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Phytochemical Analysis and Antiviral Potential of Aqueous Leaf Extract of *Psidium guajava* Against Newcastle Disease Virus *in ovo*

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ABSTRACT

Leaf extract of *Psidium guajava* were subjected to phytochemical screening and *in ovo* antiviral assay against Newcastle Disease Virus (NDV). Phytochemical analysis revealed the presence of pharmacologically active and nutritionally relevant compounds. Nine-day-old embryonated chicken eggs were divided into ten groups of fives and received various treatments. Groups 1-6 received $100EID_{50}$/0.1ml NDV pre-treated with *P. guajava* leaf extracts at final concentrations of 250, 200, 100, 50, 25 and 10mg/ml in that order. Controls were included. Embryo survival was observed daily. Allantoic fluid from treated eggs and serum from hatched chicks were collected for spot hemagglutination (HA) and hemagglutination inhibition (HI) tests to detect NDV in the eggs and antibodies against NDV in the hatched chicks respectively. Results showed that embryo survival was higher with higher extract concentrations.. Just as increase in extract concentration was directly proportional to virus death and inversely proportional to production of antibody against NDV in hatched chicks. The current findings have clearly demonstrated that *P. guajava* leaf extract has nutritional value as well as great antiviral potential against NDV *in ovo*. *In vivo* trials are needed to validate the use of the tree in controlling Newcastle disease in chickens.

INTRODUCTION

The apple guava (*Psidium guajava*) or common guava has nearly a global presence**.** It is an evergreen shrub or small tree native to Mexico, the Caribbean, and Central and South America (Psidium guajava, 1995). It is a common shade tree or shrub in door-yard gardens in the tropics. The tree is easily identified by its distinctive thin, smooth, copper-colored bark that flakes off, showing a greenish layer beneath. Lozoya et al. (2002) reported that the phytochemical analyses of guava leaf, revealed the presence of more than 20 isolated compounds with quercetin as the main active substance. Spasmolytic and antidiarrheal effects are reported to be associated with its quercetin-derived, flavonoids and glycosides, which support use of this ancient leaf remedy in treating gastrointestinal disorders (Joseph and Priya, 2011).plants remained the primary 2011). The plant is used in many different shampoo products for its scent. It is also becoming a popular Bonsai Species and is currently quite popular in India and Eastern Asia (Mark, 2011). Local preparations made from the leaves and/or bark of *Psidium guajava* are reported to be useful in treatments of diarrhea, dysentery, sore throats, vomiting, stomach upsets and vertigo. They have also been found to be effective in regulating menstrual periods throughout the tropical Amazon and India (Holetz et al., 2002). Also, tender leaves of the plants are also reported to be chewed for bleeding gums and bad breath, and it is said to prevent hangovers when they are chewed before alcohol consumption. According to the report, Indians throughout the Amazon gargle a leaf decoction for mouth sores, bleeding gums, or

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use it as a douche for vaginal discharge and to tighten and tone vaginal walls after childbirth (Holetz et al., 2002; Nwogu et al., 2007) Very recently, some plants were screened for antiviral potential against NDV. Bakari et al., (2012) examined crude extracts from *Commiphora swynnertonii* against the virus, Suleiman et al., (2011) also demonstrated that root bark of baobab (*Adansonia digitata* Linn) can inhibit NDV *in ovo.* Newcastle disease has remained unabated the world over in spite of the various intervention programs including vaccination. It is a highly infectious disease of domestic poultry and wild birds. It is caused by a virus and is widely regarded as one of the most important avian diseases. Although most avian species are susceptible to infection with the virus that causes Newcastle disease, chickens are the most susceptible to clinical disease (Young et al., 2002). Newcastle disease was first recognized in Indonesia and England in 1926 (Doyle, 1927) and Newcastle disease viruses are now found worldwide (Aldous and Alexander, 2001). NDV is classified by *Office international des Epizooties* (OIE) as a list 'A' disease: it spreads rapidly, extends beyond national borders, and has serious socioeconomic consequences and major trade implications. It is of major importance in both commercial and village chicken flocks where it may cause outbreaks with up to 100% mortality (Young et al., 2002).

