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In OVO Assessment of Antiviral Potential of *Aloe Barbadensis* Miller against Newcastle Disease Virus

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ABSTRACT

The antiviral potential of *Aloe barbadensis* Miller against Newcastle Disease Virus (NDV) was determined using 11-day old embryonated chicken eggs. Identification and harvesting of *Aloe barbadensis* Miller plant were done at Federal College of Forestry, Jos while laboratory assay was done at the Biochemistry and Viral Research Divisions of National Veterinary Research Institute Vom. Extraction and phytochemical analysis of plant extract was done using distilled water, acetone, ethanol and chloroform, while cytotoxicity studies, determination of EID_{50} as well as antiviral assay were carried out according to standard methods. Results obtained indicated that distilled water is the best solvent for extraction of *Aloe barbadensis* Miller in terms of quantity and quality of products. This is because it turned in the highest yield of extract and also retained most pharmacologically active substances, as shown by phytochemical studies. Cytotoxicity studies, on the other hand, showed that embryonated eggs could tolerate the plant extract at concentrations of 200mg/ml and below, while antiviral assay clearly revealed inhibition of virus multiplication in embryonated eggs in extract concentrations as low as 100mg/ml and above. It, therefore, implies that the *Aloe barbadensis* Miller plant can be extracted in distilled water and administered to birds infected with NDV at recommended dosage to effectively treat Newcastle Disease.

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INTRODUCTION

Newcastle disease (ND) is one of the most important infectious diseases of poultry. It is distributed worldwide and has the potential to cause large economic losses in the poultry industry [1, 2]. Its causative agent is the Newcastle disease virus (NDV), a virus that is able to infect over 240 species of birds and spreads primarily through direct contact between infected and healthy birds [3].

The Office International des Epizooties (OIE), also known as World Organisation for Animal Health,

defines Newcastle disease as an infection caused by a highly virulent avian paramyxovirus-1 (APMV-1 virus) – an isolate that has either: (1) an intracerebral pathogenicity index (ICPI) of at least 0.7 in day-old chicks, or (2) an amino acid sequence that resembles those seen in highly virulent viruses (multiple basic amino acids at the C-terminus of the F2 protein and phenylalanine at residue 117 of the F1 protein) [3, 4]. It is recognized as a list 'A' disease by OIE [5]. NDV is a single-stranded RNA-containing virus with helical capsid symmetry [6], which belongs to the family Paramyxoviridae and the genus Avulavirus [6, 7]. The disease still remains a serious economic challenge to all segments of the poultry industry [8] because of its contagious and mortality records [1, 9]. Control of Newcastle disease has been through strict implementation of biosecurity measures in farms, public enlightenment and vaccination [9–11]. Vaccination has remained the most effective control strategy as it can confer full protection against the disease. Occasionally, vaccine failure occurs, leading to widespread outbreaks, including high rates of mortality and morbidity [12, 13]. Treatment is limited to management of secondary bacterial infections using antibiotics and supportive through administration of vitamins [14, 15] as there is no known drug for the treatment of Newcastle disease virus [16, 17]. Interestingly, many claims of herbal treatment using different plant preparations abound in history [17]. Some of these claims have been analyzed scientifically for evidence [18, 19]. *Aloe barbadensis* Miller plant has been copiously documented as a medicinal plant with application cutting across a wide range of diseases, including ND [20, 21]. Specifically, the Kenyans have had a track record of ethnoveterinary use of the plant to treat ND, although they had no laboratory data at the time to back the claims [22, 23]. The aim of this research is, therefore, to verify these claims and generate scientific data in that regard.

MATERIALS AND METHODS

Collection and Identification of the Plant Material

Fresh blades (leaves) of *Aloe barbadensis* Miller were identified and harvested from the herbarium of Federal College of Forestry, Jos, Nigeria (9.8965^{o}N, 8.8583^{o}E). The plant was identified on the field according to the method of Haider (2011) [24] using standard keys and descriptions.

