

# In-vitro Antiviral Activity of *Gossypium hirsutum* on Newcastle Disease Virus.

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## ABSTRACT

The methanolic leaf extract of a medicinal plant, *Gossypium hirsutum* was analysed phytochemically. Toxicity was assayed and estimated as percentage egg mortality. In-vitro antiviral assay of the extract was also determined with  $10^{3.60}$  EID<sub>50</sub>/ml of Newcastle disease virus using three antiviral assay regimen: including pre-infection, at-infection and post infection assays. The phytochemical test revealed the presence of proactive constituents such as Flavonoids, Alkaloids, Tanins, Terpenoid, Steroides, Saponins and Phenols. However, all three antiviral assay regimen applied revealed the virus inhibition by the extract at levels between 20.3 and 80.3 percent. This study indicates that *Gossypium hirsutum* aqueous extracts have some inhibitory effect on the replication of Newcastle Disease virus in embryonated eggs.

**Keywords:** *Gossypium hirsutum*, phytochemicals, toxicity assay, in-vitro antiviral assay, regimen, Newcastle disease viral, mortality.

## 1. Introduction

Many infectious diseases are known to be treated with herbal remedies throughout the history of mankind. Indeed, the history of ancient medicine has been identified to be mainly dependent on plants and their extracts. Even today, plant materials continue to play a major role in primary health care as therapeutic remedies in many developing countries. (Jonathan and Fasidi, 2003; Jonathan and Ishola, 2005; Jonathan *et al.*, 2007). Plants are able to produce compounds which, though have no apparent function in the primary metabolism of the plant, have activity against bacterial, fungal and some viral pathogens (Udobi and Onaolapo 2009).

The demand on plant-based therapeutics is increasing in both developing and developed countries due to growing recognition that they are natural products, non-narcotic, easily biodegradable, pose minimum environmental hazards, have no adverse side-effects and are easily available at affordable prices (Kannan *et al.*, 2009). Over the last 20 years, a large number of secondary metabolites from different plant species have been evaluated for their antimicrobial activity and researches are still in progress on most African medicinal plants to evaluate their antiviral, antibacterial and pharmacological effects (Kannan *et al.*, 2009).

Currently, the global market for medicinal plants has been estimated to be around US \$62 billion and the demand is growing rapidly (Indian Council of Medical Research, 2003). The World Health Organization (2000) has estimated that 80% of the inhabitants of the world rely mainly on traditional medicines for their primary health care needs, and it may be presumed that a major part of traditional healing involves the use of plant extracts or their active principles. Infectious diseases account for approximately one-half of all deaths in tropical countries (Iwu, *et al.*, 1999). Medicinal plants have been traditionally used for different kinds of ailments including infectious diseases. Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found in vitro to have antimicrobial properties. Laboratories all over the world have found literally thousands of phytochemicals which have inhibitory effects on all types of microorganisms in vitro (Cown, 1999).

*Gossypium hirsutum* is a plant under the family Malvaceae. It is cultivated primarily for its vegetable, seed, and fibre. It is a raw material for a large volume of textile product, and this species is considered the most important of cotton yielding plants, providing the bulk of the commercial cottons. (Taiye *et al.*, 2011) Several species of Malvaceae family have been used in traditional medicine (Taiye *et al.*, 2011), for several infectious diseases however, their active principle(s) has not been elucidated. It is possible that compounds other than gossypol could be responsible for the anti-parasitic activity (Sotelo *et al.*, 2005). The pharmacological characteristics of Gossypol, a compound initially isolated from *Gossypium hirsutum*. (Malvaceae) seeds have been studied mainly in relation to its reversible antifertility effects in men (Sotelo *et al.*, 2005), effect on diverse pathogenic agents, such *Trypanosoma cruzi* (Abe *et al.*, 2004), *Plasmodium telpemerum* (Tripathi *et al.*, 2004), *Edwardsiella icaluri* (Yildirim-Aksoy *et al.*, 2004). Gossypol also inhibits the growth of numerous parasitic

organisms and shows antiviral activity against a number of enveloped viruses, including the AIDS virus (Vander Jagt *et al.*, 2000). The study is aimed at determining the inhibitory effect of *Gossypium hirsutum* extracts on Newcastle disease virus.

