Antiviral Potentials of *Gossypium hirsutum* Extracts on Yellow Fever Virus

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Abstract: *Gossypium hirsutum* used by traditional medical practitioners or in phytomedical practice in the treatment of diseases such as fevers and influenza was investigated for their inhibitory activities on the yellow fever virus in the tissue cell culture using Vero cells. The dried powdered leaf of *Gossypium hirsutum* were extracted with water and the extracts evaporated to dryness. Dry residue were dissolved in respective solvents (1:10 w/v) and tested for antiviral activity at $P<0.01$ against yellow fever virus by standard laboratory procedures. The water extract of the plants assayed for cytotoxicity in Vero cells showed that the Minimum Inhibitory Concentrations (MICs) of *G. hirsutum* was 0.079mg/ml. The extracts were used at the established MICs. The extracts were mixed with equal volumes of 100TCID50 Yellow Fever Virus (YFV) in confluent monolayer of Vero cells. The extracts showed antiviral activities against yellow fever virus. *G. hirsutum* inhibited yellow fever viruses at MICs of 0.079mg/ml. *Gossypium hirsutum* showed higher toxic dose and ceased to be cytopathic at 0.079mg/ml. The result of the study revealed that the water extract of *G. hirsutum* showed significant antiviral activity. Based on this experimental evidence, the extracts of *G. hirsutum* are considered effective against YFV as they completely inhibited the infectivity of YFV as evident in complete absence of Cytopathic effects (CPEs). It should therefore be recommended that application of extracts from *Gossypium hirsutum* could help in the treatment of yellow fever infections. It is possible that more potent components especially against YFV might reside in the polar fractions which should form the focus of future investigation.

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1.0. INTRODUCTION

Plants are used for medicinal and industrial purposes among other things. Plants are able to produce compounds which though have no apparent function in the primary metabolism of the plant, have good activity against bacterial, fungal and some viral pathogens (Udobi and Onaolapo 2009). These compounds have had an extensive history of use as therapeutic agents (Udobi and Onaolapo 2009). Higher plants have the capacity to produce a large number of organic phytochemicals with complex structural diversity that is known as secondary metabolites (Kannan *et al*., 2009). These secondary metabolites are most likely responsible for the observed activity of the plant parts (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).

Numerous studies have been conducted by several research groups concerning several biological activities of medicinal plants. 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). Combinations of two or three or more medicinal plants for the treatment of infection are mostly employed. The herbal preparation in form of powder could be swallowed, extracted in water or alcohol, used in bathing, and as an ointment. Cytotoxic effects of some of these plants at times leads to vomiting, nausea, skin rashes, unpleasant odour due to lack of proper storage.

Gossypium hirsutum is a plant under the family Malvacae. It is cultivated primarily for tits vegetable seed fiber, it is a raw material for a large volume of textile product, this species is considered the most important of cotton yielding plants, providing the bulk of the commercial cottons. The use of herbs to treat and cure diseases has been in practice among the Nigerian community for many years. This includes the flowers, fruits, seeds, leaves, stems, roots and barks. The parts of plants were harvested, sundried or powdered for preservation and subsequently sold in the herbs market or may be used

fresh. Several species of Malvaceae family have been use in traditional medicine (Aguilar *et al*., 1994), 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 antiparasitic activity (Sotelo *et al*., 2005). Mucilaginous teas of fresh or roasted seeds of *G. hirsutum* have been used for bronchitis, diarrhea, dysentery, and haemorrhage. However, plant fruits appear to have evolved complex antibiotic compounds to cure various diseases like cancer, cardiovascular, digestive and pathogenic bacteria (Kannan *et al*., 2009).

The pharmacological characteristics of Gossypol, a compound initially isolated from *Gossypium hirsutum* L. (Malvacea) seeds have been studied mainly in relation to its reversible antifertility effects in men (Sotelo *et al*., 2005), diverse pathogenic agents, such as *Trypanosoma cruzi* (Abe *et al*., 2004), *Plasmodium falciparum* (Tripathi *et al*., 2004), *Edwardsiella ictaluri* (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 toxic effects of gossypol earlier observed against several parasitic protozoa and viruses makes these findings very important, since the Malvaceae specimens studied here have been used in traditional medicine against scalp infection, dysentery, gonorrhea and as antiseptic (Sotelo *et al*., 2005).

