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In vitro Antiviral Activities of *Aframomum melegueta* leaf Extracts on Newcastle Disease Virus (NDV), Fowl Pox Virus (FPV) and Infectious Bursal Disease Virus (IBDV)

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ABSTRACT

The *in vitro* antiviral activities of aqueous, ethanolic and methanolic extracts of *Aframomum melegueta* leave was evaluated against three viral families 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 to NDV is directly proportional to the concentration of both the aqueous, ethanol and methanol extracts of Aframomum melegueta leaves; 400mg/ml, 200mg/ml and 100mg/ml of the sample yielded 86.0% 50.0% and 50.0% for aqueous extract, 89% 86% and 50% for ethanol extract, while methanolic extract gave 95% 89% and 50% respectively. However, a moderate antiviral activity against FPV and complete egg mortality against IBDV was observed. The presence of Alkaloids, Tannins, Saponins, and Flavonoids was exhibited, while Cyanogenic glycosides and Triterpenes were absent. These findings suggest that *Aframomum melegueta* could be a potential candidate in the management of Newcastle and Fowl pox diseases affecting the poultry industry. The active component responsible for the antiviral activity needs evaluation.

Keywords: *In vitro,* antiviral, *Aframomum melegueta,* bursal disease virus.

INTRODUCTION

*Aframomum melegueta (*K. Schum.*)* is specie in the ginger family, *Zingiberaceae.* The specie, commonly known as grains of paradise, *melegueta pepper, alligator pepper, Guinea grains,* or *Guinea pepper,* is obtained from the ground seed; it gives a pungent, peppery flavour. The alligator pepper is extensively used as a common ingredient of many traditional medicines. The decoction of the leaves is used for small pox and chicken pox. As a purgative, galactogogue (to increase the production of breast milk), antihelmintic and haemostatic agent (purifies the blood) in medicinal applications. Also used as a vermifuge and stimulant. Further it is used against intestinal infections, infestations, to calm indigestion and heartburn. It also posses potent anti-inflammatory activity with favourable gastric tolerability profile [1]. Many natural plants have also 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 [2, 3, 4].

The antiviral activities of aqueous, ethanolic and methanolic extracts of *Aframomum melegueta* leave was determined on Newcastle Disease Virus (NDV), Fowl Pox Virus (FPV) and Infectious Bursal Disease Virus (IBDV). Infectious Bursal Disease (IBD) also known as Gumboro disease is a highly contagious disease of young chickens caused by infectious bursal disease virus (IBDV) [5], characterized by immunosuppression and mortality generally at 3 to 6 weeks of age. Fowl pox virus (F P V) is an infectious disease of worldwide distribution that affects commercial poultry e.g. chickens, turkeys, domestic pets and free living birds of many species [6]. Newcastle disease virus causes a highly contagious and fatal disease affecting all species of birds in poultry industry. The continuous spectrum of virulence displayed by NDV strains enabled the grouping of them into three different pathotypes; Lentogenic. mesogenic and velogenic [7]. The disease has a worldwide distribution and remains a constant major threat to commercial poultry production. As with other viral disease, there is no known specific treatment for ND [8]. Hence there is need to continue the search for antiviral agents with more satisfactory results.

MATERIALS AND METHODS

Aframomum melegueta 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 plant materials were 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 [9] 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(Grimes, 2002).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).

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 [10, 11].

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 [12]. 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 [13]. 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 [14] 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.

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^{0}C 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 control.

