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Antiviral Activities of the Aqueous, Ethanolic and Methanolic Extracts of *Diospyros Mespiliformis* leaf on some pathogenic Avian viruses.

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ABSTRACT

The antiviral activities of aqueous, ethanolic and methanolic extracts of *Diospyros mespiliformis* Hochst, ex A.DC., used traditionally to treat illnesses were evaluated against three avian viruses namely, Newcastle disease virus (NDV), Fowl pox virus (FPV) and Infectious bursal disease virus (IBDV). The assay was performed in ten day old embryonated chicken eggs by chorioallantoic membrane (CAM) and the allantoic sac inoculation for FPV or IBDV and NDV respectively. The viral replication in the tests and controls were estimated by haemagglitination assay of harvested allantoic fluid for NDV and reduction in pocks formation when compared with controls as an indication of viral inhibition in FDV and IBDV. The percentage inhibition of aqueous extracts concentrations of 400mg/ml, 200mg/ml and 100mg/ml of *Diospyros mespiliformis* (DM) on the virus (NDV) were 91.0%, 86.0% and 85.0%, respectively. The percentage inhibition of the ethanolic extracts were: 95.0%, 90.5% and 89.0%; respectively. The percentage inhibition of methanolic extracts were: 100.0%, 92.8% and 90.5%; At 400mg/ml crude extract concentration of *Diospyros mespiliformis*, the challenged virus FPV recorded very high activity, A one hundred percentage (100%) egg mortality was observed in all plant extracts investigated at the end of the experiment with infectious bursal disease virus (IBDV). The phytochemical analysis of crude extract of *Diospyros mespiliformis* and fractions exhibited the presence of Alkaloids, Tannins, Saponins, Triterpenes, Flavonoids and Cyanogenic glycosides. These results obtained in this study suggest that *Diospyros mespiliformis* could be a potential candidate in the management of Newcastle and Fowl pox diseases affecting the poultry industry. However, the active components responsible for the antiviral activity need to be evaluated.

Keywords:Antiviral, *Diospyros mespiliformis,* pathogens, avian virus.

INTRODUCTION

Medical practices (Traditional and Veterinary) still play an important role in many areas of Nigeria; as a result, large number of plants is used on the continent of Africa for the treatment of various kinds of diseases and ailments [1, 2]. Many natural plants have been sourced and used as valuable medicinal agent for many years with proven potential of treating infectious

disease and with lesser side effects compared to synthetic agents [3, 4, 5]. Within the past decades therapeutic option for viral infections have improved significantly and has witnessed intensive studies on extracts and biologically active compounds isolated from plants species used for natural therapies or herbal medicine [6, 7, 8].

Extracts from leaves and barks of *Diospyros mespiliformis* have long been credited for their medicinal properties that include being antiviral, anti-bacterial, anti-inflammatory among others [9]. *Diospyros mespiliformis* (Hochs, ex.A. DC). Ebenaceac is confined to tropical and sub-tropical regions notably in Africa [10, 11, 12, 13]. Several ethno-pharmacological applications have been reported for *Diospyros mespiliformis* (Hochsr. Ex. A. DC), which include the use of leaf decoction as extraordinary remedy for fever, whooping cough and for wounds [14, 15, 16]. Barks and roots are used for serious infections such as malaria, pneumonia, syphilis, leprosy and dermatomycosis, as an anthelmintic and to facilitate delivery (WRC, 2008). Different parts of the tree are used against diarrhea, skin infections, headache, toothache and similar pains and as a psycho-pharmacological drug [17,18, 19]. The plant commonly known as Ebony tree in Nigeria is used as dressing for burns, antibacterial agent and astrigent in diarrhea. Over dose of concentrated decoction can cause abortion [20].