Preparation of Leaf Extract

Harvested blades were taken to the laboratory and weighed. Warm water extraction of plant material was done as documented by Abubakar & Haque (2020) [25]. The gel of *Aloe barbadensis* Miller blades was scraped with a sterilized spatula. The spongy gel was blended in an electric blender, and the net weight was taken. The spongy gel was dried in an oven to obtain a dry matter. The weight of the dried matter was also taken. It was labeled and stored at room temperature till use. The dried substance was divided into four equal parts and labeled A-D. A-D were extracted with distilled water, 95% ethanol, acetone and chloroform, respectively, at room temperature according to the protocol of Zhang *et al.* (2018) [26]. The extraction process was repeated till the solvents became colourless. The extracts were then filtered using Whatman No.1 paper. The filtrates were concentrated in a vacuum at 50^{o}C $\pm$ 1^{o}C in a rotary evaporator to obtain the crude extracts. The resulting extracts were reconstituted using appropriate solvents to give a final working concentration of 1000mg/ml each.

Phytochemical screening

Phytochemical screening was carried out on extracts A-D using the methods of Trease and Evans (1989).

Egg-Based Cytotoxicity Assay

The method earlier applied by Chollom *et al.* (2012) [18] was adopted. A total of forty-five (45) 11-day old embryonated chicken eggs were used for this assay. They were divided into 9 groups of fives. Groups 1-8 were inoculated through the allantoic fluid with 0.2ml Aloe vera extract at concentrations of 50, 100, 150, 200, 250, 300, 350 and 400mg/ml, respectively, while group 9 was inoculated with PBS (negative control). Inoculated eggs were sealed and incubated at 37^{o}C for 72hrs with intermittent candling after every 24hr cycle. Eggs with dead embryo within the period were isolated and stored at -20^{o}C. At the end of the experiment, a count of eggs with dead and living embryos was taken against each concentration, while a simple percentage of mortality was computed (Table 4).

Antiviral Assay

Determination of EID_{50} of the virus

A velogenic strain of NDV was sourced from the Viral Research Department of the National Veterinary Research Institute, Vom. EID_{50} of the virus was determined according to the method of Young et al. (2002) [27]. From this, 100 EID_{50}/ 0.1 ml of the virus stock was made for the experiment (Table 5). The Reed and Muench index below was used to determine the value of one EID50 in 0.1ml of the suspension

Index =

$$\frac{\%\ mortality\ above\ 50\% - 50\%}{\%\ mortality\ immediately\ above\ 50\% - \%\ mortality\ immediately\ below\ 50\%}$$

100 EID_{50} was subsequently prepared as working dilution

Preparation of inocula

A 1:2 dilution of the 100 EID_{50}/0.1 ml of the virus with predetermined extract concentrations was made to put extract final concentration in the virus/extract mixture at 50, 100, 150 and 200mg/ml.

Inoculation of eggs

This was done according to the protocol of Chollom *et al.* (2012) [18] with minor modifications. Eleven-day-old embryonated chicken eggs were divided into eight groups of fives. The embryonated chicken eggs were labeled according to the extract concentrations used. A set of plastic egg trays were thoroughly cleaned with Virkon ®, and the eggs were swabbed with 70% alcohol in cotton wool and transferred into the cleaned trays.

The swabbed eggs were placed in the biosafety cabinet, where they were punched and immediately inoculated via the allantoic route.

Groups 1 to 4 were inoculated with 0.2 ml of virus/extract mixtures at final concentrations of 50,100,150 and 200mg/ml in that order. Group 5 was inoculated with 0.2 ml 100 EID50/0.1 ml standard NDV (virus control), group 6 was inoculated with 0.2 ml extract suspension (extract control). Group 7 was inoculated with 0.2 ml phosphate-buffered saline (diluents control), while group 8 had eggs that were not inoculated (uninoculated control).

The eggs were sealed with molten wax and incubated at 37°C. Embryo survival was observed daily. Allantoic fluid from treated eggs was collected for spot and haemagglutination tests to detect NDV in the experimental eggs.

Spot Haemagglutination Test

Dead embryos earlier chilled in the refrigerator were kept at room temperature for about 30 min. The eggs were swabbed and placed in the biosafety cabinet. The shell of each egg was opened to reveal the air space and a pipette was used to dispense a drop of 10% washed chicken red blood cells on a white tile. A wire loop was thoroughly flamed and used to pick a drop of the allantoic fluid and mixed with the drop of blood. The tile was gently rocked and observed for visible agglutination, indicating viral activity [28, 29]. This was done for all eggs, and the observations were recorded.

