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Assessment of Antiviral Activity of *Curcuma longa* on Two RNA Viruses

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ABSTRACT

Major pandemics and seasonal epidemics that have ravaged the world in the past and even at present, are mostly caused by RNA viruses. This has necessitated the need for continuous research to identify important natural products, with antiviral potentials, which can be harnessed for use in the prevention and treatment of viral infections. This study therefore, evaluated the antiviral property of *Curcuma longa* on two important RNA viruses of public health importance, namely polio and measles viruses. Extraction of active ingredients from turmeric rhizomes was done with the use of Analar grade methanol and concentrated using rotary evaporator. Polio and measles viruses were isolated from their respective vaccines using Reed-Muench method. Infective doses of the viruses and toxicity profile of extract were determined. Confluent Vero cells were inoculated with the viruses at different dilutions of the extract, incubated and observed for 7 days. Methanol extract of *Curcuma longa* inhibited polio virus at the maximum non-toxic concentration (MNTC) of $0.031 \mu\text{g } \mu\text{L}^{-1}$ and inhibitory concentration (IC_{50}) of $0.067 \mu\text{g } \mu\text{L}^{-1}$ with selectivity index of 2.16. Inhibition by the extract was observed prior to infection with the viruses. Phytochemical analysis of the extract showed presence of terpenes, saponins, alkaloids, flavonoids, tannins, cardiac glycosides and phenol as the bioactive phytochemicals. This study has shown that *curcuma longa* has potent inhibitory activity, hence can be harnessed in the development of an effective antiviral agent against polio and measles viruses.

Keywords: *Curcuma longa*, Inhibition, Vero cell lines, Polio virus, Measles virus

Introduction

Viral infections are a huge burden to humans, animals and plants. A good number of human diseases do not respond effectively to current antiviral agents and some lack effective therapeutics (Tomei *et al.*, 2005). In addition, other factors include antiviral resistance (Hulgan and Haas, 2006), drug intolerance or inability of patients to procure available antiviral agents due to their exorbitant prices (Lemoine *et al.*, 2013). For

those viruses that have vaccines, numerous infections have been reported even among vaccinated individuals, either due to vaccine failure or circulation of vaccine strains of the viruses among vaccinated populations. Polio and measles virus infections have been reported among vaccinated population, especially with vaccine strains (Davidson *et al.*, 2008). Wild polio has been eradicated from Africa, however, the challenge of

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infections due to circulation of vaccine strains still persists in the region (WHO, 2020). Vaccine-derived polio virus strains (VDPVs) cause clinical symptoms including paralysis that are comparable to those by wild poliovirus. Hence, VDPVs are reported to be caused outbreaks and are responsible for over 1,200 paralysis cases between 2000–2019, which since 2017, have surpassed reported cases by wild poliovirus (CDC, 2020). Studies have reported measles outbreak among vaccinated people especially school children, not only in low- and middle-income countries, but also in some countries around the globe (Chen *et al.*, 1989; Yeung *et al.*, 2005; Davidson *et al.*, 2008). As such, harnessing natural products that have the potential to target different stages of the viral life cycle of the virus such as attachment, entry and replication may offer effective antiviral and anti-inflammatory therapy against several viral infections (Moghadamtousi *et al.*, 2014).

Numerous medicinal plants indigenous to Africa are found in all the geographical regions of Nigeria (FEPA, 1992). Most have been proven to inhibit entry and attachment of some viruses in cell cultures (Obi and Shenge, 2018). Curcumin derived from turmeric is known for possession of chemoprophylactic effects and potent pharmacological properties including anti-inflammatory, anti-pyretic and inhibitory properties against viral replication of most viruses. This inhibitory activity of Curcumin has been observed to ameliorate cytokine storm and associated pneumonia with viruses such as Middle-East respiratory syndrome (MERS), severe acute respiratory syndrome coronavirus (SARS-CoV) and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Lui and Ying, 2020).

Turmeric (*Curcuma longa*) is a widely- distributed spice, which belongs to the ginger family (*Zingiberaceae*) and it is commonly used around the globe due to its rich antioxidant profile (FEPA, 1992). The spice is widely grown and used as a condiment in Nigeria. The orange-yellow pigment derived from turmeric and used as a coloring agent

is called curcuminoid (Mazumder *et al.*, 1995). Turmeric is reported to have antiviral, anti-inflammatory, antifungal and antibacterial activities due its polyphenol component known as Curcumin (Moghadamtousi, 2014). Several studies have identified anti-inflammatory and anti-cancer properties of turmeric (Chen *et al.*, 2002; Zhou *et al.*, 2011). In a 2009 study on liver cells, turmeric halted the replication of hepatitis B virus (Kim *et al.*, 2009). Another *in vitro* study in 2010 equally reported the suppression of hepatitis C virus replication by turmeric extract (Kim *et al.*, 2010). Tomita *et al.* (2005), observed turmeric and its extracts as potential antiviral agents against HIV and HTLV-1 (Tomita *et al.*, 2005).