The antiviral activities of aqueous, ethanolic and methanolic extracts of *Diospyros mespiliformis* leave was determine on some pathogenic avian viruses such as Newcastle disease virus (NDV), Fowl pox virus (FPV) and Infectious bursal disease virus (IBDV). Newcastle disease virus causes a highly contagious and fatal disease affecting all species of birds in poultry industry. The disease can vary from clinically inapparent to highly virulent forms, depending on the virus strain and the host species. The continuous spectrum of virulence displayed by NDV strains enabled the grouping of them into three different pathotypes; Lentogenic. mesogenic and velogenic [21]. Fowl pox virus (FPV) is an infectious disease of worldwide distribution that affects commercial poultry e.g. chickens, turkeys, domestic pets and free living birds of many species [22, 23, 24]. Infectious bursal disease (IBD) is a highly contagious disease of young chickens caused by infectious bursal disease virus (IBDV) [25] also known as Gumboro disease, characterized by immunosuppression and mortality generally at 3 to 6 weeks of age.

Materials and methods

Diospyros mespiliforms leaves were collected from their natural habitats in Dekina, Dekina Local Government Area of Kogi State and Eziani village, Ichi in Ekwusigo Local Government Area of Anambra State respectively. This sample was collected for extraction in the rainy

season (May 2010 and July 2010). The plant is indigenous and the specimen was identified by Prof. C.U. Okeke of the Department of Botany, Nnamdi Azikiwe University, Awka.

Preparation and Extraction

The sample was rinsed with clean water; air dried at room temperature in locally designed and constructed wire trays for two weeks. The dried leaves were then blended to fine powdered forms using a household electric blender. Phytochemical analysis were carried out on some Quantity of each of the ground crude samples while the remaining dried leaves homogenized powder were stored in sealed air-tight plastic containers at room temperature, pending commencement of extraction.

Distilled water was used for aqueous extraction, while Soxhlet extraction method [26] was used to obtain the ethanol and methanol extracts of the plants.

Preparation of Phosphate Buffered Saline (PBS): The following salts were carefully weighed out: Sodium chloride (Nacl) 8g, Potassium chloride (Kcl) 0.20g, Potassium dihydrogen phosphate (KH_2 Po_4) 0.20g and Disodium hydrogen phosphate (Na_2 HPo_4) 0.92g. These were added to a 2500ml capacity bottle, and 1000ml (1 liter) of distilled water was added to it. The solution was allowed to dissolve and was autoclaved for 15mins at 121^0c and 151bs per square inch (Pis) pressure. The PH was taken as 7.2 [27]. The lid of the bottle was tightened, labelled and stored in the refrigerator at 4^0C until ready for use.

Preparation of Antibiotic Solution (PSGA): The following reagents were carefully weighed out: Benzyl penicillin 6g, Streptomycin 500mg, Gentamicin 250mg, and Amphotericin B 4mg. These were dissolved in 200ml capacity bottle and 1000ml (1liter) of sterile phosphate buffered saline (PBS) was added to it and allowed to dissolve. The solution was sterilized by passing through a 0.2micron filter. The solution was dispensed into 100ml sterile glass bottles, lid and labelled.

Preparation of washed chicken red blood cells (RBCs) for haemagglitination Assay (HA): The washed RBCs from a 10weeks – old ND unvaccinated chicken were used to carry out haemagglitination test to quantify the virus in allantoic fluid and ascertain the level of inhibition of virus replication by the different plant extracts used for the study.

Concentration and Reconstitution of Extracts: Phosphate buffered saline (PBS) was used to reconstitute the aqueous, methanol and ethanol extracts. Three (3) different concentrations in dilutions were made namely: 400mg/ml, 200mg/ml and 100mg/ml. The reconstituted extracts were sterilized by filtration using Millipore micro-filter (0.45um pore size).

Viruses

Three (3) common animal viruses, Newcastle disease virus (NDV) thermo stable strain, its EID_{50} was $10^{8.6}$ /ml, Fowl pox virus (FPV) and Infections Bursal Disease Virus (IBDV) – which were prepared from infected tissues – were supplied by the Avian Viruses Research Disease Laboratory Section, Viral Research Division, National Veterinary Research Institute Vom, Plateau State, Nigeria. The virus was transported in an ice pack in its lyophilized form and stored at -20^{0}C deep freezer.

