International Scholars Journals

African Journal of Infectious Diseases Research ISSN 2756-3340 Vol. 12 (1), pp. 001-007, January, 2025. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Exploring the *In-Ovo* Biological Effects of *Phyllanthus amarus* Leaf Extracts on Newcastle Disease Virus

Faeji, C. O.[1*], Oladunmoye, M. K.[2], Adebayo, I. A.[3] and Adebolu, T. T.[2]

[1]Department of Medical Microbiology and Parasitology, CHMS, Afe Babalola University, Ado Ekiti, Ekiti State, Nigeria.
[2]Department of Microbiology, Federal University of Technology, Akure, Ondo State, Nigeria.
[3]Department of Animal Production and Health, Federal University of Technology, Akure, Ondo State, Nigeria.

Accepted 15 October, 2024

Newcastle disease virus is a paramyxovirus which causes Newcastle disease in birds. Investigation was done on the effect of leaf extract of *Phyllanthus amarus* against Newcastle disease virus (NDV) using an *in-ovo* assay. Nine to eleven day-old viable embryonated chicken eggs (ECE) were used for the assay, these were divided into six groups of six eggs each. Methanol, aqueous and n-hexane extracts of the plant leaves were administered to the various groups at concentrations varying from 50 to 5 mg/ml. Embryonated eggs were incubated and embryo survival was monitored daily. Negative control and diluents control groups received phosphate buffer saline (PBS) and dimethly suphoxide (DMSO), respectively. The other group was uninoculated while a virus control group received 100 EID_{50}/0.1 ml NDV alone. Bacteria free allantoic fluid from the embryonated eggs in different treatment groups were harvested and collected for spot hemagglutination (HA) test and HA assay to detect the presence of NDV viral particles and the viral titre, respectively. Leaf extracts were assayed for presence of phytochemicals and antioxidant potentials. It was observed from the results that the extract was toxic to the embryo at a concentration above 50 mg/ml and further results showed that the HA viral titre reduction was directly proportional to increasing extract concentration. The phytochemical assays of leaf extract revealed the presence of phytochemicals including alkaloids, tannins, saponins, flavonoids, phenols, steroids, glycosides. The current findings have demonstrated that leaf extract from *P. amarus* has potentials of medicinal value as well as antiviral activity against NDV *in-ovo*. Further experimental assays using live animal models are recommended to validate the use of *P. amarus* plant extract in therapeutic measure in chickens.

Key words: Antiviral, *Phyllanthus amarus*, Newcastle disease virus (NDV), embryonated chicken eggs (ECE).

INTRODUCTION

For the past years, viruses remains a threat to human and animal lives, the prevention and alleviation of these viruses and its infections is necessary and cannot be overemphasized (Fajimi and Taiwo, 2005). However,

*Corresponding author. E-mail: faejicharles@gmail.com.

vaccines have been developed for some of these viral diseases but there is need for pursuit in discovery of therapeutic agents for cases of unvaccinated individuals or animals exposed to these infections. Most of the antiviral agents that are currently being used are limited in therapeutic potencies and may possess several other problems in the clinical practices such as tolerability, contraindications, toxicity amongst others not to mention the availability and cost of the drugs, hence the need for an alternative approach or therapeutic measures (Asres et al., 2005; Verma et al., 2014).

Plants on the other hand are a rich source of essential nutrients (Abad et al., 2000), chemicals and other active component (Babich et al., 2003). Some of the existing therapeutic agents or drugs were developed based on chemical compounds isolated from plants (Babich et al., 2003). These plants exist in different parts of the world such as Asia, Europe and Africa. However, these plants and many more may have not been exhaustively explored which brings us to the ideas of possibilities of creating or discovering new products or antiviral agents from plants as the use of medicinal plants in treatment of animal and human diseases has become more prominent. In Nigeria and many other African countries, rural and urban communities have continued to use or consult local herbs/medicine-men for remedies to a variety of diseases. Such plants include *Phyllanthus amarus* which is one of the species belonging to a large family, Euphorbiaceae. It is found in the tropical and sub-tropical region worldwide (Joseph and Raj, 2011). It is commonly called “iyin olobisowo” in yoruba and said to possess claims of medicinal values which include hepatoprotective, anti-diabetic, anti-hypertensive, analgesic, anti-inflammatory and anti-microbial properties (Adeneye et al., 2006; Okiki et al., 2015). Chemical compounds including alkaloids, flavonoids, lignans and phenols have been reportedly isolated from this plant (Adeneye et al., 2006). However, the antiviral potentials of this plant have not been exhaustively explored.