RESULTS

Sample Collection, Processing and Weighing Results

Fresh *Aloe barbadensis* Miller blades with herbarium numbers (FCF/H/003 to FCF/H/010) were collected at the herbarium of Federal College of Forestry, Jos. Altogether 26, 500g of the plant material was collected. From this, 14,500g spongy mass was harvested and on drying, it yielded 189.99g (dry weight) (Table 1).

The 189.99g Aloe vera was divided into 4 parts of 47.25g each and extracted with the trial solvents. Extract yield was highest with water (14.32g) and least with acetone (1.72g) (Table 2).

Outcome of Plant Extraction

The extracted yield of *Aloe barbadensis* Miller vary from one extract to another, as seen in Table 2.

In descending order, Water gave the highest yield of 14.32g, followed by ethanol with 7.76g. Chloroform yielded 4.85g of the extract, while Acetone yielded the least, 1.72g.

Phytochemistry Results

Interestingly, phytochemistry revealed the presence of pharmacologically active substances with the solvents. Only chloroform interfered most with the extraction of key products such as tannins, saponins and flavonoids (Table 3).

Cytotoxicity Results

Egg-based cytotoxicity assay carried out on varied concentrations of extract from 50 to 400mg/ml. Live embryos were recovered at extract concentrations of 50mg/ml to 200mg/ml.

Unfortunately, embryo mortalities were observed from 250mg/ml to 400mg/ml in increasing order. It thus revealed that the extract is tolerated in embryonated eggs to a maximum concentration of 200mg/ml, as revealed in Table 4

Results of EID_{50}

EID_{50} was determined based on standard procedure. 1EID_{50}/0.1ml was determined to be $10^{-5.5}$ while 100EID_{50} /0.1ml was calculated to be $10^{-5.3}$. Equivalent of 1:100,000 (Table 5)

Antiviral assay showed inhibition of virus growth in 2 eggs, 3 eggs and 5 eggs in 100mg/ml, 150mg/ml and 200mg/ml virus- extract concentrations respectively (Table 6). This was confirmed by the HA spot test results in Table 7

Results of Antiviral Assay

Antiviral assay showed inhibition of virus activity from extract concentration of 100mg/ml to

200mg/ml in increasing order. 40% of embryos survived at 100mg/ml, 60% survived at 150mg/ml while 100% of embryos survived at 200mg/ml extract concentration (Table 6)

Result of HA Spot Test

Assessment of virus multiplication in embryonated eggs was done using Haemagglutination (HA) spot testing. The presence of virus was detected from eggs inoculated with 100mg/ml to 200mg/ml virus-extract mixture (Table 7). These findings confirm results in Table 4, Table 5 and Table 6.

DISCUSSION

Newcastle disease (ND) is a viral disease of poultry caused by a single-strand, nonsegmented, negative-sense RNA virus known as Avian paramyxovirus 1 (APMV-1). The disease is present worldwide and affects many species of birds, causing severe losses in the poultry sector. In developing countries, where the majority of chickens are reared under "backyard" subsistence conditions, ND can drastically limit the amount of dietary protein as well as damage the micro-economy due to loss of ability to sell off extra chickens or eggs. Where chickens are raised commercially, either in developing or developed countries, outbreaks have occurred in many locations, causing massive economic losses [1, 9, 30]. Since ancient times, plants and plant parts have been indispensable sources of medicine for indigenous poultry production [31, 32]. Although modern medical science has developed to a great extent, many farmers in parts of Africa depend on plant parts and herbal remedies for indigenous poultry health management. *Aloe barbadensis* Miller is one plant that has come under the searchlight following its successful use as a concoction by poultry farmers in Kenya [22, 23].