Newcastle disease virus (NDV): The procedure applied in the egg inoculation were as described by [28]. Using asceptic technique, the harvested allantoic fluids were pooled, centrifuged at 5000rpm for 5mins and aliquots of the supernatant prepared and there after the Haemagglunation (HA) titre of the viral suspension was established using the standard method described by Office International des Epiziooties [29]. The concentration of Newcastle disease virus in a suspension was expressed as an infectivity titre. The infectivity titre was established by carrying out a titration. The end point of the titration was used to calculate the infectivity titre of the original suspension of virus. The [30] mathematical technique was used to calculate this end point from the result of the HA test on each of the inoculated eggs.

(% infected at dilution immediately above 50%) – (% infected at dilution immediately below 50%), The virus was quantified by HA using the harvested allantoic fluid from the inoculated fertilized eggs. The aim was to check if the virus actually grew in the eggs and if so, determine the percentage inhibition by the extract using the HA titres of positive control and test. The HA titres of the tests (virus inoculated with extracts) were compared with those of positive control (virus alone) and the difference used to compute the percentage inhibition of virus replication by the extracts.

Newcastle disease virus (wild type), obtained from the National Veterinary Research Institute, vom, was transported in an ice pack in its lyophilized form. The NDV strain supplied was further expanded in ten-day old embryonic chicken eggs and diluted with phosphate buffered saline (PBS) to obtain a titre of 1024 Haemagglutination unit HA (10log2). The eggs used for cultivation must be sterile and the shell should be intact and healthy. Candling is the process of holding a strong light above or below the egg to observe the embryo. The crude extracts of the test sample at 400mg/ml 200mg/ml 100mg/ml were prepared in sterile PBS containing autibiotics (PSGA). Blank controls and positive controls were included in the experiment.

Antiviral testing of the plant extracts were carried out invitro using allantoic sac routes of developing chick embryos. NDV: Inhibition of the haemagglitination by each extract was calculated as follows:

$$\text{HA inhibition \%} = \frac{C - T}{C}$$

Where C = the HA titre (GM) of the virus control, and T – the HA titre (GM) treated with the extracts.

Partial Chromatographic Separation of Extract: The crude methanolic extract (8ml) of *Diospyros mespiliformis* was centrifuged at 5000rpm for 30min at 4^0C in an Eppendorf centrifuge. It was evaporated to dryness by using stream of cold air (16^0C for 36hrs). This was achieved by exposing the extract in small amounts (2ml) in petridishes to cool air emanating from an air conditioner at 16°c. The stream of cold air was complimented with air from a standing fan. About 165mg of the dry extract was dissolved in 3ml of 20mM phosphate buffered saline, PH 7.2. About 2.5ml of the re-dissolved extract was applied to a high purity silica gel (220 – 440 mesh, 35 – 75um, Sigma – Aldrich) column (1.2 x 45cm) previously equilibrated with dimethyl sulphoxide (DMSO). The extract was eluted with the above solvent at a flow rate of 2ml/hour. A total of 17 (seventeen) fractions (each containing 10ml) were collected. The active fractions were evaporated to dryness by exposing them to a stream of cold air (16^0C, 36hours). The dry extracts were weighed to 3 – decimal places and re-suspended in 2ml of phosphate buffered saline (PH 7.2). Fraction 9 which exhibited the highest antiviral activity was selected for further studies. The partially purified extract of *Diospyros mespiliformis* was also examined for the presence of chemical constituents as illustrated in crude extract method.

Fowl Pox Virus (FPV): It cultivation was done by inoculating about 0.1ml virus suspension on the chorioallontoic membranes (CAMs) of 10days old developing chicken embryo. These were incubated at 38^0C for 7days and then examined for focal pock lesions or generalized thickening of the CAMs.

Infectious Bursal Disease Virus (IBDV): The virus was cultivated by inoculation of a viral suspension containing 10^5 EID_{50} onto the chorioallontoic membranes of 10day old chicken embryos .The eggs were incubated and observed daily for viability. Dead embryos during the first 24hours post inoculation (Pl) were discarded. Mortality was recorded between 2 - 4days Pl. At the end of 4thday Pl effects were compared with controls. The reduction or no pock/lesion formation was observed as an indicator for antiviral activity. The results were compared to the sample without treatment as a positive and PBA solution as a negative controls.