Newcastle disease (ND) is a highly infectious disease of domestic and wild birds which is caused by Newcastle disease virus (NDV) and is widely regarded as one of the most important avian diseases belonging to the family paramyxoviridae (Young et al., 2002). It is a threat to most avian species but chickens are the most susceptible with clinical disease (Young et al., 2002). Newcastle disease virus can be a causative agent for conjunctivitis in humans usually in persons exposed to large quantities of the virus.

Newcastle disease currently can be prevented or managed by due and proper vaccination but has no treatment. Beyond this, the cost, challenges faced in availability and distribution of vaccine and lack of treatment is a major setback in alleviation of this disease. In view of aforementioned, this study evaluated the antiviral potentials of plant extracts from *P. amarus* against NDV which will provide information that may serve as basis for further researches and also may lead to development of cheaper, more available and effective alternative approach to control and or novel drugs for therapeutic purposes.

MATERIALS AND METHODS

Collection and identification of the plant material

Leaf parts of the study plant were collected from their natural habitats in Ado-Ekiti, Nigeria through the months of April and June, 2016. It was identified at the Department of Biological Sciences, Afe Babalola University, Ado-Ekiti using standard keys and description.

Preparation of plant extract

Leaves of study plant were air dried under shade and ground into powdery form prior to extraction process. The extraction was carried out according to Oladunmoye (2012). The resulting weight of the powdered form was 500 g which was exhaustively extracted at a ratio of 1:4 (powder weight to solvent volume) with multiple solvents namely n-hexane, water and methanol. 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 in 10.5 ml of dimethyl sulphoxide (DMSO) to give a stock of a concentration of 1000 mg/ml, which was used for further testing at varying concentrations.

Antiviral assay

Acute toxicity assay of leaf extract

This was done to evaluate the effect of extract toxicity on the embryo of the embryonated chicken egg (ECE). Egg toxicity assay was used to estimate the minimum toxic concentration of the extract in nine days old ECE using 0.2 ml of each concentration to inoculate five viable ECE and the control group received 0.2 ml DMSO. Inoculated eggs were incubated for 4 days at 37°C and monitored twice daily for mortality by the process of candling. Extract toxicity was determined by examination of embryo for lesions and hemorrhages and by the percentage mortality of embryos.

Source of virus and 9-day old embryonated chicken eggs (ECE)

Velogenic strain of NDV was obtained from Viral Research Department, NVRI, Vom and embryonated chicken eggs (ECE) aged nine days were obtained from poultry division, NVRI, Vom, Nigeria.

Determination of EID_{50} of the virus

Fifty percent embryo infectious dose (EID_{50}) of the virus was determined according to the method of Young et al. (2002). From this, 100 EID_{50}/0.1 ml of the virus stock was made for the experiment.

