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RESEARCH ARTICLE

# In Vitro Evaluation of the Antiviral Activity of Extracts of *Gynostemma pentaphyllum* against Poliomyelitis Virus

Okoye EL

Department of Applied Microbiology and Brewing, Nnamdi Azikiwe University, PMB 5025, Awka, Anambra State, Nigeria

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***Corresponding Address:**
Dr. Okoye EL
ebyemma2006@yahoo.com

**ABSTRACT**

Antiviral activity of the crude extracts of *Gynostemma pentaphyllum* on Poliomyelitis virus was evaluated. The dried and powdered leaves of the plant - *Gynostemma pentaphyllum* were extracted with methanol, water and petroleum ether using standard methods. The cytotoxicity of the extracts was evaluated using the end-point Cytopathic effect assay on L20B cell lines. Phytochemical evaluations of the extracts were also carried out. The Poliovirus was titrated and determination of the 50% tissue culture infective dose ($TCID_{50}$) done on L20B cells. The 50% tissue culture infective dose was later calculated using standard methods. The antiviral properties were determined against the three sero-types of Poliovirus ($SL_1$, $SL_2$ and $SL_3$) using the end-point Cytopathic effect assay on L20B cell lines. Phytochemical analysis of the extracts revealed the presence of saponins, alkaloids, glycosides, tannins, flavonoids, carbohydrates, reducing sugar, resins, fats and oil, acidic compounds and proteins. Poliomyelitis viral infectivity was inhibited by the extracts giving a range of specificity indices of 25-150. This shows that the extracts selectively inhibited the virus and that their activity against the virus was not just a consequence of toxicity to the cells. The research has shown that the plant possesses potent antiviral potentials and could serve as a possible source of lead antiviral drug against Poliomyelitis since the disease has no known drug for treatment.

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## INTRODUCTION

Poliomyelitis, often called polio or infantile paralysis, is an acute viral infectious disease spread from person to person, primarily via the feacal-oral route (Cohen, 2004). The causative agent of the disease is a positive stranded RNA virus belonging to the family of Picornaviridae (Ryan and Ray, 2004). Poliomyelitis is highly contagious and spreads easily by human-to-human contact (Kew *et al.*, 2005). In endemic areas, wild polioviruses can infect virtually the entire human population. In fact Poliomyelitis has been a public health concern. There is no drug for the treatment of the disease but prevention has been by the use of Inactivated Poliovirus Vaccine (IPV) and Oral Polio vaccine (OPV). Oral Polio vaccine (OPV) has been a vaccine of choice for controlling Poliomyelitis in many Countries but on very rare occasions, the attenuated virus in OPV reverts into a form that can paralyze (WHO, 2007). There is therefore need for an antiviral agent that will be used for the treatment of this disease.

Plants and plant products present some hope to scientists, serving as an avenue to discover lead antiviral drugs. Currently, medicinal plants are being increasingly projected as suitable alternative sources of antiviral agents because of their multiple targets, minor side effects, low potentials to cause resistance and low costs (Esimone *et al*., 2010). In this report, the results of the determination of the antiviral activity of crude extracts of the plant: - *Gynostemma pentaphyllum* are presented. *G. Pentaphyllum* is an herbaceous climber with slender stem and two branched tendrils. The leaves are compound consisting of three to five leaflets but rarely seven leaflets (Warunee *et al.,* 2005). *Gynostemma pentaphyllum* belongs to the family Cucurbitaceae. It is also called jiaogulan by the Chinese, Five-leaf ginseng or Miracle grass in English and "Ugu Ohia" in Igbo language (Nigeria) (Blurnert *et al*., 2003). *Gynostemma Pentaphyllum* has been claimed to possess antiviral

activities in complementary Medicine. It has also been reported to have various activities such as Cholesterol-reducing effect (Huang et al., 2005) and hypoglycemic effects (Samer *et al*., 2006). Locally, the traditional medicine practitioners use it for the treatment of Bacterial and Viral infections. These claims led to the screening of this medicinal plant for its antiviral activity against Poliomyelitis virus which is a major public health concern.

## MATERIALS AND METHODS

### Collection and Extraction of plant materials

The leaves of *Gynostemma pentaphyllum* (G.P) plant were collected from Nibo in Awka south Local Government Area, Anambra State, Nigeria. The leaves were identified by Prof. C.C .Okeke of the Department of Botany, Nnamdi Azikiwe University, Awka. They were oven-dried at $50^0$C for 24 hrs and ground to powder using a mechanical grinder.