Phytochemical Analysis of Plant Materials: The ground samples of *A. melegueta* was examined for the presence of chemical constituents such as tannins, alkaloids, flavonoids, terpenoids, cyanogenic glycosides, saponins, following the descriptions of [31 and 32].

RESULTS

Table 1: Phytochemical Analysis of *Diospyros mespiliformis* Extracts from Various Solvents.

Phytochemical	Aqueous	Ethanol	Methanol
Alkaloids	---	(++)	(++)
Saponins	(+)	(++)	(++)
Triterpenes	---	(+)	(+++)
Flavonoids	(+)	(+)	(++)
Tanins	(+)	(++)	(++)
Cyanogenic glucosides	---	(+)	(+)

Key: +++ = high concentration, ++ medium concentration, + = low concentration, - = not detected

Table 2: Result of some phytochemical compounds in *Diospyros mespiliformis*

Phytochemical	Crude extract	Partial chromatographic separation of extract
Alkaloid	++	+
Saponin	++	+
Triterpenes	+++	++
Flavonoids	++	+
Tannins	++	+
Cyanogenic glucoside	+	_

Key: +++ = high concentration present; ++ = moderate concentration present; + = mild concentration present; - = absence

Table 3: Toxicity of *Diospyros mespiliformis* extracts under study in embryonated chicken eggs, estimated as percentage egg mortality

Extraction	Extract concentration mg/ml	Egg mortality			Percentage Egg mortality (%)
		Inoculated	Dead	Alive	
Aqueous Extraction	500	5	2	3	40.0
	400	5	0	5	0.0
	200	5	0	5	0.0
	100	5	0	5	0.0
Ethanol Extraction	500	5	3	2	60.0
	400	5	0	5	0.0
	200	5	0	5	0.0
	100	5	0	5	0.0
Methanol Extraction	500	5	3	2	60.0
	400	5	0	5	0.0
	200	5	0	5	0.0
	100	5	0	5	0.0

Table 4. Reduction of Newcastle Disease Viral Load by Extracts of *Diospyros mespiliformis (DM)* leaves in Embryonated chicken eggs.

Extraction	Test Extract concentration	Inoculums	No of Eggs inoculated	No of Eggs survived	Percentage egg mortality	Geometric mean virus titre	Percentage (%) inhibition
Aqueous	400mg/ml	0.2ml(Virus+Extract)	5	4	20%	36.0	91.0
Extract	200mg/ml	0.2ml(Virus+Extract)	5	3	40%	55.7	86.0
	100mg/ml	0.2ml(Virus+Extract)	5	3	40%	56.7	85.0
	Positive control	0.2ml(Virus+PBS)	5	0	100%	388.0	0.0
	Negative control	0.2ml PBS only	5	5	0.0%	1.0	==
Ethanol	400mg/ml	0.2ml(Virus+Extract)	5	5	0%	18.4	95.0
Extract	200mg/ml	0.2ml(Virus+Extract)	5	5	0%	36.8	90.5
	100mg/ml	0.2ml(Virus+Extract)	5	4	20%	42.0	89.0
	Positive control	0.2ml(Virus+PBS)	5	0	100%	388.0	0.0

	Negative control	0.2ml PBS only	5	5	0%	1.0	==
Methanol	400mg/ml	0.2ml(Virus+Extract)	5	5	0%	==	100.0
Extract	200mg/ml	0.2ml(Virus+Extract)	5	5	0%	27.8	92.8
	100mg/ml	0.2ml(Virus+Extract)	5	5	0%	36.8	90.5
	Positive control	0.2ml(Virus+PBS)	5	0	100%	388.0	0.0
	Negative control	0.2ml PBS only	5	5	0.0%	1.0	1.0

Table 5. Virucidal Effect of 4mg/ml concentration of Various Fractions of Methonolic Extracts of Diospyros mespiliformis on Newcastle Disease virus