Polio virus is responsible for polio infection, which predominantly occurs among children below age five. It is a highly contagious disease that is transmitted through the faeca-oral route and affects mostly unimmunized persons (Wallace *et al.*, 2016). Polio (also known as poliomyelitis) affects the nervous system, leading to acute flaccid paralysis. According to World Health Organization, 1 in every 200 polio infections will result in permanent paralysis (WHO, 2014). The virus is small, non-enveloped or ‘naked’, icosahedral single-stranded RNA genome of *Picornaviridae* family and genus enterovirus. It is same genus with 32 human echoviruses, 29 coxsackie viruses and a few other enteroviruses (ICTV, 1990).

Measles derived from the Latin word "misellus" meaning ‘miserable’, is an infection caused by measles virus (WHO, 2007). It is a member of the genus *Morbillivirus* of the family *Paramyxoviridae*, that causes acute disease, a common infection in children (Griffin, 2001; Kingsbury *et al.*, 1988). Measles virus is a negative, single stranded, enveloped virus with a non-segmented RNA genome (Barrette, 1999). The virus is highly contagious and transmission is air-borne through respiratory droplet nuclei spread, or by direct contact with infected nasal or throat secretions (Joe *et al.*, 2004).

Polio and measles diseases persist despite availability of vaccines especially among individuals that are not vaccinated. Deadly outbreaks with serious complications in infected persons, still occur, especially in developing nations (WHO, 2014). Wild Polio virus was recently eradicated from Africa; however, it is important to note that vaccine-derived strains of the virus still cause infections (WHO, 2020). This study therefore, was designed to evaluate the antiviral and phytochemical property of turmeric, a widely-grown and readily available spice on polio and measles viruses, which still circulate in the region as vaccine strains, even among vaccinated persons.

Materials and Methods

Study design

This is an *in vitro* tissue culture study of antiviral properties of *Curcuma longa* using Vero cells in minimum essential medium (MEM).

Ethical statement

The study was considered by research ethics committee of Dominican University, Ibadan.

Study site

This study was carried out in Ibadan, Nigeria. The turmeric rhizomes used were locally sourced; and extraction done at the Department of Pharmacognosy, University of Lagos, while antiviral evaluation was done at the Department of Virology, University of Ibadan.

Collection of plant samples

Fresh and healthy rhizome of *Curcuma longa* was purchased from Bodija, a local market in Ibadan, Nigeria. Fresh turmeric rhizomes that are insect or fungus-free were selected for use in the study. The plant was authenticated at the University of Ilorin Herbarium situated at the Department of Plant Biology, University of Ilorin, where voucher

number (001/1105/2021) was issued following deposition of Herbarium specimen.

Extraction of plant materials

Each turmeric rhizome was thoroughly washed and afterwards spread in an airy environment. With Kottermann hot air oven, the rhizomes were allowed to dry for 24 hrs at 45°C. Blending of the rhizomes into smooth powder using Christy and Norris laboratory milling machine set at 8000 rpm followed. Resulting powdery product was weighed using Sauter SM 1000 electronic weighing balance and the weight was recorded in grams.

Thereafter, with Soxhlet extractor (Nahalito), extraction from the blended product followed using 99% Analar grade of methanol (Merck KGaA, Germany) as described by Wang and Weller (2006). Then rotary evaporator (Buchi) the extract was concentrated *in vacuo* at 45°C to a final volume of 3 mL. The extract was subjected to evaporation. Dried solid residue obtained was weighed and recorded. It was then preserved at -20°C in an airtight sterile McCartney bottle in Pharmacognosy Laboratory, University of Lagos, till further use as recommended by Patrick-Iwuanyanwu (2011).

Reconstitution of extract

About 10 mg of the pasty solid extract was weighed and dispensed into sterile calibrated centrifuge tubes. It was then reconstituted in 0.5% of dimethyl sulfoxide (DMSO) (Sigma) and shaken vigorously to ensure complete dissolution. After complete homogenization of the extract with the dissolving solvent, it was brought to a final volume of 10 mL, with the addition of 9.95 mL of sterile distilled water. Initial filtration with 0.45 µm followed using 0.22 µm membrane syringe filters (Cell Treat USA). The filtrate was aliquoted unto sterile plain bottles which were tightly closed and stored as stock at -20°C till further use. One hundred microliter of the extract concentration was thereafter used to evaluate the cellular toxicity of the extracts as well as antiviral assay.