Forty gram (40 g) portion of the plant powder was macerated in 400 ml of distilled water in a conical flask and left at room temperature for 24 hours. For the methanolic and petroleum ether extract, the forty gram (40 g) portion of plant powder was macerated in 200 ml of either methanol or petroleum ether and left at room temperature for 48 hours. These were filtered using whatman No 1 filter paper. The filtrates were concentrated to dryness in the oven at $50^0$C

### Cell line and Virus

The continuous cell line used - L20B cells (a genetically engineered mouse cell line expressing the human poliovirus receptor) was propagated using Eagles Minimum Essential Medium (EMEM) (Gibco, Germany) supplemented with 10% (maintained with 2%) heat-activated fetal calf serum (FCS),100 U/ml penicillin and 100µg/ml streptomycin. The L20B cells were obtained from the WHO Polio laboratory, Department of Virology, University College Hospital (UCH) Ibadan, Nigeria.

Stock suspensions of the three sero types of poliomyelitis virus namely:-P1 ($SL_I$3978 (LR)), P2 ($SL_2$4493 (LR)) and P3 ($SL_3$4785 (RLR)) were obtained from the World Health Organization (WHO) polio laboratory in the University College Hospital (UCH) Ibadan, Nigeria.

### Phytochemical analysis of plant extracts

The extracts were first reconstituted in the respective solvents used for their extraction and then tested by standard phytochemical methods (Harborne, 1998) for presence of alkaloids, flavonoids, tannins, saponins, glycosides, protein, carbohydrate, terpenoids, resins, fats and oil, acidity, steroids and reducing sugar.

### Preparation of Virus Stock

A 0.2ml aliquot of each type of stock polio suspension was measured out using a micro pipette and used to infect a confluent monolayer of L20 B cells in 25ml tissue culture flask (T25). The three flasks were incubated at $37^o$C and observed daily for 7 days until full cytopathic effect was seen on the cells. The virus was harvested and further passaged twice and the harvested virus stored in well labeled cryo vials at -$86^o$C (Ultralow) until used.

### Cytotoxicity assay of the extracts

The cytotoxicity assay was performed before the antiviral screening using the end point cytopathic effect assay method on L20B cells. In this assay, L20B cells were seeded onto a 96-well plate at a concentration of $10^4$ cells/well and a volume of 100 µl per well. A volume of 100µl of the different concentrations of test extracts (5mg/0.01ml, 2.5mg/0.01ml and 1.25mg/0.01ml) were added to culture wells in quadruplicate. Culture medium without any drug was used as the "no-drug" control. The plates were incubated at 37°C under 5% $CO_2$ and observed daily under the inverted microscope for cytopathic effect for 7 to 10 days before termination.

### Titration of the Virus and determination of the 50% tissue culture infective dose ($TCID_{50}$)

Stepwise 10-fold (1/10) dilutions of the virus suspensions were made up to $10^{-9}$ in tissue culture tubes using 2% Eagle's Minimum Essential Medium (Maintenance medium). Then, 100µl aliquots of each dilution step was inoculated into the wells of a 96 well tissue culture plate containing confluent L20 B cells and 100µl of 2% Eagle's Minimum Essential Medium (EMEM) bringing the overall volume to 200µl. Each dilution step was seeded into four separate (quadruplicate) wells. The plates were incubated at $37^O$C and scored for cytopathic effect daily for 7 to 14 days before terminating the readings. The end point titres were calculated using the method of Kinchinton *et al*. (1995)

### Assay of antiviral activity

Various concentrations of the extracts (0.02mg/ml, 0.01mg/ml and 0.005mg/ml) were mixed in equal volumes (100 µl) with 100 $TCID_{50}$ of the virus, all in 2% EMEM. These were incubated for 1hour before aliquots of 200 µl of each mixture were used to infect a confluent monolayer cells in a 96 well tissue culture plate. The virus and cell controls were also set alongside these. They were later incubated at $37^O$C and scored daily using an inverted microscope for cytopathic effect for 10 days.