Fraction no	No of Eggs inoculation	No of Eggs survived	Percentage Egg mortality	Geometric mean virus	Percentage inhibition	Virucidal effect
7	5	0	100%	230	33.3%	1.5 times
8	5	3	40%	46.6	86.4%	7.4 times
9	5	5	0%	37.1	89.2%	9.3 times
10	5	3	40%	50.7	85.3%	6.8 times
11	5	2	60%	86.3	75.0%	4.0 times
12	5	0	100%	265.5	23.1%	1.3 times
Positive control(virus+DMSO)	5	0	100%	345	0%	
Negative control (DMSO only)	5	5	0%	1.0	==	

Table 6. Reduction of Fowl Pox Virus (FPV) pocks extracts of *Diospyros mespiliformis* (DM) leaves in Embryonated chicken Eggs.

Extract	Test Extract concentration	FPV pock lesion formation	FPV virus inhibition
Aqueous Extract	400mg/ml	Present	++
	200mg/ml	present	++
	100mg/ml	Present	+
Ethanol Extract	400mg/ml	Absent	++++
	200mg/ml	Absent	++++
	100mg/ml	Present	++
Methanol Extract	400mg/ml	Absent	++++
	200mg/ml	Absent	++++
	100mg/ml	Present	++
Positive control	Virus+PBS	Present	0
Negative control	PBS only	Absent	== ==

Key:++++=complete inhibition,++=moderate inhibition, += mild inhibition, 0 = no inhibition.

Table 7. Reduction of infectious Bursal Disease virus (IBDV) viral load by Extracts of *Diospyros mespiliformis* (DM) leaves in Embryonated Chicken Eggs.

Extracts	Test Extract concentration	IBDV pock lesion formation	IBDV virus inhibition
Aqueous Extract	400mg/ml	Present	0
	200mg/ml	present	0
	100mg/ml	present	0
Ethanol Extract	400mg/ml	Present	0
	200mg/ml	present	0
	100mg/ml	present	0
Methanol Extract	400mg/ml	Present	0
	200mg/ml	present	0
	100mg/ml	present	0
Positive control	Virus+PBS	Present	0
Negative control	PBS only	Absent	== ==

Key: ++++ = complete inhibition, 0 = no inhibition.

DISCUSSION

The results of the antiviral activities of ***Diospyros mespiliformis*** leaf extracts studied with various degree of inhibitory properties invitro have been reported and the extracts have some inhibitory activity against Newcastle disease virus. A hundred percent (100%) inactivation has been used to define an extract with antiviral activity [26]. However, partial antiviral activity was observed in extracts used in this study when tested with Fowl pox virus whereas when tested with Infectious bursal disease virus, no activity was recorded. Preparations which exert antiviral effects invivo may not be detected with invitro assays because of the extremely low concentrations of extracts tolerated by cells in the artificial system. Even with this limitation, the extracts studied exhibited various degrees of antiviral activity against NDV, FPV at various concentrations.

In this study, extracts of *Diospyros mespiliformis* (African Ebony) exhibited antiviral results in both aqueous, ethanolic and methanolic conditions. At 400mg/ml concentration the aqeous, ethanolic and methnolic extract achieved antiviral inhibitions of 91%, 95%, and 100% respectively against Newcastle disease virus. Studies have shown that *D. mespiliformis* has antimicrobial activities, especially antibacterial, antifungal and antiviral [33].

The Phytochemical analysis of the plant materials unravelled a number of chemical substances with antiviral activity. Flavones, for instance which are known to be synthesized by plants in response to microbial infection, inhibit the initiation, promotion and progression of tumours [34, 35, 36]. Flavonones exhibit inhibitory effects against viruses [37,38, 39], including HIV and respiratory syncytial virus [40,41]. Terpenoids were reported to be active against bacteria, fungi, viruses and protozoa [42, 43]. It is believed to be active against viruses by envelope disruption by the lipophilic compounds. Alkaloids have been commonly found to have antimicrobial properties [10]. It is also useful against HIV infection as well as intestinal infections associated with AIDS [11]. Tannins are found in almost every plant part; wood, leaves, bark, roots and fruits [15] and tannin containing beverages can cure or prevent a variety of viral infections [18]. At least two studies [13, 16, 18] have shown tannins to be inhibitory to viral reverse transcriptases. Tannins (hydrolysable) also show anti- carcinogenic and anti-mutagenic effects [11]. These phytochemical were significantly demonstrated in methanolic extracts of *Diospyros mespiliformis.*