Preparation of leaf extract/inoculum

This experiment was carried out in three groups, extracts were

treated with antibiotics (Penicillin, Streptomycin, Gentamycin, Amphotericin B (5x PSGA). The embryonated chicken eggs were properly labeled according to the extract concentrations and groups used. Sets of plastic egg crates/trays were thoroughly disinfected with Virkon and the embryonated chicken eggs were adequately swabbed with 70% alcohol before transferring into the disinfected trays to avoid contamination. Under a properly sterilized bio-safety cabinet, nine-day-old embryonated chicken eggs were divided into groups of six, the eggs were punched and inoculated with the extract and virus according to the grouping through the allantoic route. The predetermined concentrations studied were 50, 25, 10 and 5 mg/ml. In the first group designed to test the prophylactic effect, each predetermined concentration of extract at 0.2 ml was inoculated into the ECE and incubated for 90 min before the inoculation of 100 EID_{50}/0.1 ml of the virus. In the second group, the therapeutic effect was tested by inoculating 100 EID_{50}/0.1 ml of virus and incubated for 90 min before inoculation of the extracts after the 90 min at the different concentrations studied. The third group, a mixture containing 100 EID_{50}/0.1 ml of virus and 0.2 ml of the extracts at varying concentration was incubated at 4°C for 90 min, after which the mixture was inoculated at the same time into the ECE to determine the inhibitory potentials. A group inoculated with standard NDV 100 EID_{50}/0.1 ml was used as virus control, another group inoculated with 0.2 ml DMSO served as diluent control and another group of eggs also was not inoculated nor punched and this serves as un-inoculated control. The ECE were sealed with nail polish and incubated at 37°C in a humidified incubator. The ECE were candled daily and embryo survival observed. The experiment was terminated by chilling at +4°C after 96 h when the virus control group have all died (after 48 h). Bacteria free allantoic fluid from the different test groups was harvested for spot test and haemagglutination assay to detect the presence of NDV in the ECE.

Harvesting and spot haemagglutination test

Harvesting was done after chilling the eggs overnight in a 4°C refrigerator. Embryos that had been chilled were brought out of the refrigerator and kept at room temperature for about 30 min to thaw. The eggs were swabbed and put in the bio-safety cabinet. The shell of each egg was cracked opened at the air space and the allantoic fluid was siphoned with a syringe while using an harvesting spoon to prevent obstruction of the fluid, this was dispensed into a sterile universal bottle (which was labelled accordingly). Sterility of allantoic fluid was checked by culturing on blood agar and nutrient agar to confirm absence of bacteria. To spot test, a pipette was used to dispense a drop of 1% washed chicken red blood cells on a clean white tile while clean and sterile rubber wire loops was used to pick a drop of the allantoic fluid which was mixed with the drop of blood. The tile was gently rocked and observed for visible agglutination to indicate viral presence (OIE, 2012).

Haemagglutination assay

Haemagglutination assay was used to determine the presence and quantity of virus/viral particle in the allantoic fluid of the eggs. The haemagglutination assay was performed in V bottom shaped microtitre plates using 25 µl each of phosphate buffered saline (PBS), allantoic fluid and 1% chicken red bloods cells (OIE, 2012). This was performed in replicates and the mean titre value was recorded.

Qualitative phytochemical analysis of plant extracts

This was carried out in order to determine the presence of glycosides, tannin, saponin, phenol, alkaloid and flavonoid in the plant extracts (Hadi and Bremner, 2001; Wadood et al. (2013; Desphande and Kadam, 2013).

Determination of total phenol content and antioxidant capacity of plant sample

Total phenols were determined using Folin Ciocalteu reagent (McDonald et al., 2001) and antioxidant capacity assayed according to the method of Serpen et al. (2012) using inhibition of 1,1 diphenyl-2-picryl-hydrazyl (DPPH) radicals expressed in trolox equivalent antioxidant capacity (TEAC) and ferric reducing antioxidant power (FRAP) of extract expressed in gallic acid equivalents (GAE).

Data analysis

Data were analysed using SPSS version 21(IBM, USA). Values were reported in mean ± SEM. The mean differences were analysed using one way analysis of variance (ANOVA) for comparison between different extract types and significance reported at $P<0.05$. Further comparison within concentrations was done using a post hoc test (Turkey HSD).

