

2.3 Preparation of Leaf Extract

Leaves of *P. amarus* were air dried under shade and ground into powdery form prior to extraction process. The extraction was carried out according to Oladunmoye [12]. The resulting weight of the powdered form was 500 g which was exhaustively extracted at a ratio of 1:4(w/v) with n-hexane as solvent. The leaf extract was concentrated *in vacuo* using a rotary evaporator at 40°C, while the un-evaporated solvent remaining in the extract was left to air-dry which gave a residue weighing 10.50 g. The concentrated extract was reconstituted to give a stock concentration of 1000 mg/ml, which was used for further testing at varying concentrations.

2.4 Source of Challenge Strain

Virus stock of Kudu strain was obtained from National Veterinary Research Institute, Vom and was transported under cold chain to the research facility at FUTA where challenge was carried out. The Virus contains $1x10^8$ EID_{50} /ml and was reconstituted for the challenge protocol.

2.5 Haemaglutination Assay

HA tests were carried out in U-shaped plates with 96 wells. 25ul of PBS was added into wells 1 to 12 and the antigen suspension was added in 25ul volume in well 1. The content of well 1 was serially diluted in 2-fold serial dilution to well [11]. A total of 25ul of 1% chick RBC was added to all wells including the controls and the plates were incubated at 40ºC for 45 minutes after which the titer was taken.

2.6 Haemaglutination Inhibition (HI) Test

The HI test was done in U-shaped plate with 96-wells. 25 ul of PBS was added in well 1 through to well 12. The test serum was added in well 1. The content of well 1 was serially diluted in 2-fold serial dilution to well 11. A total of 25ul of the antigen suspension (4HA unit) of previously titrated antigen was added to the test wells and controls. The plates were incubated at 40°C for 30 minutes to allow antiserum antigen reaction to take place. 25ul of 1% chick red blood cells was added to all wells including controls and the plates well re-incubated at 40ºC for 45 minutes after which the titer was taken [4].

2.7 Statistical Analysis

Data were analyzed using SPSS version 21.0 (IBM Corp, 2012) and mean values ± standard deviation were recorded.

3. RESULTS AND DISCUSSION

Table 1 shows distribution and grouping of birds according to the virus challenge. Four days post experimental infection with NDV challenge strain, it was observed that 50 percent of all infected birds started showing symptoms of inappentence and greenish diarrhoea followed by death in one

Table 1. Experimental design

Groups	Group No.	Ext. dose (mg/l)	keys
Vaccinated Prophylactics	1	250	VP250
Vaccinated Prophylactics	2	500	VP500
Vaccinated Therapeutics	3	250	VT250
Vaccinated Therapeutics	4	500	VT500
Unvaccinated Prophylactics	5	250	UVP250
Unvaccinated Prophylactics	6	500	UVP500
Unvaccinated Therapeutics	7	250	UVT250
Unvaccinated Therapeutics	8	500	UVT500
Vaccinated positive Controls	9	0	VPC
Unvaccinated positive Controls	10	0	UPC
Vaccinated Negative Controls	11	0	VNC
Unvaccinated Negative Controls	12	0	UNC

Table 2. Percentage mortality of chickens

Group name	Group	No. of birds	Ext. dose (mg/l)	% Mortality rate of chickens
Vaccinated prophylactics	1	10	250	50
Vaccinated prophylactics	2	10	500	20
Vaccinated therapeutics	3	10	250	50
Vaccinated therapeutics	4	10	500	20
Unvaccinated prophylactics	5	10	250	80
Unvaccinated prophylactics	6	10	500	50
Unvaccinated therapeutics	7	10	250	100
Unvaccinated therapeutics	8	10	500	80
Vaccinated positive Control	9	10	0	0
Unvaccinated positive control	10	10	0	100
Vaccinated negative Control	11	10	0	0
Unvaccinated negative control	12	10	0	0

group (Group 10) on the 5th day. By day 8 post-infection (P.I) 80% of the chickens in the positive control group which consist of unvaccinated birds had severe clinical signs of the disease and 75% mortality while these clinical signs were less conspicuous in chickens in the Vaccinated experimental groups. However, mortality was recorded in subgroups which consist of unvaccinated experimental chickens.