The ethanolic extracts and methanolic extracts appeared to have had more antiviral phytochemical than the aqueous extracts which accounted for better reduction in the Newcastle disease viral load for the most part of the experiment. Thus the efficacy of plant extract evaluated as antimicrobial agents was dependent on the solvent of extraction. This finding is in agreement with the finding of [16] who examined a variety of extractants for their ability to solubilize antimicrobials from plants and ranked them in the order; methanol, ethanol, and water and posited also that most active inhibitors extracted are not water soluble. It is also in agreement with results of [18] who found that ethanolic extracts of some Nigerian spices were more potent than the aqueous extracts against common food borne microorganisms including Staphylococcus aureus, Klesbsiella pneumonia, Proteus vulgaris and Streptococcus faecalis. In this study, a good number of the ethanolic and aqueous extracts of the plants employed portrayed some level of antiviral activity. Nevertheless, less than 100% activity was obtained in all cases, and might be taken to be that the antiviral compounds present were in amounts insufficient to inactivate all infectious virus particles.

The extracts and the fractions delayed embryo mortality. The low embryo mortality exhibited from some extracts concentrations could be explained by the corresponding arrested virus multiplication which was reflected by the low virus titres harvested from the eggs by the end of day- 5 PI. The results of embryo mortality also clearly showed that both the crude *Diospyros mespiliformis* extract and some phytochemical fractions and controls were not lethal to the chicken embryonated eggs as all the embryos were alive by day-5 when the experiment was terminated. The results also showed that within the experimental group, methanolic crude extract of *D. mespiliformis* and phytochemical fractions delayed embryo mortality.

D.mespiliformis correlate well with the present findings. The results gave evidence that a good number of the aqueous ethanolic and methanolic extracts of the plants employed in this study portrayed some level of antiviral activity. Preliminary results of this investigation appear to indicate that leaves of *D.mespiliformis* have high potential antiviral activity. Further investigations are necessary in order to draw solid conclusion. The bioactive compounds from the leaves need to be isolated and screened for their pharmaceutical and biotechnological applications in order to cure chronic and infectious diseases.

REFERENCES

1. Adzu, B., Amos, S., Dzarma, 1., Mauzzam, and Gamaniel, K. S. (2002). Pharmacological Evidence favouring the folkloric use of Diospyros mespiliformis Hochst in the relief of pain and fever. *Journal of Ethnopharma.* **82**: 191-195.
2. Agatemor, C. (2009). Antimicrobial activity of aqueous and ethanol extracts of nine Nigeria species against four food borne bacteria. *Eletronic Journal of Environmental, Agricultural and Food Chemistry.*8(**3**):195 – 200.
3. Alexander, D. J. (1997). Newcastle disease and other avian paramyxoviridae infection. In: Disease of poultry. B. W. Calnek H. J. Barnes., C. W. Beard., L. R. Mcdougald, and Y. M. Saif, ed. *Iowa State University Press, Ames*, I.A. Pp541 – 569.
4. Ayatoa, J.F., Tchendem, M.H.K. and Nyasse, B. (1994). Novel bioactive diterpenoids from *Aframomum aulacocarpos. Journal of Natural Products* **57**: 917-923.
5. Bernard, D.L., Huffman, H.J., Meyerson, L.R. and Sidwell, R.W. (1993). Mode of Inhibition of respiratory syncytial virus by plant flavonoid. Chemotherapy **39**: 21-217.