RESULTS AND DISCUSSION

Table 1 shows the maximum toxic concentration of the methanol, aqueous and n-hexane leaf extracts against the embryonated chicken egg. It was observed that the maximum tolerable concentration of extract as measured by the embryonic death and morphology of the embryo in comparison with the controls was 50 mg/ml, therefore test were subjected to a concentration of 50 mg/ml and below. The diluent was safe for the embryo as no mortality was recorded. The antiviral assay revealed decreasing HA viral titre with increase in concentration of the extracts. It was observed that at a concentration of 50 mg/ml, the n-hexane extracts reduced the viral HA titre to 2, while the methanol and aqueous extracts reduced the HA titre to 56 and 128, respectively, however at a low concentration of 5 mg/ml, the leaf extracts exhibited insignificant reduction of viral titre in comparison with the viral control (Figure 1). Figure 2 shows the therapeutic activities of the leaf extracts of *P. amarus* against NDV, it was observed that n-hexane extracts reduced the viral HA titre from 1024 to 4 at a concentration of 50 mg/ml, while at 25 mg/ml, the viral HA titre was reduced to 32 and 128 at the lowest concentration of 5 mg/ml, however methanol and aqueous extracts did not show reduction in viral titre below 64 at the highest concentration studied. *P. amarus* n-hexane leaf extracts exhibited some inhibitory antiviral potentials by reducing the viral HA titre to 4 at 50 mg/ml and 128 at 5 mg/ml, while methanol and aqueous leaf extracts reduced the viral HA titre to 128 and 64 at a concentration of 50 mg/ml respectively. The methanol and aqueous extracts showed little to insignificant reduction in the viral titre value at concentration of 5 mg/ml (Figure 3). The phytochemicals screening revealed the qualitative presence of bioactive

Table 1. Acute toxicity assay of leaf extract of test plant.

Extract	Concentration (mg/ml)	No of eggs	No. of embryonic death/hrs				
			24 h	48 h	72 h	96 h	Total
***P. amarus* Aq**	250	5	0/5	0/5	1/5	2/5	3/5
	100	5	0/5	0/5	0/5	1/5	1/5
	50	5	0/5	0/5	0/5	0/5	0/5
	25	5	0/5	0/5	0/5	0/5	0/5
	10	5	0/5	0/5	0/5	0/5	0/5
***P. amarus* Hex**	250	5	0/5	0/5	1/5	2/5	3/5
	100	5	0/5	0/5	0/5	1/5	1/5
	50	5	0/5	0/5	0/5	0/5	0/5
	25	5	0/5	0/5	0/5	0/5	0/5
	10	5	0/5	0/5	0/5	0/5	0/5
***P. amarus* meth**	250	5	0/5	1/5	4/5	0/0	5/5
	100	5	0/5	1/5	2/5	1/2	4/5
	50	5	0/5	0/5	0/5	0/0	0/5
	25	5	0/5	0/5	0/5	0/0	0/5
	10	5	0/5	0/5	0/5	0/0	0/5
Control	PBS	5	0/5	0/5	0/5	0/5	0/5
	DMSO	5	0/5	0/5	0/5	0/5	0/5
	Uc	5	0/5	0/5	0/5	0/5	0/5

B- *Phyllanthus amarus,* Vc- virus control, Uc- uninoculated control, Dc- diluent control, Meth- methanol, Hex- hexane, Aq- aqueous.