No clinical sign of ND was observed in the negative control group which was not infected. There was difference in mortality rates among the positive control, prophylactic and therapeutic trials within the vaccinated and unvaccinated groups (Table 2).

Administration of the extract before experimental infection of chickens with NDV reduced mortality rates of chickens by 50% in group which received extract concentration of 500 mg/l and by 20% in groups which received extract concentration of 250 mg/l in comparison with the control group which received no extract and had a mortality rate of 100%. Mortality rates of chickens in therapeutic groups were reduced by 20% at administration of extract concentration of 500 mg/l (Table 2). The Vaccinated group showed reduced mortality rate compared to the unvaccinated group. Post-mortem examination of dead chickens revealed petechial haemorrhage in the proventriculus (Plate 1). Proventriculus was swollen with severe bleeding (Plate 2). The gizzard was filled with green content (Plate 2). Haemorrhage normally found in caecal-tonsils of chicken (Plates 3).

HI titer was assayed on days 7 and 14 post infection in all the groups. The levels of antibody titer between the positive control and others which received no extract differs from the groups in which extract was administered. The HI titers of chickens in the positive group rose to $\log_2 8$ by day 7 post-infection. However, in the prophylactic trial, results showed that administration of the extract reduced antibody titers in the survivors in the groups by the 14th day to $\log_2 5$. (Fig.1). For the therapeutic trial, the antibody titers

decreased slightly in a dose dependent pattern. There was no significant difference in the titer of the negative control groups through the experiment (Fig. 2).

Comparison of ND antibody titers between the various groups showed that the levels of titers were significantly lower in the prophylactic than in the therapeutic trial which is in line with the study of Bakari et al. [13] who reported reduction of antibody titer using plant extract. This observation suggests that administration of the extract before the infection helped to reduce/interfere with virus multiplication which consequentially reduced immunological response towards the virus. Mortality rates have also been reportedly reduced post ND infection following administration of leaf extracts *in-ovo* [14].

Reduction in antibody titer, including mortality rates and pathological lesions of Newcastle disease suggested that the leaf extract had significant antiviral effect during the chicken trial. *P. amarus* has been implicated in several potentials such as antibacterial, antifungal and some viral infection in humans [15].

The antibody titer observed in the vaccinated group and controls were in line with reports of Akele et al. [11] who reported a titer range of log27 to log29 in birds vaccinated with NVRI produced vaccine and the survivor of birds in the group could be attributed to the efficacy and immunogenicity of the vaccine as also shown by Chukwuedo et al. [16].

These compounds exert their virucidal effect by interfering with viral multiplication [17]. Specifically, some of these compounds are speculated to exhibit protease inhibition, hence interferes with cleavage of haemaglutinin neuramidase and fusion protein, which are important glycoproteins for ND virus attachment and multiplication [18]. Other classes of compounds such as flavonoids from plant extract have been reported to act by inhibiting production of prostaglandin (signaling molecule) and phosphosdiesterases involved in cell activation [13].

Plate 1. Petechial haemorrhage in the proventriculus (marked by arrow)

Plate 2. Haemorrhage seen in the proventriculus (a) and green coloured content in the gizzard (b)

Plate 3. Haemorrhage in caecal-tonsils (marked by arrow)