## 2. Materials and Methods

### 2.1 Experimental Plants

The leaves of *Gossypium hirsutum* were collected from bush in Awukuzu, Oyi Local Government Area of Anambra State. The plant, was identified by Professor C.U Okeke a plant taxonomist at the Department of Botany, Nnamdi Azikiwe University, Akwa.

### 2.2 Pre-Extraction Preparation of Plants

The plant materials were rinsed in sterile distilled water, air dried at room temperature for 7-20 days and, pulverized using pestle and mortar, ground into fine texture in electric grinder and stored at room temperature in air-tight container until further processing.

### 2.3 Preliminary Phytochemical Analysis of Plant Materials

Preliminary qualitative phytochemical tests were performed on the ground plant sample using the method of Harbone(1998).

### 2.4 Virus

Newcastle virus Kudu strain was obtained from Dr.Ponman of the National Veterinary Research institute, Vom, Plateau State, Nigeria. It is a velogenic, strain and of titre  $10^{8.2}$  MLD<sub>50</sub>/ml.

### 2.5 Embryonated Chicken Eggs

Nine to eleven day old pre-incubated embryonated chicken eggs were obtained from Guffons Veterinary Centre Hatchery, Owerri, Imo State and used immediately on arrival in the laboratory for the study.

### 2.6 Virus Propagation and Quantification

Virus stock was passaged four times in 9-11 day old embryonated chicken eggs by inoculation of 0.2ml fraction into the allantoic sac. Inoculated eggs were incubated for four days at 37°C in an egg incubator, chilled in the refrigerator overnight and the allantoic fluid was harvested as virus. Virus in allantoic fluid was quantified by cultivation of tenfold serially diluted stock in embryonated chicken eggs and virus titre estimated as egg infectious dose fifty per millilitre of allantoic fluid by the end point assay of Reed and Muench (1938).

### 2.7 Extraction of Plant Material

#### 2.7.1 Methanol Extraction

The pulverized material was wrapped in a porous thimble and placed in the extraction chamber of the soxhlet extractor. Extraction was performed using 250ml methanol (60/80°C boiling point) for 4 – 5 hours until the solvent dripping from the extraction chamber became clear.

Methanol extracts was concentrated by drying in an evaporating dish at 50°C under the fan. Concentrated extract was weighed and stored in the refrigerator until used for antiviral study.

### 2.8 Assay of Toxicity of Extract

Graded concentration of plant extract in sterile phosphate buffered saline were inoculated into 9 – 12 day old embryonated chicken eggs by the allantoic route . Inoculated were incubated for five days at 37°C in an egg incubator and monitored for egg mortality. Toxicity of extract was estimated as percentage egg mortality.

### 2.9 In-Vitro Antiviral Assay of Extracts

#### 2.9.1 Reconstitution of Concentrated Extracts

Extract previously concentrated and stored was reconstituted in sterile phosphate buffered saline (PBS) (pH 7.2) at concentration of 200; 20; 2.0 milligrams per millilitre.

### 2.9.2 Purification of Extracts

Prior to egg inoculation, reconstituted extracts were purified by filtration through milipore filters 0.45 $\mu$ m pore size to prevent contamination.

### 2.9.3 Experimental Design

Quantified virus ( $10^{3.60}$  EID<sub>50</sub>/ML) and the various concentration of reconstituted extract were inoculated (0.1ml extract and 0.1ml virus) into 9 days old embryonated chicken eggs by the allantoic route. Three inoculation regimen were used namely pre-infection assay (extract inoculated one hour before virus), at infection assay (virus inoculated followed immediately by extract), post infection assay (extract inoculated one hour after virus). Five eggs were used per inoculation regimen. Inoculated eggs (including tests and controls) were incubated for four days at 37°C in an egg incubator, chilled overnight at 4°C and harvested allantoic fluids assayed by virus quantification using the haemagglutination test (Beard 1980). Three controls involving five eggs each were also included. These were virus control (eggs inoculated with 0.1ml of virus and 0.1ml PBS only), extract control (eggs inoculated with 0.2ml of extract only) and diluent control (egg inoculated with 0.2ml of PBS only). Virus haemagglutination titre in tests and controls were compared statistically.