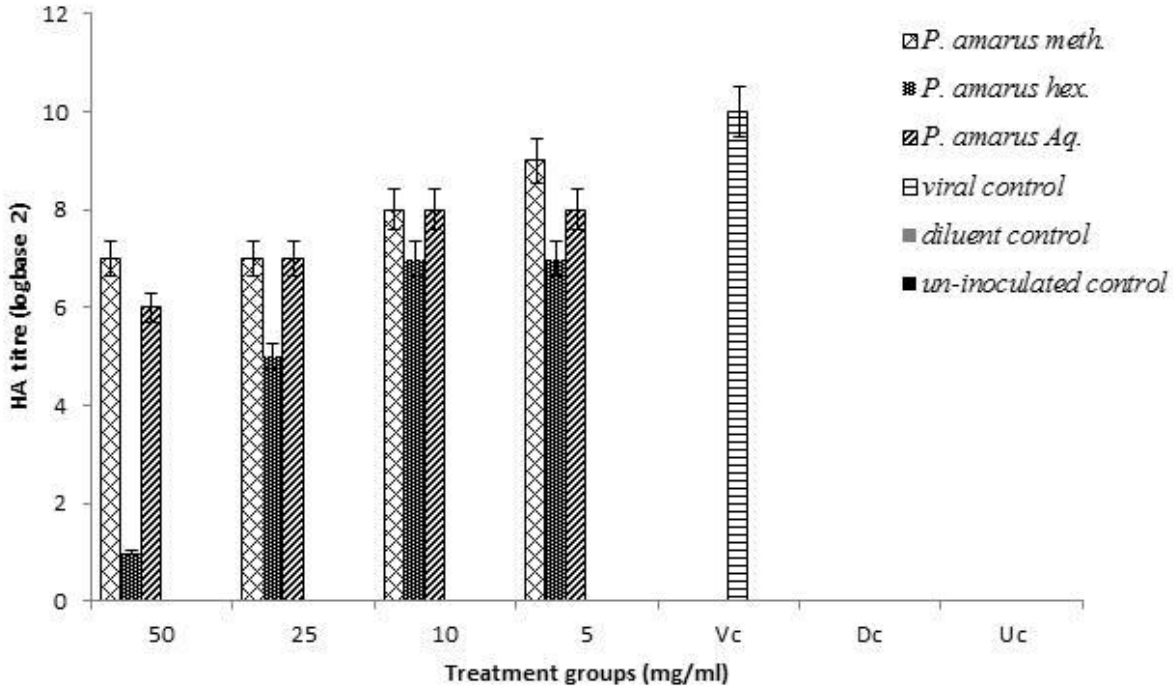

Figure 1. Prophylactic potentials of different extracts of test plant.

Table 2. Qualitative phytochemical properties of plant extracts.

Metabolites	B_{aq}	B_{Hex}	B_{Meth}
Alkaloids	+	+	+
Tannins	++	++	++
Phenol	++	++	++
Saponin	++	++	++
Terpenoids	+	+	+
Steroids	+	+	+
Cardiac glycosides	-	-	-
Glycosides	+	+	+
Flavonoid	+	+	+
Phlobatannin	+	-	-

B- *Phyllanthus amarus,* Meth- methanol, Hex- hexane, Aq- aqueous, - not detected, + slightly present, ++moderately present, +++ strongly present.

Table 3. Quantitative pytochemical values of plant leaf extracts.

Extracts	Alkaloids (mg/ml)	Phenols (mg/ml)	Saponin (mg/ml)	Tanins (mg/ml)	Flavonoids (mg/ml)
B(Aq)	0.248± 0.18	1.044± 0.02	0.252± 0.14	0.599± 0.10	0.696± 0.15
B(Hex)	0.290± 0.14	1.127± 0.19	0.288± 0.14	0.621± 0.01	0.590± 0.11
B(Meth)	0.263± 0.19	1.010± 0.17	0.264± 0.15	0.615± 0.11	0.593± 0.11

Results reported in mean of replicates ± SEM, n=3. B- *P. amarus,* Meth- methanol, Hex- hexane, Aq- aqueous.

ingredients in varying degrees which may be attributed to the bioactivity seen in the antiviral assay (Table 2). Table 3 revealed the quantitative presentation of the important active ingredients in the extracts. The study also revealed that the extract possesses antioxidant capacity as indicated by the value 38.943 ± 0.11 (mmol TEAC/100 g of sample) obtained from its percentage DPPH free-radical scavenging activity as well as the percentage ferric reducing antioxidant power of 165.076± 0.24 (mgGAE/100 g sample).