MATERIALS AND METHODS

Collection and Identification of the Plant Material

Fresh leaves of *P. guajava* were randomly collected from different areas in Tapo, Barkin- Ladi Local Government Area of Plateau State, Nigeria. The plant was first identified at the field using standard keys and descriptions (Dalziel, 1956; Keay, 1989). It's botanical identity was further confirmed and authenticated at the Federal College of Forestry in Jos.

Preparation of Plant Extract

The leaves of *P. guajava* were sorted to eliminate any dead matter and other unwanted particles after which they were air-dried for 2 weeks and pulverized before the commencement of the extraction. The extraction was carried out as described by Njar et al. (1993) and Raji (1995). The pulverized leaves weighing 245g was exhaustively extracted with distilled water by means of cold extraction and extract evaporated *in vacuo*. The leaf extract was then concentrated *in vacuo* using a rotary evaporator at 40^{o}C. The solvent remaining in the extract was finally removed by placing the extract in porcelain dishes in temperature-controlled oven to give a residue weighing 9.55g. The resulting extract was reconstituted in 9.55ml of sterile distilled water to give a final concentration of 1000mg/ml.

Phytochemical Screening

Phytochemical screening was carried out as earlier described by Sofowora(1993) as documented earlier by Dalen et al., (2009).

ANTIVIRAL ASSAY

Source of Virus and 9-Day Old Embryonated Chicken Eggs

A velogenic strain of NDV was obtained from Viral Research Department while embryonated chickens eggs were obtained from Poultry Division both of National Veterinary Research Institute, Vom, Nigeria.

Determination of EID_{50} of the virus

The EID_{50} of the virus was determined as recorded by Young et al., (2002). From this, $100EID_{50}$/ 0.1ml of the virus stock was made for the experiment.

Preparation of Inoculum (Virus/Extract mixture)

A 1:2 v/v dilution of the $100EID_{50}$/0.1ml of virus with predetermined extract concentrations was made to put extract final concentration in the virus/extract mixture at 250mg/ml, 200mg/ml, 100mg/ml, 50mg/ml, 25mg/ml and 10mg/ml. The virus/extract mixtures were kept at 4^{o}C for 1hr to react.

Inoculation of Eggs

Nine-day-old embryonated chicken eggs were divided into ten 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 ®, the eggs were swabbed with 70% alcohol in cotton wool and transferred into the cleaned trays. The swabbed eggs were placed in the micro-safety cabinet where they were punched and immediately inoculated with the extract/virus mixture via the allantoic route. Groups 1-6 were inoculated with 0.2ml of virus/extract mixtures at final concentrations of 250mg/ml, 200mg/ml, 100mg/ml, 50mg/ml, 25mg/ml and 10mg/ml in that order. Group 7 was inoculated with 0.2ml $100EID_{50}$/0.1ml standard NDV (virus control), group 8 was inoculated with 0.2ml extract suspension at 250mg/ml concentration (extract control). Group 9 was inoculated with 0.2ml phosphate buffered saline (diluent control) while group 10 had eggs that were not inoculated with anything (uninoculated control). The eggs were sealed with molten wax and incubated at 37°C. Embryo survival was observed daily while a few eggs from selected groups were left to hatch. Allantoic fluid from treated eggs were collected for spot test and haemagglutination test to detect NDV in the eggs while serum from hatched chicks were collected for hemagglutination inhibition (HI) tests to detect antibodies against NDV in the hatched chicks.

Spot haemagglutination test

Dead embryos that had been chilled were brought out of the refrigerator and 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 1% 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 which was mixed with the drop of blood.

The tile was gently rocked and observed for visible agglutination, indicating viral activity (Thayer and Beard, 1998; Murakawa et al., 2003). This was done for every egg and the observations were recorded.