RESULTS

Table 1. Phytochemical Analysis of *Aframomum melegueta* (AM) leave 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: Toxicity of *Aframomum melegueta* (AM) 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	1	4	20.0
	400	5	0	5	0.0
	200	5	0	5	0.0
	100	5	0	5	0.0
Ethanol 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
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 3. Reduction of Newcastle Disease Viral Load by Extracts of *Aframomum melegueta* (AM) 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 Extract	400mg/ml	0.2ml(Virus+Extract)	5	3	40%	55.7	86.0
	200mg/ml	0.2ml(Virus+Extract)	5	4	20%	194.0	50.0
	100mg/ml	0.2ml(Virus+Extract)	5	0	100%	194.0	50.0
	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 Extract	400mg/ml	0.2ml(Virus+Extract)	5	4	20%	42.0	89.0
	200mg/ml	0.2ml(Virus+Extract)	5	3	40%	55.7	86.0
	100mg/ml	0.2ml(Virus+Extract)	5	1	80%	194.0	50.0
	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 Extract	400mg/ml	0.2ml(Virus+Extract)	5	5	0%	18.4	95.05
	200mg/ml	0.2ml(Virus+Extract)	5	1	20%	48.5	87.5
	100mg/ml	0.2ml(Virus+Extract)	5	3	60%	194.0	50.0
	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	==

Table 4. Reduction of Fowl Pox Virus (FPV) pocks extracts of *Aframomum melegueta* (AM) 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	0
Ethanol Extract	400mg/ml	present	++
	200mg/ml	Present	+
	100mg/ml	Present	0
Methanol Extract	400mg/ml	present	++
	200mg/ml	Present	+
	100mg/ml	Present	0
Positive control	Virus+PBS	Present	0
Negative control	PBS only	Absent	== ==

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

Table 5. Reduction of infectious Bursal Disease virus (IBDV) viral load by Extracts of *Aframomum melegueta* (AM) 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

Aframomum melegueta (alligator pepper) is extremely used as a common ingredient of many traditional medicines. It is extensively used as a purgative, antihelminthic, haemostatic agent and has a potent anti- inflammatory activity with favourable gastric tolerability profile [15, 16]. The results of the antiviral activities of plant extracts studied with their various degree of inhibitory properties invitro have been reported and the extract have shown some inhibitory activity against Newcastle disease virus. A hundred percent (100%) inactivation has been used to define an extract with antiviral activity [17]. 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. At the concentration of 400mg/ml, using aqueous, ethanolic and methanolic extract, the plant achieved viral inhibitions of 86%, 89% and 95.05% respectively against Newcastle disease virus, this is slightly higher to that of *Quisquali indica* extract which yielded 50%, 86% and 94.6% for aqueous, ethanolic and methanolic extracts respectively at the concentration of 400mg/ml as observed. 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 plant extracts studied exhibited various degrees of antiviral activity against NDV and FPV at various concentrations.

The Phytochemical analysis of the plant materials unravelled a number of chemical substances with antiviral activity. Flavonones exhibit inhibitory effects against viruses [18] including HIV and respiratory syncytial virus [19]. Terpenoids were reported to be active against bacteria, fungi, viruses and protozoa [20, 21, 22, 23, 24]. It is believed to be active against viruses by envelope disruption by the lipophilic compounds. Alkaloids have been commonly found to have antimicrobial properties [25]. It is also useful against HIV infection as well as intestinal infections associated with AIDS [26]. Tannins are found in almost every plant part; wood, leaves, bark, roots and fruits [27] and tannin containing beverages can cure or prevent a variety of viral infections [28]. At least two studies [29, 30] have shown tannins to be inhibitory to viral reverse transcriptases. Tannins (hydrolysable) also show anti-carcinogenic and anti-mutagenic effects [31]. These phytochemical were significantly demonstrated in methanolic extracts of Aframomum melegueta.

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 [32] 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 [33] 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 various traditional uses of the plants tested in this study correlate well with the present findings. The results gave evidence that a good number of the aqueous ethanolic and methanolic extracts of the plant employed in this study portrayed some level of antiviral activity. Nevertheless, less than 100% activity was achieved and might be taken to be that the antiviral compounds present were in amounts insufficient to inactivate all infectious virus particles. 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. The development of more potent antiviral agents for human race may be enhanced, perhaps, by harvesting these plant constituents and harnessing their potencies.

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