This antiviral investigation of *P. amarus* is suggestive of potentials present in the plant leaf extracts. It was observed that the leaf extracts exhibited no acute toxicity against the embryo at the concentration of 50 mg/ml and below, as no death was recorded and embryo appears normal which prompted further investigation on the extract's antiviral potencies (Table 1). The extracts were safe to the embryo at the concentration studied. However, it was revealed that the different extracts possess antiviral activities against NDV at different degrees. The extract demonstrated reduction in viral titre with increase in extract concentration. The treatment group exhibited a significant decrease ($F(4,10)=33.0$, $p=0.001$) in HA viral titre (Figures 1 to 3). This reduction led to the survival of some of the embryo of the ECE in the treatment group beyond the viral control group that has a titre of 1024 which all died 48 h post viral inoculation.

Further analysis showed that there was no significant mean difference between the viral titre at concentrations of 5 and 10 mg/ml ($P>0.05$), however at the concentration of 50 mg/ml, there was significant reduction ($P < 0.05$) in titre to a value of 2. It was observed that the prophylactic test group showed most significant reduction ($P< 0.05$) in viral titre than the other groups which could suggest that this plant extract when introduced into ECE before NDV infection could alienate the virus and serve as a prophylaxis. In the therapeutic and inhibitory groups, the leaf extracts also showed antiviral activities measured by reduction in the HA titre in comparison with the virus control group. The hexane extract showed significant reduction in the HA titre at all groups studied at the concentration of 50 mg/ml, indicating more potency when compared with the methanol and aqueous extracts (Figures 1 to 3). The diluents and un-inoculated control groups showed no reaction/agglutination as well as zero HA titre as no viral particle nor extract were inoculated into it. This biological properties exhibited by *P. amarus* could be attributed to the phytochemicals revealed in the plant extracts (Sofowora, 1993), however the varying degrees of the presence of this phytochemicals could be due to the ability of the solvents to extract some of the active ingredient or substances from the plant leaf based on its polarity (Table 3) (Simon et al., 2015). The free-radical scavenging activity of the leaf extracts may also possibly contribute to the antiviral effect exerted by the

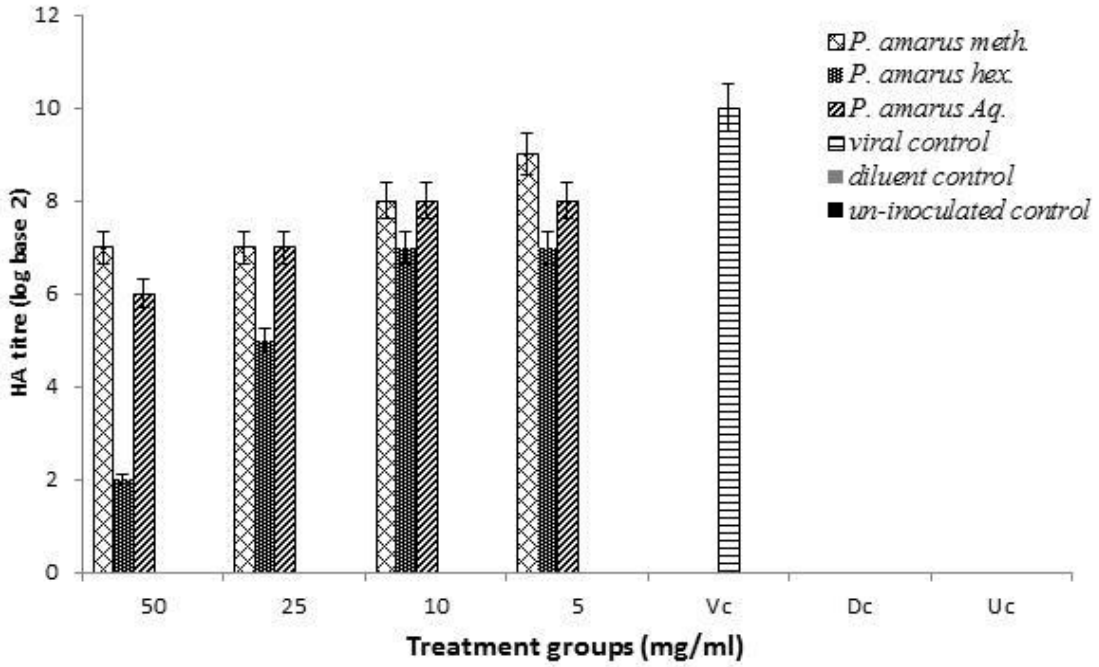

Figure 2. Therapeutic potentials of different extracts of test plant.