## 3. RESULTS

### 3.1 Phytochemical Analysis

Phytochemical analysis of methanol extract showed the presence of Flavonoids, Alkaloids, Tannins, Terpenoids, Steroids, Saponins and Phenols. Cyanogenic glycosides, was absent. (Table 1)

### 3.2 Toxicity Assay of Extract in Embryonated Eggs

The methanol extract of *Gossypium hirsutum* caused 100 percent lethality of embryonated chicken eggs when inoculated at concentrations of 75-200mg/ml (15-40mg/egg). It decreased from 20% at concentration of 50mg/ml (10mg/egg) to zero at 20mg/ml (4mg/egg) (Table 2).

### 3.3 Inhibition of Newcastle Disease Virus Replication

When extract was inoculated one hour before the virus (Pre-infection assay), concentration of 20mg/ml (2mg/egg) decreased virus geometric mean haemagglutination titre (GMHT) in allantoic fluid from 1261 to 304 giving a percentage inhibition of 75.9 whereas at the concentration of 10mg/ml (1mg/egg) virus GMHT was reduced to 249 with a percentage inhibition of 80.3.

When both extract and virus was inoculated at same time (at infection assay), 20mg/ml concentration (2mg/egg) yielded 74.6% inhibition of virus whereas at a lower concentration of 10mg/ml (1mg/egg) virus titre was rather increased by 28.3% (Table 3).

In the post-infection assay (extract inoculated one hour after virus inoculation) percentage inhibition of virus was 20.5 and 37.6 when concentrations of 20mg/ml and 10mg/ml were inoculated respectively (Table 3).

**Table 1: Phytochemical Analysis of *Gossypium hirsutum* leaves**

<u>Phytochemicals</u>	<u>presence (+)/ Absence (-)</u>
Flavonoids	+
Alkaloids	+
Tannins	+
Terpenoids	+
Cyanogenic glycosides	-
Steroids	+
Saponins	+
Phenols	+

**Table 2: Egg lethality Assay of Methanol Extract of *Gossypium hirsutum* leaves**

Eggs	Concentration of Methanol Extract (mg/ml)					
	200	150	100	75	50	20
Inoculum/egg (mg)	40	30	20	15	10	4
Number inoculated	5	5	5	5	5	5
Number dead	5	5	5	5	1	0
Percent lethality	100	100	100	100	200	

**Table 3: Inhibition of Newcastle Disease Virus replication in embryonated chicken eggs by concentration of methanolic extract of *Gossypium hirsutum***

Egg inoculation regimen	Extract concentration (mg/ml)	Inoculums Per egg (mg)	Geometric mean of Allantoic virus HA titre			Percent inhibition
			virus only	Virus+ Extract	Extract only	
Extract hour	20	2	1261	304	0	75.9
Pre-infection	10	1	1261	249	0	80.3
Extract at infection	20	2	1261	320	0	74.6
	10	1	1261	1618	0	-28.3
Extract 1 hour post-Infection	20	2	1261	1003	0	20.5
	10	1	1261	787	0	37.6

#### 4.

#### Discussion

The phytochemical analysis of *Gossypium hirsutum* reveals the presence of bioactive compounds such as Flavonoids, Alkaloids, Tanins and Terpenoids, Steroids, Saponins, and Phenols. This is in line with previous finding that crude extracts of some plants have anti-microbial activity, in-vitro (Udobi and Onaolapo, 2009).

All the three antiviral assay regimen applied in the study resulted in virus inhibition by *Gossypium hirsutum* extract at levels between 20.3 and 80.3 percent. The virus inhibition at a level of -28.3% at 10mg/ml extract concentration showed that the extract has a negative effect on virus or probably the concentration of the extract was not sufficient to inhibit the virus, when introduced at the point of infection. Extracts of *Gossypium hirsutum* are widely used in ethno-medicine even in the treatment of viral diseases including HIV and Herpes viruses (Brown 2001). There was evidence of reduction of viral load, increase in body weight and CD4 lymphocyte count when extracts were used in AIDS patient volunteers in Anambra State before the advent of antiretroviral therapy (Chukwuezi, 2007). Fasola; et al (2011) also reported significant antiviral activity of aqueous extracts against Yellow fever virus in Vero cells. Gossypol, a polyphenolic compound found in *Gossypium* species has anti-fertility, anticancer, antioxidant, anti-trypanosomal, antiviral and antimicrobial activities (Wang et al, 2009). It is likely that the methanolic extracts of the *G. hirsutum* contains such active ingredient that can inhibit viral infectivity.

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