Haemagglutination Inhibition Test

This was conducted on serum of hatched chicks to ascertain the titre of antibody against NDV. The protocol of Young et al., (2002) was adopted.

RESULTS

Phytochemical analysis of the extract revealed the presence of Pharmacologically active substances (Table I). Elemental analysis on the other hand showed the presence of important mineral salts (Table II). *P. guajava* leaf extract at 250mg/ml and 200mg/ml concentrations completely inhibited virus growth in embryonated eggs as revealed by the survival of embryos of the inoculated eggs. Also, Spot HA test of allantoic fluids of eggs inoculated with extracts at these concentrations did not show any agglutination. Furthermore, chicks that hatched out from some eggs at these concentrations were apparently healthy and had no antibodies against the virus. The negative results of spot heamagglutination test in these concentrations buttress this point (Table III). Embryos of inoculated eggs with extract at concentration of 10mg, 25mg 50 and 100mg partially inhibited virus growth as various degrees of mortality in the embryos of the eggs were observed. The control groups were also perfect in their outcomes. The extract and diluent controls were safe for the experiment in the eggs as revealed by embryo survival while the virus control was potent enough as it killed all the embryos within 48hrs post inoculation. Uninoculated controls on the other hand had live embryos throughout the duration of the experiment.

The outcome of HI test on hatched chicks showed increasing NDV antibody titres with decreasing extract concentrations (Table III).

Table. 1: Result of Phytochemical screening of *P. guajava* leaf extract.

Compound	Present
Alkaloids	++
Tannins	+
Flavonoids	++
Saponin	++
Glycosides	+
Reducing compounds	+

KEY
++ = Highly present, + = Present

Table. 2: Result of Elemental studies of *Psidium guajava* leaf extract.

Elements	Concentration (ppm)
Calcium	10.00 ± 0.011
Zinc	0.70 ± 0.021
Iron	0.45 ± 0.011
Manganese	45.10 ± 0.022
Sodium	130.30 ±0.010
Potassium	200.40 ±0.013
Phosphorus	2.20 ± 0.012

DISCUSSION

P. *guajava* tree has a long history of medicinal uses that are still employed today (Nwinyi *et al.*, 2008). The reason behind this is not far – fetched as revealed by phytochemical analyses of the leaf in this study. The presence of pharmacologically useful substances such as alkaloids, tannins, flavonoids, saponins among others in the leaves confirm the diverse claims and application of parts of the plant in local treatment of ailments. Previously, it has been documented that guava contains broad spectrum of phytochemicals including polysaccharides, vitamins, essential oils (Smith and Siwatibau,

Table. 3: Results of Antiviral Activity of *Psidium guajava* leaf extract against NDV.

Extract(mg/ml)	Mortality (pi)							HA Test	
	No Eggs	24hr	48hr	72hr	96hr	+ve	-ve	% Mortality Due to Virus	Mean Ab Titre
250	5	0/5	0/5	0/5	0/5	0	5	0	≤1/2
200	5	0/5	0/5	0/5	0/5	0	5	0	≤1/2
100	5	0/5	0/5	1/5	¼	2	3	40	1/8
50	5	0/5	1/5	2/4	½	4	1	80	1/32
25	5	0/5	2/5	3/3	-	5	0	100	1/128
10	5	0/5	5/5	-	-	5	0	100	1/512
Vc	5	0/5	5/5	-	-	5	0	100	ND
Ec	5	0/5	0/5	0/5	0/5	0	5	0	ND
Dc	5	0/5	0/5	0/5	0/5	0	5	0	ND
Uc	5	0/5	0/5	0/5	0/5	0	5	0	ND

KEY
Vc: Virus Control
Ec: Extract Control
Dc: Diluent Control
Uc: Uninoculated Control
ND: Not Done
Pi: Post Inoculation
Ab: Antibody

1975, Macleod and Troconis, 1975), minerals, enzymes, proteins (Deo and Shastri, 2003), sesquiterpenoid alcohols and triterpenoid acids (Smith *et al*, 1975; Wilson111 and Shaw, 1978; Begum *et al.*, 2002), alkaloids, glycosides, steroids, flavanoids, tannins, saponins (Cho *et al.*, 2003; Narayana *et al.*, 2001; Geidam *et al.*, 2007).