Yellow fever virus is the causative agents of yellow fever disease. Yellow fever is one of the viral haemorrhagic fevers, with classical features of hepatitis, which is the reason for the yellow colouration of the skin (jaundice) and the name yellow fever of the disease. Chemotherapeutic agents or the use of antiviral therapy is to inhibit viral replication in the host cells. Antiviral agent is that which can produce either protective advantage of the virus infected host of any material and can significantly enhance antibody formation and improve antibody activities. One approach that has been used for the discovery of antimicrobial agents from natural sources is based on the evaluation of traditional plant extracts (Uysal-Gökçe et al., 2004; Özçelik *et al*., 2005; 2006; 2008, 2009, Koca et al., 2009; Orhan *et al*., 2009; Kan *et al*., 2009).

Many workers have extensively investigated antiviral and chemotherapeutic effect of different chemicals and extracts of plants or biological bye products. Chiang *et al*. (2003a, b) reported on the in vitro anti-herpes simplex viruses and anti-adenoviruses activity of twelve traditionally used medicinal plants in Taiwan. Sher (2009) reviewed the antimicrobial activity of natural products from medicinal plants.

This current study was designed to evaluate the antiviral effects of leaf extract of *G. hirsutum* plants on Yellow Fever Virus (YFV) in vitro using Vero cell line, to determine the minimum tolerance dose of this plant extract on Vero cells, and to justify in this regard, the administration of these medicinal plants by the traditional medicine practitioners.

2.0. MATERIALS AND METHODS

2.1. Collection and Identification of Plant Materials

The leaves of *G. hirsutum* free from disease were collected from various locations. In the University of Ibadan; *G. hirsutum* was collected from the Department of Botany and Microbiology nursery, and from the botanical garden. The leaves were washed thoroughly 2-3 times with running tap water and once in sterile water, dried, powdered and used for extraction (Kumaraswamy *et al*., 2008).

2.2. Preparation of Extracts

Extraction of the *G. hirsutum* leaves was carried out in the Department of Pharmacognosy, Faculty of Pharmacy, University of Ibadan, Ibadan, Nigeria. The method described by earlier works (Yakubu et al., 2005; Ojiako and Nwanjo 2006; Kumaraswamy *et al*. 2008; Adebayo and Ishola 2009; Obi *et al*. 2009; Obi *et al*. 2009) was employed. After complete solvent evaporation, spurred each of these solvent extract was weighed and preserved at 5°C in an airtight bottle until further use. One gram of water extract was dissolved in 10ml of water which served as a test extract for antiviral activity assay (Kumaraswamy *et al*. 2008; Adebayo and Ishola 2009).

2.3. Cells and media

African green monkey kidney (Vero) cells were passaged in Minimum Essential Eagle Medium (MEM) (GibcoBRL, Scotland, UK), supplemented with 5% Fetal Calf Serum (FCS) (BioWhittaker Europe, Germany and antibiotics; cell cultures were cultivated at 37 ^{O}C in the presence of 5% CO_2 till the formation of confluent monolayers. In the antiviral experiments 0.5% FCS was added.

2.4. Viral propagation

The hyperattenuated vaccine strain of Yellow Fever Virus (YFV) from Dakar, Ghana was grown in mouse brain of 2 day old mice at the Department of Virology, Faculty of Basic Medical Sciences, College of Medicine, University of Ibadan, Ibadan, Nigeria. The YFV harvested from the mice were grown in Vero cells in the presence of 2 µg/ml

trypsin (Sigma); the infectious titre was $10^{5.7}$ - 10^{7} TCID50/ml (50% tissue culture infectious doses/ml). The virus stocks were stored at -80 ^{O}C.

2.5. Antiviral Assays

The technique described is a modified form of procedures previously described in detail (Ojo *et al*., 2009). Vero cells were grown in Dulbecco's Modified Eagle Medium (MEM) containing 5% fetal bovine serum (all cell culture reagents were obtained from GIBCO Life Sciences, Ontario) in 96-well microtest trays (Falcon), 0.2 ml per well. When the cells formed confluent monolayers, they were used for the assays. Each plant extract was diluted 1:100 in MEM plus 0.1% serum and filtered through a sterile syringe filter 0.2 µ pore diameter. The filter equivalent to 1,000 µg/ml dried plant material and 1% of the 70% ethanol was the starting test material. In the standard procedure, serial 2-fold dilution of the extract were made (in duplicate) in MEM plus 0.1% serum across a row of wells in an empty 96-well microtest tray. With the aid of a multipipettor, these diluted extracts were transformed to the aspirated Vero cell monolayers of another 96-well tray, 0.1 ml per well. The cultures were incubated at 37°C for 60 min and examined microscopically for possible immediate cytotoxic effects. Then 0.1ml of virus (yellow fever virus), comprising 100 pfu in MEM + 0.1% serum was added to each well. Controls induced cells with no virus and cells infected with untreated virus. Cultures were inspected periodically in the microscope for viral CPE.