6. Caston, J.A. (2008), "Infections Bursal Disease virus (IBDV)." (HTTP: //WWW. horizon press. Com/Ma). Segmented Double Stranded RNA viruses: structure and Molecular Biology. Caster *Academic Press*. ISBN 978 – 1 – 90 4455 – 21 - 9
7. Coates Palgrave, M. (2002). Keith Coates Palgrave of Southern African, ed.3.Struik Cape Town.
8. Dalziel, J. M., (1995). The useful plants of West Tropical Africa. *Crown Agents,* London. Pp. 364.
9. Ekpa, O. D. (1996). Nutrient composition of three Nigeria medicinal plants.
10. Eloff, J.N. (1998). Which extractant should be used for the screening and isolation of antimicrobial components from plants? *Journal of Ethnopharmacology* **60** : 1-8. *Food Chemistry*. 57(2): 229 – 232
11. Gbile, G.O.and Adesina, S.K. (1986). Nigeria flora as pharmaceutical potentials. *Journal of Ethnopharmacology.* **19**: 1 – 16.
12. Ghoshal, S., Krishna , B.N. and Lakshmi, V. (1996). Anti amoebic activity of Piperlongum fruits against Entamoeba histolytica *in vitro* and *in vivo. Journal of Ethnopharmacology.* **50**: 167-170.
13. Gill, L.S. (1992). Ethnomedical uses of plants in Nigeria. Uniben press, University of Benin, Benin – city, Edo State, Nigeria. ISBN 978 – 2027 – 20 – 0 Pp. 102 – 103.
14. Grimes S. E (2002).A basic laboratory manual for the small-scale production and testing of 1-2 news cattle diseases – vaccine RAP publication Pp 1-20.
15. Hasegawa, H. Matsumiya, S., Uchiyama, M., Kurokawa, T., Inovye, Y., Kasai, R., Ishibashi, S. and Yamasaki, K. (1994). Inhibitory effect of some triterpenoid saponins on glucose transport in tumor cells and its application *to invitro* cytotoxic and antiviral activities. *Planta Medica*. **6**: 240 - 243
16. Kaul , T.N., Middle town, E.Jr. and Ogra, P.L. (1985). Antiviral effect of flavonoids on human viruses. Journal of Medical Virology. **15**: 71-79
17. Kerharo, J. and Adam, J.C.(1974). La pharmacopee senegalaise traditionnelle, vigot, Paris.
18. Kirmse, P:, (1969): Host specificity and pathogenicity of pox viruses from wild birds. *Bull wildlife Disease Association*. **5**: 376 – 389.
19. Krishnaiah, D., Devi, T., Bonno, A. and Sarbartly, R. (2009) Studies on phytochemical constituents of six Malaysian plants. *Journal of medicinal Plant Research*. **3**: 67 – 72.
20. McDevitt, J.T. Schneider, D.M., Katiyar, S.K. and Edlina , T.D. (1996). Berberine: a candidate for the treatment of diarrhea in AIDS patients Abstr, 175 In program and Abstracts of the 36th Interscience conference on Antimicrobial Agent and Chemotherapy. American Society for Microbiology. Washington D.C.