## RESULTS

The phytochemical analysis of the crude extracts of the plant *Gynostemma pentaphyllum* (Table 1) showed the presence of saponins, alkaloids, glycosides, tannins, carbohydrates, flavonoids, resins, acidic compounds, proteins, reducing sugar and fats and oil.

The antiviral assay against the three sero-types of poliovirus ($P_1$, $P_2$, $P_3$) were evaluated on the L20B cells. The cytotoxicity effect of the extract on the L20B cells was also evaluated. The result shows the concentration of extracts that was toxic to 50% of the cells ($TC_{50}$) and also the concentration of extracts that inhibited viral infectivity (cytopathic effect) by 50% ($IC_{50}$). The selectivity indices of the extracts were calculated by dividing the $TC_{50}$ by the $IC_{50}$. The selectivity indices that ranged between 2.5 and 150 is worthy of note. This shows that the extracts selectively inhibited the virus without having much effect on the extracts.

**Table 1:** Phytochemical constituents of *Gynostemma pentaphyllum* extracts

| Constituents | Type of extract | | |
|---|---|---|---|
| | Ether | Aqueous | Methanol |
| Saponins | + | +++ | ++ |
| Alkaloids | ++++ | - | + |
| Glycosides | - | +++ | +++ |
| Tannins | - | ++ | + |
| Carbohydrates | - | +++ | +++ |
| Reducing sugar | - | ++ | ++ |
| Flavonoids | ++++ | - | +++ |
| Resins | ++ | - | + |
| Steroids | - | - | - |
| Terpenoids | - | - | - |
| Fats and oil | + | - | - |
| Acidic compounds | - | +++ | - |
| Proteins | - | + | +++ |

**Key:** (-) - not present; (+) - present in small concentration; (++) - present in moderately high concentration; (+++) - present in very high concentration; (++++) - Abundantly present.

**Table 2:** Antiviral activity against Polio 1 (Sl 1) Virus

| | AG | EG | MG |
|---|---|---|---|
| $TC_{50}$ (µg/ml) | 3.75 | 0.94 | 7.50 |
| $IC_{50}$ (µg/ml) | 0.09 | 0.19 | 0.05 |
| Selectivity index ($TC_{50}/IC_{50}$) | 41.67 | 4.95 | 150 |

**Key:** AG = Aqueous extract of *Gynostemma pentaphyllum;* EG = Ether extract of *Gynostemma pentaphyllum;* MG = Methanolic extract of *Gynostemma pentaphyllum*; $IC_{50}$ = Concentration of extract that inhibited viral infectivity (Cytopathic effect) by 50%; $TC_{50}$ = Concentration of extract that is cytotoxic to 50% of cells; The result represents mean values of quadruplicate experiment

**Table 3:** Antiviral activity against Polio 2 (Sl 2) Virus

| | AG | EG | MG |
|---|---|---|---|
| $TC_{50}$ (µg/ml) | 3.75 | 0.94 | 7.50 |
| $IC_{50}$ (µg/ml) | 1.50 | 0.19 | 0.75 |
| Selectivity index ($TC_{50}/IC_{50}$) | 2.50 | 4.95 | 10.00 |

**Key:** AG = Aqueous extract of *Gynostemma;* EG = Ether extract of *Gynostemma;* MG= Methanolic extract of *Gynostemma;* $IC_{50}$ = Concentration of extract that inhibited viral infectivity (Cytopathic effect) by 50%; $TC_{50}$ = Concentration of extract that is cytotoxic to 50% of cells; The result represents mean values of quadruplicate experiment

**Table 4:** Antiviral activity against Polio 3 (Sl 3) Virus

| | AG | EG | MG |
|---|---|---|---|
| $TC_{50}$ (µg/ml) | 3.75 | 0.94 | 7.50 |
| $IC_{50}$ (µg/ml) | 1.50 | 0.38 | 0.75 |
| Selectivity index ($TC_{50}/IC_{50}$) | 2.50 | 2.47 | 10 |

**Key:** AG = Aqueous extract of *Gynostemma;* EG = Ether extract of *Gynostemma;* MG= Methanolic extract of *Gynostemma;* $IC_{50}$ = Concentration of extract that inhibited viral infectivity (Cytopathic effect) by 50%; $TC_{50}$ = Concentration of extract that is cytotoxic to 50% of cells; The result represents mean values of quadruplicate experiment.