Our findings show that *Aloe barbadensis* Miller is readily available in Jos and environs. It is cultivated in gardens, homes and institutions of learning. It is consumed locally as a medicinal remedy or sold out for industrial production of beverages, cosmetics and drugs and nutritional supplements. The availability of the plant in most gardens in Jos has laid credence to the fact that it is widely distributed as an ornamental plant that is highly succulent. Its succulent nature was aptly depicted in the weight dynamics of the plant material, where a field weight of 26,500.00g yielded 14,500g spongy mass, out of which only 189.99g dry weight was obtained, indicating that 14,499.81g evaporated. Mathematically, only about 1:140 of plant material obtained from the field is available for laboratory-based assay. This supports earlier literature relating to its succulent nature [33]. Researchers must therefore harvest large volumes of the plant from the field to enable them to get a reasonable amount for laboratory assays. The 1:140 ratio of available *Aloe barbadensis* Miller product for laboratory-based research as obtained in this study can be used as a guide for researchers interested in further work on *Aloe barbadensis* Miller as previous studies did not provide data on weight dynamics from field to extracted products [34].

The behavior of the plant material against the various extracting solvents is interesting as the quality and quantity of extracted products varied from one extracting solvent to another. Distilled water turned in the highest yield quantity (14.32g) while acetone turned in the least quantity (1.72g). Also, distilled water was best in terms of the quality of extracted products as it retained most pharmacologically active substances against denaturation/deactivation by other extraction solvents. It, therefore, goes to say that distilled water is the best solvent for extraction of the plant material judging from extracted yield quality and quantity. This is not unconnected to the age-long confirmation that water is a universal solvent [35]. On the other hand, acetone, ethanol and chloroform all have unsaturated functional groups that can readily combine with free hydrogen radicals when mixed with other compounds [36]. Consequently, distilled water is strongly advocated for the extraction of biological products since it retains them in their natural composition. This discovery will also be heart-warming to farmers as water is readily available and accessible at almost no cost. It, therefore, presents a very good option to farmers who would simply dilute the required purified product in water and present it to their birds for the treatment of NDV. Also, since phytochemistry revealed that most nutritional and pharmacologically active products were retained in water extraction, the farmer stands to benefit more as this would serve as some form of nutritional supplement to his bird for rapid growth and competitive market value of poultry products. The nutritional supplement obtained in the process may be crude, but they are natural and usually safer for animal and human consumption relative to the commercially formulated supplements, which may be genetically modified with attendant health challenges of cancerous cell formation and obesity in the long run [37].

The fact that embryonated chicken eggs tolerated the extract in a dose-dependent manner is in tandem with the physiological disposition of biological cells. As living entities, the cells have tolerance levels to biochemical products when exposed to them.

Table 1: Dry weight of Aloe vera Spongy mass relative to Aloe vera weight from field

Weight	
Total weight of harvested Aloe vera blades	26,500. 00g
Total weight of chaff after harvest of a spongy mass	11,700. 15g
Total weight of spongy mass	14,500.00g
The total dry weight of spongy mass after evaporation	189.99g

Table 2: Relative Yield of Extract from Solvents Used

	Water	Ethanol	Acetone	Chloroform
The dry weight of Aloe vera	47.25g	47.25g	47.25g	47.25g
Weight loss due to extraction	32.93g	39.49g	45.53g	42.40g
Net Weight of extract	14.32g	7.76g	1.72g	4.85g

Table 3: Outcome of Phytochemistry

	Tan	Sap	Cardiac Gly	Steroids/Terp	Alkaloids	Arthroquir	Flavonoids
Acetone	+	+	+	+	-	+	+
Ethanol	+	+	+	+	-	+	+
Chloroform	-	-	+	-	+	+	-
D/Water	+	+	+	+	+	-	+

Table 4: Outcome of Cytotoxicity Assay (Water Extract) with Embryonated Chicken Eggs

Dosage (mg/ml)	No of eggs Inoculated	No of eggs alive after 24 hrs	48 hrs	72 hrs	No of eggs dead after 24 hrs	48 hrs	72 hrs	% mortality
50	5	5	5	5	0	0	0	0
100	5	5	5	5	0	0	0	0
150	5	5	5	5	0	0	0	0
200	5	5	5	5	0	0	0	0
250	5	4	4	4	1	0	0	20
300	5	4	3	3	1	1	0	40
350	5	2	2	2	3	0	0	60
400	5	0	-	-	5	-	-	100
0 (-ve control)	5	5	-	-	0	0	0	0