Also, the presence of key elements in *P. guajava* leaves as revealed by elemental analysis is no doubt responsible for the high nutritional value of the fruit. Hassimotto *et al. (*2005) reported that guavas are often included among superfruits, being rich in dietary fiber, vitamins A and C, folic acid, and the dietary minerals, potassium, copper and manganese. Having a generally broad, low-calorie profile of essential nutrients, a single common guava (*P. guajava*) fruit contains about four times the amount of vitamin C as an orange (Joseph and Priya, 2011).

Results of antiviral assay of *P.guajava* from this study confirm that the leaf had antiviral potential against NDV. This was revealed by the complete inhibition of virus growth *in ovo* at 200mg/ml and 250mg/ml. At these concentrations, all the inoculated eggs had live embryos just as sera of hatched chicks had no antibodies against NDV implying that the plat extract at these concentrations inhibited the growth of the virus.

Also, HI test results showed increasing NDV antibody titre with decreasing extract concentration. This implies that the antiviral potential of the plant was concentration based. It is therefore safe to conclude that the higher the extract concentration, the more the antiviral activity.

This is not strange considering the phytochemical composition of the leaf and previous citations of the antiviral potential of the leaf extract against some pathogenic microorganisms.

In several studies, guava showed significant antibacterial activity against common diarrhea-causing bacteria such as *Staphylococcus*, *Shigella*, *Salmonella*, *Bacillus*, *E. coli*, *Clostridium* and *Pseudomonas*. Lozoya et al. (2002) reported that a double-blind clinical study of the effects of a Phytodrug (QG- 5) developed from guava leaf showed a decrease in duration of abdominal pain, which is attributed to antispasmodic effect of quercetin present in leaf extract (Joseph and Priya, 2011).

The microbicidal activity of *Psidium guajava* is attributable to guajaverine and to psydiolic acid (Berdy *et al.*, 1981). The leaves of guava have been found to contain large amounts of tannin, triterpernoids (crategolics, guaijavolic, oleanolics and ursolic acids) and essential oils containings sitosterol, s-bisabolene, scariophyllene, aromadendrene, s-salinene, guaijaverine, nerolidiol and sel-11-en-4_-ol (Morton,1981).

Also, investigation on the antiviral potential of the plant has previously been documented: Direkbusarakom et al. (1997) tested *Psidium guajava* extract for antiviral activity against the fish pathogenic viruses, infectious, haematopoietic necrosis virus (IHNV) infectious pancreatic necrosis virus (IPNV) and *Oncorhynchus masou* virus (OMV) using plaque reduction in CHSE-214 cell lines. Antiviral tests against the shrimp pathogenic virus, yellow-head virus (YHV), they tested the efficacy of guava extract using MIC of the extract against 24 strains of pathogenic bacteria including *Vibrio harveyi* (9 strains), *V. splendidus (*7 strains), *V. parahaemolyticus* (2 strains) and 1 strain of each *V. mimicus*, *V. vulnificus*, *V. fluvialis*, *V. cholerae*, *V. alginolitycus* and *Aeromonas hydrophila*. They found that the extract of guava demonstrated antiviral activity against IHNV, OMV and YHV but was not effective for IPNV (Joseph and Priya, 2011).

These findings are scientific and relevant judging from the performance of the control groups. The virus control was potent enough to cause embryo death within 48 hrs post inoculation just as extract, diluents and uninoculated controls did not interfere with embryo survival signifying the acceptability of the outcome of the tests groups.

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