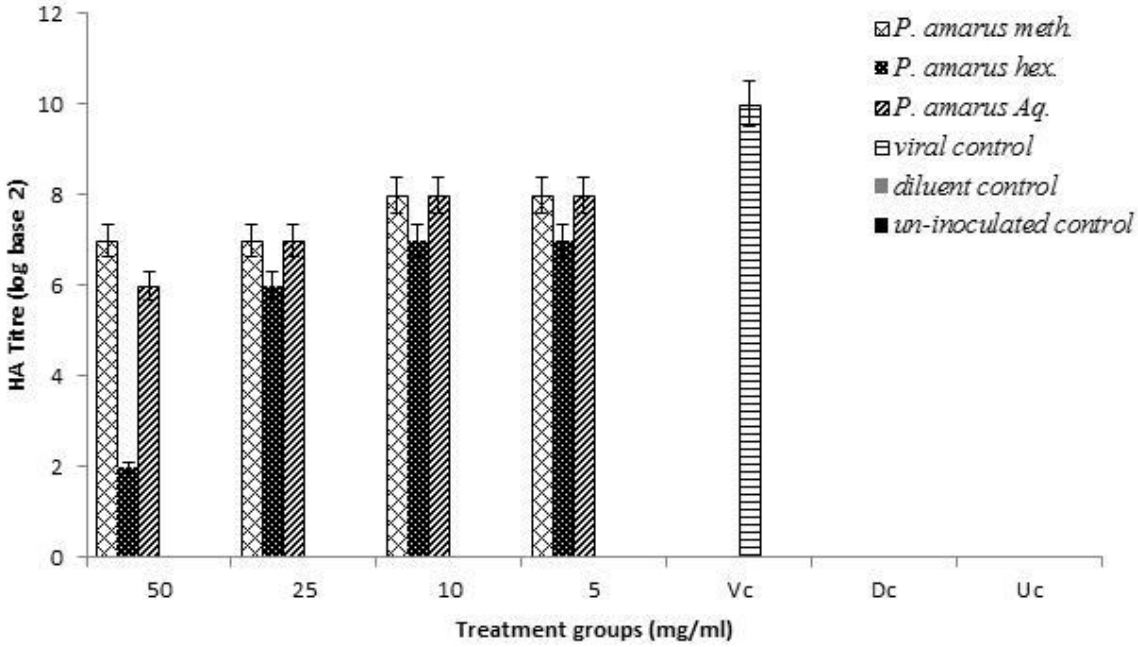

Figure 3. Inhibitory potentials of different extracts of test plant.

bioactive compounds present in the extract (Ayo et al., 2009), however this require validation by further investigation.

This research shows that the study plant may emanate as an alternative approach to prophylactic and therapeutic effect of viral infection which is in agreement with studies conducted by Chollom et al. (2012) and Bakari et al. (2013) that showed the anti-NDV potentials

Moringa olifera and C*ommiphora swynnertonii*, respectively.

Based on the result obtained from this study, it can be said that *P. amarus* extracts possess antiviral activity against NDV which can be a baseline for larger and further researches into tapping the antiviral potentials of this plant. It is recommended that a larger study and an *in vivo* study using live chickens that could possibly accommodate higher and varying concentrations be conducted in order to further validate the efficacy of the plant extract on anti-NDV and antioxidant activities.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Abad MJ, Guerra JA, Bermejo A, Iruzun A, Carrasco L (2000). Search for antiviral activity from higher plant extracts. Phytother. Res. 14:604-607.

Adeneye AA, Amole OO, Adeneye AK (2006). The Hypoglycemic and Hypocholesterolemic Activities of the Aqueous Leaf and Seed Extracts of *Phyllanthus amarus.* Filoterpia 77:511-514.