2.6. Cytotoxicity assays

The procedure was similar to the antiviral assay except that no virus was added to the wells and following light exposure, the trays were returned to the incubator for periodic microscopic assessment of changes in cell morphology or visible toxic effect (Ojo *et al*., 2009). The cells grown in the absence of the extracts were used as 100% cell survival. The concentration at which the cell number were reduced to 50% of that when compared with the cell controls was taken as 50% cytotoxic dose (CD50). The concentration which had no effect on the cell number (maximum tolerated concentration or minimum cytotoxic dose (MCD)) was also observed.

3.0. RESULTS

The screening of the crude water extracts of plants were tested for maximum tolerance dose on Vero cells using different concentration. The results of the cytotoxicity assays are shown in Table 1. The water extracts of *G. hirsutum* leaves were found to have maximum tolerance (MTD_{50}) or minimum cytotoxic dose (MCD_{50}) at 0.079mg/ml on Vero cells. *G. hirsutum* leaves demonstrated a lower level of toxic effect on the cell line as shown in Table 1.

Table 1: Cytotoxicity assays of *Gossypium hirsutum*

***Gossypium hirsutum* extract Concentration (mg/ml)**	**Cytotoxicity**
79.0	+
7.9	+
0.79	+
0.079	-

Key: **-** = absence of cytotoxicity; + = presence of cytotoxicity

Tables 2a -2b show the propagation of yellow fever virus (YFV) in two sets of mice. The YFV grows readily in the brain of 1-2 day-old mice. The results showed that at day 1, 2 and 3, the mice showed no visible signs and symptoms of the YFV inoculated intracerebrally at day 0. At day 4, two (16.7%) of the mice showed signs of sickness which included sluggishness, crouching distinctively in one corner by the mice involved. At day 5, two (16.7%) of the mice became visibly sick but were not dead yet. At day 6, the two (16.7%) sick mice were eaten by their mothers and all the others were visibly sick.

Antiviral activities were measured as complete or partial inhibition of viral CPE (cytopathic effects) at concentrations of 79mg/ml, 7.9mg/ml, 0.79mg/ml, and 0.079mg/ml (Tables 3).

Table 2a: Virus propagation (1st passage)

Incubation period (days)	No. of mice inoculated	No. showing signs of sickness (%)	No. confirmed sick (%)	No. missing/lost (%)	No. eating (%)	No. dead (%)	No. survived (%)
0	12	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	12(100.0)
1	12	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	12(100.0)
2	12	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	12(100.0)
3	12	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	12(100.0)
4	12	2(16.7)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	12(100.0)
5	12	0(0.0)	2(16.7)	0(0.0)	0(0.0)	0(0.0)	12(100.0)
6	12	0(0.0)	10(83.3)	0(0.0)	2(16.7)	0(0.0)	10(83.3)
Total	**12**	**2(16.7)**	**3(25.0)**	**0(0.0)**	**2(16.7)**	**0(0.0)**	**10(83.3)**

Table 2b: Virus propagation (2nd passage)

0	12	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	12(100.0)
1	12	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	12(100.0)
2	12	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	12(100.0)
3	12	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	12(100.0)
4	12	2(16.7)	2(16.7)	2(16.7)	0(0.0)	0(0.0)	10(83.3)
5	12	2(16.7)	7(58.3)	2(16.7)	0(0.0)	1(8.3)	09(75.0)
Total	**12**	**4 (33.3)**	**9(75.0)**	**4 (33.3)**	**0(0.0)**	**0(0.0)**	**09(75.0)**

Table 3: Antiviral activities of the *Gossypium hirsutum* extracts on yellow fever virus

Well	Log of virus dilution	EXTRACTS CONCENTRATION (mg/ml)	CPE	NO CPE	CPE	NO CPE	Cumulative value	
							RATIO	%
1	10^{-1}(+++)	0.79(-)	4	0	18	0	4/4	100
2	10^{-2}(+++)	0.13(-)	4	0	14	0	4/4	100
3	10^{-3}(+++)	0.079(-)	4	0	0	0	4/4	100
4	10^{-4}(++)	0.013(-)	4	0	6	0	4/4	100
5	10^{-5}(++)	0.006(-)	2	2	2	2	2/4	50
6	10^{-6}(-)	0.003(-)	0	7	0	16	0/4	0
7	CC(-)	CC(-)	CC(-)	CC(-)	CC(-)	CC(-)	CC(-)	CC(-)
8	VC(++)	EC(-)	EC(-)	EC(-)	EC(-)	EC(-)	EC(-)	EC(-)

