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Determination of Antiviral Activities of *Quisqualis indica* leaf Extracts on Three Selected Avian Viruses

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ABSTRACT

The determination of antiviral activities of *Quisqualis indica* leaf extracts on three selected avian viruses was evaluated. 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 repectively. 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 FPV and IBDV. At a concentrations of 400mg/ml, 200mg/ml and 100mg/ml *Quisqualis indica* yielded a percentage inhibition of 50.0%, 50.0% and 43.0% for aqueous extract; 86.0%, 50.0% and 50.0% for ethanol extract; and 94.6%, 90.5% and 42.6% for methanol extraction respectively on NDV. The challenged virus FPV recorded no activity with Quisquali indica and A one hundred percentage (100%) egg mortality was observed at the end of the experiment with infectious bursal disease virus (IBDV).The phytochemical analysis of the crude extracts reveals the presence of Alkaloids, Tannins, Saponins, and Flavonoids, while Cyanogenic glycosides and Triterpenes were absent. These results suggest that Quisquali indica could be use for the management of Newcastle and Fowl pox diseases affecting the poultry industry. However, further research is needed to determine the active components responsible for the antiviral activity.

Keywords: *Quisqualis indica*, antiviral, chorioallantoic membrane, avian viruses.

INTRODUCTION

Large number of plants have been sourced and used in Africa as valuable medical agent for the treatment of various kinds of diseases and ailments with lesser side effects compared to synthetic agents [1, 2]. Plants have long been used as remedies and many are now being collected and examined in an attempt to identify possible sources of antiviral [3, 4, 5, 6]. Studies conducted in laboratories around the world revealed that traditional medicinal plants can provide a rich source of antiviral activities. There are reports on the use of ethno- veterinary herbal practices in the management of diseases of chickens caused by infectious viral pathogens [7, 8, 9]. *Quisqualis indica* A. L., also known as the Rangoon creeper belongs to the family *Combretaceae.* The plant is mainly used for traditional medicine. Decoction of the root, seed or fruit can be used as antihelminthic to expel parasitic worms or for alleviating diarrhea. The fruits and leaves can be used to combat nephritis and to relieve pain caused by fever [10]. Studying medicinal plants with ethnobotanical importance and folklore reputation has become the more important need in recent times in order to promote the use of herbal medicines and to determine their potential as source of new drugs [11]. Plant materials are known as source of new antimicrobial agents as a result search has been to discover new antiviral drugs of plant origin. A number of compounds like quinine, Garlic acid, morphine, codeine, vitexin and lupeol, etc, have been derived from plants which are having enormous therapeutic potentials [12]. There are fewer substances available for the treatment of viral infection when compared with the large amount of available antibiotics for the treatment of bacteria infections [13].

This study investigated the antiviral effects of the crude extracts of *Quisqualis indica* , invitro, and the phytochemical constituents of it leaves extract against three animal viruses representing different viral families using 9 - 11 day – old embryonated chicken eggs . These diseases have worldwide distribution and remains a constant major threat to commercial poultry production. Hence there is need to continue the search for antiviral agents with more satisfactory results. The plant extracts were screened for their activities against representatives of three viral families namely, Newcastle disease virus (NDV), Fowl pox virus (FPV) and Infectious bursal disease virus (IBDV). Infectious bursal disease (IBD) is a highly contagious disease of young chickens caused by infectious bursal disease virus (IBDV) (Caston et al, 2008) also known as Gumboro disease, characterized by immunosuppression and mortality generally at 3 to 6 weeks of age. 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. 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 [14].

Materials and methods

The Collection of *Quisqualis indica* leaves was done from it natural habitats in Dekina, Dekina Local Government Area of Kogi State and Eziani village, Ichi in Ekwusigo Local Government Area of Anambra State respectively. Collection for extraction was done during rainy season

(May 2010 and July 2010). The indigenous plant 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 [15] 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 10 weeks-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 [16, 17].

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 [18] 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 (OIE), 1996. 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 [19] 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 (10 log2). 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 38^0C 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 control.

Results

Table 1. Phytochemical Analysis of *Qusquali Indica* 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 *Qusquali Indica* 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	2	3	40.0
	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 *Qusquali Indica* 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	0	100%	194.0	50.0
Extract	200mg/ml	0.2ml(Virus+Extract)	5	0	100%	194.0	50.0
	100mg/ml	0.2ml(Virus+Extract)	5	0	100%	222.0	43.0
	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	2	60%	55.7	86.0
Extract	200mg/ml	0.2ml(Virus+Extract)	5	1	80%	194.0	50.0
	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	400mg/ml	0.2ml(Virus+Extract)	5	5	0%	21.1	94.6
Extract	200mg/ml	0.2ml(Virus+Extract)	5	1	20%	36.8	90.5
	100mg/ml	0.2ml(Virus+Extract)	5	4	80%	222.9	42.6
	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 *Quisquali indica* leaves in Embryonated chicken Eggs.

Extract	Test Extract concentration	FPV pock lesion formation	FPV 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,++=moderate inhibition, += mild inhibition, 0 = no inhibition.

Table 5: Reduction of infectious Bursal Disease virus (IBDV) viral load by Extracts of *Quisquali indica* 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

Quisquali indica leaves are reputed for use as an antihelminths to expel parasitic worms and for alleviating diarrhea, combating nephritis and to relieve pains caused by fever [20]. The determination of the antiviral activities of *Quisquali indica* leaves extract studied with their various degree of inhibitory properties invitro have been reported. At the concentration of 400mg/ml, using aqueous, ethanolic and methanolic extract, the plant achieved viral inhibitions of 50%, 86% and 94.6% respectively which is lower than virus inhibition achieved by Afromomum melegueta extracts with 86%, 89% and 95.05% respectively aqueous, ethanolic and methanolic extracts at the concentration of 400mg/ml aganist Newcastle disease virus. A hundred percent (100%) inactivation has been used to define an extract with antiviral activity [21]. However, no 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. The Phytochemical analysis 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 [22, 23]. Flavonones exhibit inhibitory effects against viruses [24] including HIV and respiratory syncytial virus [25,26]. Terpenoids were reported to be active against bacteria,

fungi, viruses and protozoa [27, 28, 29, 30]. It is believed to be active against viruses by envelope disruption by the lipophilic compounds. Alkaloids have been commonly found to have antimicrobial properties [31, 32, 33]. It is also useful against HIV infection as well as intestinal infections associated with AIDS [34, 35, 36]. Tannins are found in almost every plant part; wood, leaves, bark, roots and fruits and tannin containing beverages can cure or prevent a variety of viral infections [36]. At least two studies [37, 38] have shown tannins to be inhibitory to viral reverse transcriptases. Tannins (hydrolysable) also show anti-carcinogenic and anti-mutagenic effects [39].

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. 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 [40] 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 [41, 42, 43] 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 extract of Quisquali indica exhibited no activity at various concentrations (400mg/ml, 200mg/ml and 100mg/ml) when tested with FPV and IBDV. 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 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. Nevertheless, less than 100% activity was achievedand 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.

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