Table 2 shows the result of the antiviral activity against polio 1 virus by the three extracts. The methanolic extract of *Gynostemma pentaphyllum* gave the lowest concentration for $IC_{50}$ and the highest concentration for the $TC_{50}$, thus giving the highest selectivity index. This showed that at such a low concentration (0.05mg/ml) the extract was able to inhibit viral infectivity by 50% and the selectivity index of 150 shows that before the extract will be toxic to 50% of the cells, it will be at a concentration 0.05mg/ml multiplied by 150. This suggests that the extract can serve as a potential antiviral drug with minimal toxicity to the cells. The same thing applies to the aqueous extract that gave a selectivity index of 41.67. The Ether extract gave the least selectivity index of 4.95.

The methanolic extract gave the highest selectivity index of 10 for P2 while the Ether extract gave 4.95. The aqueous extract gave the least selectivity index of 2.50 (Table 3). In Table 4, the methanolic extract also gave the highest selectivity index of 10 for P3 followed by the aqueous-2.50 and the Ether which gave the least-2.47.

**Table 5:** Result of titration of the $Tcid_{50}$ of Poliovirus

| Virus dilution | Polio 1 (SL) | | | | Polio 2 (SL2) | | | | Polio 3 (SL3) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Well 1 | 2 | 3 | 4 | Well 1 | 2 | 3 | 4 | Well 1 | 2 | 3 | 4 |
| $10^{-1}$ | + | + | + | + | + | + | + | + | + | + | + | + |
| $10^{-2}$ | + | + | + | + | + | + | + | + | + | + | + | + |
| $10^{-3}$ | + | + | + | + | + | + | + | + | + | + | + | + |
| $10^{-4}$ | + | + | + | + | + | + | + | + | + | + | + | + |
| $10^{-5}$ | + | + | + | + | + | + | + | + | + | + | + | + |
| $10^{-6}$ | + | + | + | + | + | + | + | + | + | + | + | + |
| $10^{-7}$ | + | + | - | - | - | - | - | - | + | + | + | + |
| $10^{-8}$ | - | - | - | - | - | - | - | - | - | - | - | |
| $10^{-9}$ | - | - | - | - | - | - | - | - | - | - | - | |

$TCID_{50}$; $P_1 = 10^7$; $P_2 = 10^{6.5}$; $P_3 = 10^{7.5}$

**Key:** + = Cytopathic effect; - = No cytopathic effect

## DISCUSSION

The phytochemical screening of the extracts of *Gynostemma pentaphyllum* (GP) showed the presence of saponins, alkaloids, glycosides, tannins, flavonoids, carbohydrates, reducing sugar, resins and proteins. The presence of saponins, glycosides and flavonoids in the extracts of *Gynostemma* is consistent with other findings about the leaves of the plant (Xin *et al*., 2004; Cui *et al*., 1999) The leaves of *Gynostemma pentaphyllum* have been shown to contain more than ninety (90) saponins and more than one hundred (100) dammarane-type glycosides have been isolated and identified from it (Zhang and Huang, 1993; Cui *et al*., 1999).

The extracts have shown varying degrees of antiviral activities against the Poliomyelitis virus assayed. The antiviral activities may be attributed to the rich phytochemicals contained in the extracts since various studies have shown that phytochemicals like tannins found in almost all plant parts cure or prevent a variety of viral infections (Serafini *et al*., 1994; Nonaka *et al*., 1990)

One emerging fact from the titration of the viruses/determination of the $TCID_{50}$ is that the polio3 virus ($SL_3$) had the highest titre (7.5) followed by polio 1 ($SL_I$) (7.0) and polio 2 ($SL_2$) that had 6.5 titre. These findings show the relative infectivity and the amount of virus particles per specimen.

The extract inhibition of the polio virus-induced cytopathic effect on L20B cells is highly plausible. The selectivity indices ranging from 25 – 150 showed that the extracts were very potent against the different polio viruses ($SL_I$, $SL_2$ and $SL_3$). This suggests that the antiviral activity against the polio viruses is really specific and not just a consequence of its influence on cell metabolism and/or toxicity. Based on this, one can recommend the plant (*Gynostemma pentaphyllum*) as a possible source of lead antiviral drug against poliomyelitis since it can

selectively inhibit the virus without having much toxic effect on the host cells.

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