Asres K, Seyoum A, Veeresham C, Buca F, Gibbons S (2005). Naturally derived anti-HIV agents. Phytother. Res. 19:557-581.

Ayo RG, Amupitan JO, Ndukwe IG, Audu OT (2009). Some chemical constituents of the leaves of *Cassia nigricans* Vahl. Afr. J. Pure Appl. Chem. 3(11):208-211.

Babich H, Visioli F (2003). Invitro cytotoxicity to Human cells in culture of some phenolics from olive oils. Pharmacology 58:406-408.

Bakari GG, Max RA, Mdegela R, Phiri ECJ, Mtambo MMA (2013). Efficacy of resinous extract from Commiphora swynnertonii (Burrt) against newcastle infection in chickens. Int. J. Med. Plants Res. 2:156-61.

Chollom SC, Agada GOA, Gotep JG, Mwankon SE, Dus PC, Bot YS, Nyango DY, Singnap CL, Fyaktu EJ, Okwori AE (2012). Investigation of aqueous extract of *Moringa oleifera* lam seed for antiviral activity against Newcastle disease virus *in-ovo*. J. Med. Plants Res. 6(22):3870-3875.

Desphande SN, Kadam DG (2013). Preliminary phytochemical analysis of some medicinal plants.DAV. Int. J. Sci. 2(2):61-65.

Fajimi AK, Taiwo AA (2005). Herbal Remedies in animal parasitic diseases in Nigeria. A Review. Afr. J. Biotechnol. 4:303.

Joseph B, Raj SJ (2011). An overview: Pharmacognostic properties of *Phyllanthus amarus* Linn. Int. J. Pharm. 7:40-45.

Hadi S, Bremner B (2001).Initial studies on alkaloids from Lombok medicinal plants molecules 6: 117-129.

McDonald S, Prenzler PD, Autolovich M, Robards K (2001). Phenolic content and antioxidant activity of olive extracts. Food Chem. 73:7384.

OIE (2012). Manual of diagnostic tests and vaccines for terrestrial animals:mammals, birds and bees. Biological Standards Commission. World Organization for Animal Health. Paris. pp.1-19.

Okiki PA, Olatunji BP, Egbebi A, Asoso S, Ojo O (2015). A comparative study of Nutritional and phytochemical composition of *Phyllanthus amarus* leaf and seed. Am-Eur. J. Toxicol. Sci. 7(4):321-327.

Oladunmoye MK (2012). Antioxidant, free radical scavenging capacity and antimicrobial activities of Mirabilis jalapa. J. Med. Plants Res. 6(15):2909-2913.

Serpen A, Capuano E, Fogliano V, Gökmen V (2007). A new procedure to measure the antioxidant activity of insoluble food components. J. Agric. Food Chem. 55:7676-7681.

Verma S, Sharma H, Gary M (2014). A review: *Phyllanthus amarus*. J. Pharm. Phytochem. 3(2):18-22.

Simon BIA, Lidianys MLL, Claudia LL, Armida AGS, Daniela FA, Jose LR, David DH (2015). Solvent effects on phytochemical constituent profiles and antioxidant activities, using four different extraction formulations for analysis of *Bucida buceras* L. and *Phoradendron californicum*. BMC Res. Notes. 8:396.

Sofowora AE (1993). Medical plants and traditional medicine in Africa. Spectrum Books limited, Ibadan, Nigeria. P 289.

Wadood A, Ghufran M, Jamal SB, Naeem M, Khan A, Ghaffar R, Asnad C (2013). Phytochemical analysis of medicinal plants occurring in local area of Mardan. Biochem. Anal. Biochem. 2(4):1-4.

Young M, Alders R, Grimes S, Spradbrow P, Dias P, da Silva A, Lobo Q (2002). Controlling Newcastle Disease in Village Chickens - A Laboratory Manual. Available at: https://www.mysciencework.com/publication/show/663226be7dc072c6e0ca8b4709d7a923