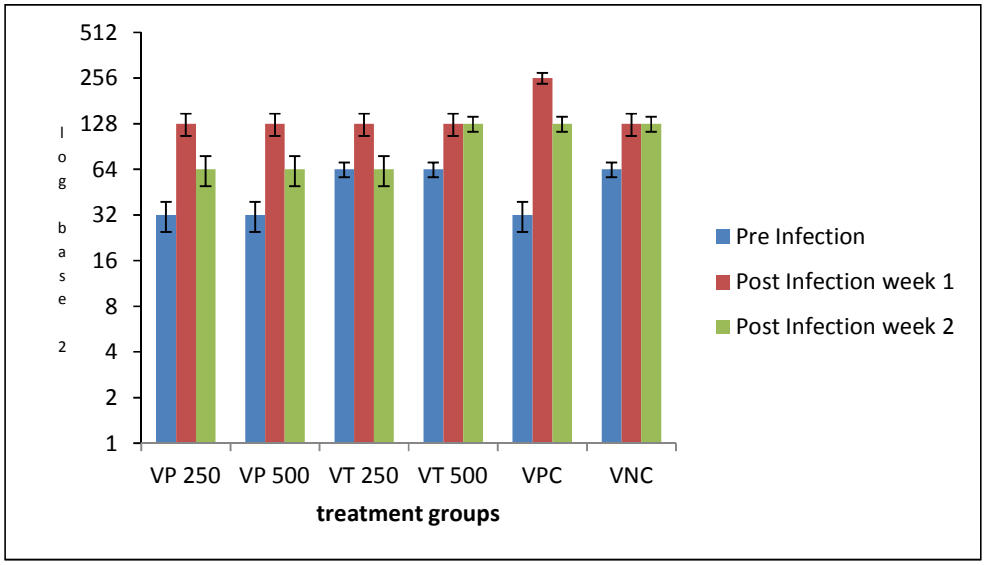

Fig. 1. HI titer profiles of vaccinated chickens infected with ND virus and treated with different concentrations of *Phyllanthus amarus* leaf extracts before and after the experimental infection

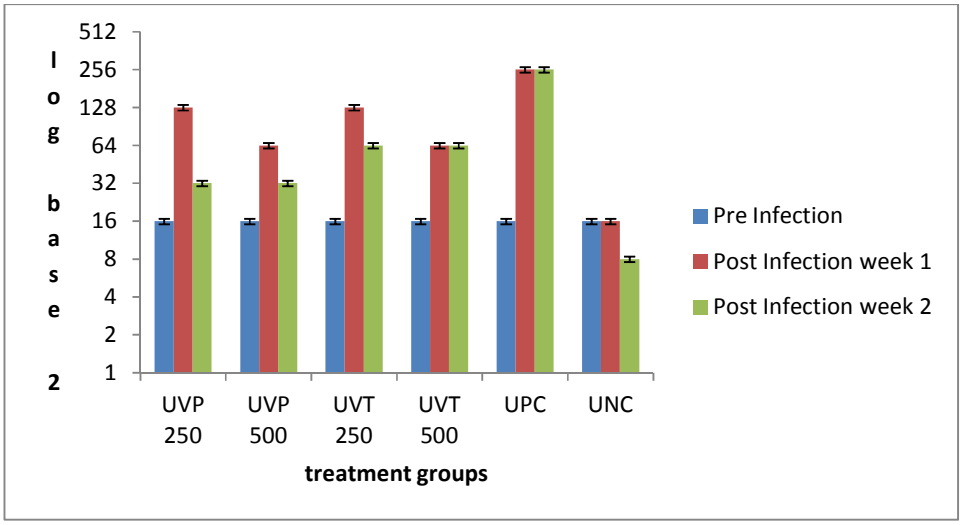

Fig. 2. HI titer profiles of unvaccinated chickens infected with ND virus and treated with different concentrations of *Phyllanthus amarus* leaf extracts before and after the experimental infection

However, many traditional medicinal plants used to treat viral diseases have been shown to contain high levels of compounds such as flavonoids, alkaloids, and tannins. Same classes of compounds have been found in *P. amarus* [14,19].

4. CONCLUSION

The current study has shown significant antiviral potential of n-hexane leaf extract of *P. amarus* against experimental Newcastle disease in broiler chickens. The typical clinical signs which were observed following infection were a clear indication that the ND was established and a virulent virus strain was used. Findings were suggestive that prophylactic administration of n-hexane leaf extract of *P. amarus* against experimental Newcastle Disease in broilers could be a more promising approach in mitigating the effects and replication of ND in endemic areas. Furthermore, the administration of extract after

infection could also be used to reduce disease severity and mortalities.

Field trials are however, recommended as a way of validating the use of *P. amarus* extract against Newcastle disease in chickens as well as studies at molecular level is recommended.

ETHICAL APPROVAL

Experiment was carried out in accordance with the ethical guidelines of the University.

SIGNIFICANCE STATEMENT

This study discovered the possible prophylactic effect of n-hexane leaf extract of *Phyllanthus amarus* which can be an alternative approach to alleviate Newcastle Disease in broiler chickens. This study will also help researchers to uncover the importance of phyto-constituents in antiviral treatments. Thus, the probable emergence of a new drug from alternative medicine have been needed.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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