Table 5: Determination of EID50

D/F	No of Eggs	No alive after 72 hrs	No dead after 72 hrs	% Mortality
10^{-1}	5	0	5	100
10^{-2}	5	0	5	100
10^{-3}	5	0	5	100
10^{-4}	5	1	4	80
10^{-5}	5	2	3	60
10^{-6}	5	3	2	40
10^{-7}	5	4	1	20
10^{-8}	5	5	0	-
10^{-9}	5	5	0	-
10^{-10}	5	5	0	-

Table 6: Antiviral Assay

Conc (mg/ml)	No of Eggs	Mortality 24 hrs	Mortality 48 hrs	Mortality 72 hrs	Alive After 72 hrs	% Survival
50	5	0	5	-	0	0
100	5	0	2	1	2	40
150	5	0	1	1	3	60
200	5	0	0	0	5	100
Virus Control	5	0	5	0	0	0
Extract Control	5	0	0	0	5	100

Table 7: HA Spot

Conc (mg/ml)	No of Eggs inoculated	-ve Spot Test	+ve Spot Test
50	5	0	5
100	5	2	3
150	5	3	2
200	5	5	0
Virus Control	5	0	5
Extract Control	5	5	0

KEY:
Conc: Concentration, -ve : Negative, +ve : Positive

They remain alive and multiply rapidly at concentrations friendly to their metabolic and physiologic wellbeing but begin to show pathology and eventually die when such exogenous products interfere adversely with their physiology [38]. At this level, the product is said to be toxic at such concentrations.

Aloe barbadensis Miller extracts at a concentration of 200mg/ml and below was tolerated by embryonated eggs hence its acceptability for the research at tolerable concentrations. This finding is therefore critical to the successful and scientific use of *Aloe barbadensis* Miller products in the treatment of ND in poultry. For farmers to get optimum results, researchers must determine acceptable doses per body weight of birds to ensure that lower doses are not administered because they will be highly ineffective against the virus although tolerable to host cells, while very high doses beyond the tolerance threshold of host cells will highly toxic and deleterious. A scientifically derived formula must therefore be generated to ensure the effectiveness of the *Aloe barbadensis* Miller product against the virus as well as its safety on host cells.

The determination of $1EID_{50}$/0.1ml of the virus suspension as $10^{-5.5}$ and $100EID_{50}$ /0.1ml as $10^{-5.3}$ means that the suspension has sufficient viral load to serve as a challenge virus for the experiment. The EID_{50} of our challenge virus is also higher than that obtained elsewhere in other experiments [39]. It is critical to determine this value to ensure there are sufficient viable viruses in the challenge viral suspension to cause disease in the experimental birds as well as stand the inhibitory potentials of the plant extract with the same charisma or virulence as a field virus would in the event of natural infection with NDV.

Interestingly, the antiviral activity of the extract against the virus was detected from a 100mg/ml concentration. The activity increased as the extract concentration increased, as seen in the number of embryonated eggs that survived the challenge by the virus (Table 6). This outcome is authenticated by the outcome of the control groups as all eggs inoculated with virus alone died just as those inoculated with extract alone survived. This has therefore supported the earlier claims by Okitoi *et al*. (2007) [22] and Adams et al. (2014) [23] about the continuous and successful use of *Aloe barbadensis* Miller plant in Kenya for the treatment of animals with symptoms synonymous with ND. Also, previous reports by Surjushe *et al*. (2008) [40] had elaborately identified the plant as a highly medicinal and nutritive cactus-like shrub growing freely in the tropics.

The significant antiviral and nutritional properties of this plant single it a good candidate for drug development. This is because the nutritional components would support the growth and market value of birds, while the antiviral components would rid the animal of viral challenges, thereby increasing the productivity and profitability in the poultry business.

Also, the edge that water extract has over extracts from other extracts is a comparative advantage to the farmers. This is because water is user-friendly without the need for extra precautionary measures for safety, as would be of chemicals like chloroform or acetone. This, therefore, means that local farmers can by themselves prepare these extracts and administer them to their animals when clear instructions are provided, thereby cutting the cost of labour outsourcing.

CONCLUSION

Empirical data generated from this research endeavor has revealed that *Aloe barbadensis* Miller has antiviral activity against ND. It has therefore added a layer on existing evidence on the possible use of this plant to control the epidemic of ND in poultry. Also, there is the need to begin further work on formulating and packaging this product for field trials and eventual drug development.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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