21. McMahon, J.B., Currens, M.J., Gulakowaki. R.J., Buckheit. R.W.J., Lackman-Smith, C., Hallock, Y.F. and Boyd, M.R. (1995). Mochellamine B, A Novel plant alkaloid, inhibits human immunodeficient virus–induced cell killing by at least two distinct mechanisms. *Antimicrobial Agents Chemotherapy*. **39**:484-488.
22. McMillan, J.K., Cochran, M.D. Junker, D.E., Redd, D.N., and Valencia, D.M., (1994). The safe and effective use of fowl pox virus as a vector for poultry vaccines. *Development Biological Standard.* **82**: 137 – 165.
23. Mendooza, L., Wikkens, M. and Urzua, A. (1997). Antimicrobial study of the resinous exudates and of diterpenoids and flavonoids isolated from some Chilean Pseudognaphalium(Asteraceae). *Journal of Ethnopharmacology.* **58**: 85-88.
24. Mikail, H.G. (2010). Phytochemical screening, elemental analysis and acute toxicity of aqueous extract of *Allium sativum* L. *bulbs* in experimental rabbits. *Journal of Medicinal Plant Research* **4**: 322 – 326.
25. Mohamed, I.E., EI Nur, E.E., and Abdelrahman, M.E., (2010). The antibacterial, antiviral activities and phytochemical screening of some Sudanese medicinal plants. *Eur Asia journal of Biosciences* 4, 2, 8, - 16.
26. Mtambo, M.M.A., Mushi, E.J., Kinabo, L.D.B., Maeda-Machangu, A., Mwamegele, G.L.M., Yongolo, M.G.S. and Temu.R.P.C.(1999). Evaluation of the efficiency of the crude extracts of *Capsicum frustescens, citrus limon* and *Opuntia vulgaris* against Newcastle disease in domestic fowl in Tanzania. *Journal of Ethnopharmacology*. **68**:55-61.
27. Nascimento – Gislen, G. F., Lukatelli, J. Frentas, P. C., and Silva, G. L. (2000). Antimicrobial activity of plant extracts and phytochemical on antibiotic resistant bacteria, *Brazillian Journal of Microbiology*, **31**: 247 – 256.
28. Nonaka, G.I., Nishioka, I., Nishizawa, M., Yamagishi, T., Kashiwada, Y., Dutschman, G.E., Bodner, A.j., Kilkushie, RE., Chang, Y.C. and Lee, K.H.(1990). Anti-AIDS agents, 2. Inhibitory effects of tannins on HIV reverse transcriptase and HIV replication in H9 Lymphocyte cells. *Journal National Products*. **53**:587-595.
29. Nwude, N., and Ibrahim, M. A., (1980). Plants used in traditional veterinary practice in Nigeria. *Journal of Veterinary Pharmacology*. Pp 261 – 273.
30. O.I.E., (1996). The Newcastle disease In: Manual of standards for diagnostic Test and vaccines. 161 – 169.
31. Okwu, D.E. (2004). Phytochemicals, Vitamins and mineral contents of two Nigerian Medicinal plants. *International Journal of Molecular Medicine and Advanced Sciences*. 1(**4**):375-381.

32. Okwu, D. E. (2004). Phytochemicals and vitamine content of indigenous spices of South Eastern Nigeria. *Journal of Sustaining Agricultural Environment*. **6**: 30 - 34
33. Omulokoli, E., Khan, B. and Chhabra, S.C. (1997). Antiplasmodial activity of four Kenyan Medicinal Plants. *Journal of Ethnopharmacology*. **56**: 133-137.
34. Pengsuparp, T., Cai, L., Constant , H., Fong, H.H., Lin, L.Z., Kingborn, A.D., Pezzuto, J.M., Cordell, G.A., Ingoifsdottir, K. and Wagner, H. (1995). Mechanistic evaluation of new plant–derived compounds that inhibit. HIV-I reverse transcriptase. *Journal of Natural Products*. **58**: 1024- 1031.
35. Reed, L.J.AND Muench, L.H (1938) a simple method of estimating fifty percent end points American journal of hygiene 27:493-497.
36. Ros, J. L., Recio, M. C., (2005). Medicinal plants and Antimicrobial Activity, *Journal of Ethnopharmacology*, **100**: 80 – 84.
37. Scalbert, A (1991) Antimicrobial properties of tannins. *Phytochemistry* **30**: 38753883.
38. Serafini, M., Ghiselli, A. and Ferro-Luzzi, a (1994) Red wine, tea and antioxidants. *Lance*t. 344:626.
39. Sofowora, E. A., (1982). Medicine plants and Traditinal Medicine in Afircan. Wiley, New York.
40. Tripathy, D. N., Reed, W. M. (2003). Pox, in Saif. Y., Barnes, H., Gilson, J. et al (Eds): Disease of poultry (ed. 11). Ames, IA, *Lowa State University Press*, Pp: 253 – 265.
41. Uruquiaga,I.and Leighton, F.(2000). Plant polyptenol antioxidants and oxidative stress. *Biological Research* **33**: 159 – 165.
42. Venter , F. and Venter , J.A. (1996) .Making the most of indigenous trees. Briza publications, Pretoria. 1(**1**): 24 – 30.
43. Vijayan, P., Raghu,C., Ashok, G., Dhanaraj, S.A.and Suresh, B.(2004). Antiviral activity of medicinal plants of Nilgiris. *India Journal of Medical Research*. **120**: 24 – 29.