Key:
CPE = Cytopathic Effect of the virus on Vero cells
+++ = Complete cells CPE
++ = 75% CPE
+ - = partial inhibition of viral CPE (incomplete CPE)
\- = Inhibition of viral CPE
CC = Cell control containing cell lines in maintenance medium.
EC = Extract control at MCD_{50}
VC = Virus control

4.0. DISCUSSION

The widespread of viral infections in Africa and the limited number of available drugs which are effective against them led to investigations on antiviral potentials of plants easily grown in Africa (Ojo *et al*., 2009). There are still many viral infections that are of public health importance in Nigeria of which yellow fever is one of them. Regardless of the socioeconomic status of the population at large, the major focus on the control of some of these viral infections has been that of prophylaxis, whereby every effort has been made to prevent infections through the judicious use of vaccines (Ojo *et al*., 2009).

This protocol involving continuous exposure of the cells to extract for 5 days, permits detection of cytotoxic effects leading to cell death as well as more subtle effects on the cells that may not be deleterious e.g. alteration of cell shape to a more rounded morphology (Ojo *et al*., 2009). The extracts produced such changes in cell morphology at concentration higher than 0.079mg/ml. The effectiveness of the *G. hirsutum* against the yellow has been observed. Extract of *G. hirsutum* were however inhibitory on yellow fever viruses. Yellow fever virus was inhibited at a minimum concentration of 0.079mg/ml by *G. hirsutum* respectively. This is in line with other scientists who established that crude extracts of some plants and some pure compounds from such plants can potentiate the activity of antibiotics in-vitro (Udobi and Onaolapo, 2009; Ojo *et al.*, 2009).

Plants are able to produce compounds which though have no apparent function in the primary metabolism of the plant, have good activity against bacterial, fungal and some viral pathogens when they are able to find their way into and accumulate in them. These compounds have had an extensive history of use as therapeutic agents (Udobi and Onaolapo, 2009). In the course of our search for the antiviral properties of *G. hirsutum* extracts having various polarities, remarkable antiviral activities were determined against the YFV. This study has shown the antiviral effect of water extract from fresh leaves of *G. hirsutum*. The cytotoxicity assay of the extract on Vero cells showed that *G. hirsutum* is less toxic with minimum concentration of 0.079mg/ml. This shows that generally the phytochemical constituents in *G. hirsutum* is less toxic to Vero cells. This justifies the raw application of the crude water extracts of the plant when taken orally or applied directly on the body. The leaves extract of *G. hirsutum* was active against the YFV. It inhibited the virus 100% at minimum inhibitory concentration (MIC) of 0.079mg/ml. *G. hirsutum* showed antiviral effect against the YFV. It may be that the extracts of the *G. hirsutum* equally contains such alkaloid or

active ingredient that can inhibit viral infectivity. *G. hirsutum* extracts was also active against the YFV. Influenza virus is activated by treatment with extracts of this plant and it results in a 96-100% protection rate. The plant extracts were also been found to have antiherpetic action in infected mice on both oral and subcutaneous administration. It was however found to be more effective against dermotropic than keratogenic strains of the HSV (Vichikonova and Gorunyuova, 1972).

Water extract of *G. hirsutum* fresh leaves completely inhibited the infectivity of the YFV against the Vero cell at concentration 0.079mg/ml of $100TCID_{50}$ of the virus. The antiviral potential of the plant samples was screened in 2 model systems: reproduction of 2 vaccine strains of YFV in mice brain and of 2 YFV in Vero cells. The water extract of *G. hirsutum* leaves showed good anti-YFV effects; the growth of YFV were reduced significantly. These extracts exhibited anti-YFV activity; evident by the complete absence of CPE when compared with the cell control. It may be that these extracts acted directly on the virus or coated the surface of the cell thereby preventing penetration and/or inhibiting the stages of viral replication. There is therefore, the need for further studies to elucidate and determine the active ingredients, pharmacological properties, mode of actions, chemospects and other therapeutic properties or values of these plants as antiviral agents. To this effect, there must be cooperation between the various modern health workers, researchers, scientists and the traditional system. Plants produce many organic substances which have value in the treatment of viral infection. It is essential to establish these active substances which act on the viral infections and replication, so that they can be used more effectively in treatment and prevention of viral diseases.

5.0. CONCLUSION

The outcome of the antiviral screenings of *G. hirsutum* was impressive as the extracts possess activity against the yellow fever virus which was tested. From the findings of this study, it was confirmed that water extracts of *G. hirsutum* leaves, have shown promising but differential *in-vitro* antiviral activity. It should therefore be recommended that application of extracts from these plants could help in the treatment of yellow fever infections. It is possible that more potent components especially against YFV could form the focus of future investigations.

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