Phytochemical screening

The extract was first reconstituted in methanol extraction solvent and then tested for the presence of alkaloids, cardiac glycosides, flavonoids, saponins, tannins and terpenes by standard phytochemical methods according to Evans (1999).

Evaluation of cellular toxicity

The method used was based on cellular morphologic changes. Vero cells were prepared at a density of 8×10^4 cells mL^{-1} (containing 8000 cells) in a 10% MEM medium in 75 cm^3 tissue culture flasks (Cell Treat, USA). One hundred microliter of this cell suspension (containing 8000 cells) was then dispensed into each well of a 96-well tissue culture plate (Cell Treat, USA) and incubated for 24 hr at 37°C. The growth medium was aspirated after this period, discarded and replaced with 100 μL of 1% MEM medium. Then a 2-fold serial dilution of the extract was carried out using 1% MEM medium as the diluent. The 96-well plates containing the Vero cells were labeled with the different dilution of the extract. Changing pipette tips each time, 100 μL of each extract dilution was introduced into each of the wells in duplicates. The last row of wells containing cells with no extracts was used as negative control, while the row containing the acyclovir (antiviral drug) and cell was used as positive control. Time and date were indicated on the plates which were then incubated at 36.5°C. Cell viability was monitored daily for 14 days, while observing for morphological changes or cytopathic effect (CPE), in comparison with the control wells containing only medium and no extract, using an inverted microscope (Inverskop 40C) (Omilabu *et al.*, 2010). Complete (100%) CPE was scored as 4⁺, 75% as 3⁺, 50% as 2⁺, 25% as 1⁺ and 0 when there is no CPE.

Isolation of test viruses

Measles virus was isolated from measles vaccine (Edmonston-Zagreb strain, Serum Institute,

Hadaraba, Pune, India), obtained from Institute of Child Health, University College Hospital (UCH), Ibadan; while Polio (types 1, 2 & 3, Serum Institute, Hadaraba, Pune, India). The viruses were titrated and using Reed-Muench method, the tissue culture infective doses of the viruses were calculated to be $10^{-3.5}$ TCID₅₀ mL^{-1} for measles virus and $10^{-6.5}$ TCID₅₀ mL^{-1} for Polio viruses. The respective 100 TCID₅₀ for both viruses which was used for the screening was $10^{-1.5}$ and $10^{-4.5}$ TCID₅₀ mL^{-1} .

Test for virucidal activity

Vero cells were prepared at a density of 8×10^4 cells mL^{-1} in a 10% MEM medium in 75 cm^3 tissue culture flasks (Cell Treat, USA). One hundred microliter of this cell suspension (containing 8000 cells) was dispensed into each well of tissue culture plate (Cell Treat, USA) and incubated for 24 hr at 37°C. Then (a) 200 μL of 100 TCID₅₀ virus titer +200 μL of minimum non-toxic concentration (MNTC) of the test extract and (b) 200 μL of 100 TCID₅₀ virus titer +200 μL of 1% medium as a control were prepared and incubated for 1 hr at 37°C in 5% CO₂. The 10% medium in the 96-well plate was aspirated, discarded and replaced with 100 μL of 1% MEM medium [Zandi *et al.*, 2010]. After 1hr incubation period, 100 μL of (a) Virus and extract mixture was inoculated in triplicate unto the 96-well tissue culture plate seeded with Vero cells.

Similarly, 100 μL of (b) Virus+1% medium mixture was dispensed in triplicate into the last three wells of each row to serve as control. Then two-fold serial dilutions were made using separate pipette tips for each dilution, starting from the first row downwards, keeping the last two rows of wells as cell control and extract control. All the mixtures were incubated at 37°C in 5% CO₂ (Omilabu *et al.*, 2010; Zandi *et al.*, 2010).

Cells were examined daily for 7 days, under the inverted microscope (Inverskop 40C) and scored while observing for presence of virus-induced syncytia. The wells containing virus and extract were scored and compared with the wells

containing virus but no extract (Hierholzer and Killington, 1996; Omilabu *et al.*, 2010).

Pre- infection inhibition test

About 100 μL of Vero cells was added to each of 96-well of a microtiter plate and incubated for 24 hr at 37°C. The medium was aspirated and discarded afterwards. Then 100 μL of different concentrations of the plant extract was added to each well and incubated at 37°C for 2 hr in a 5% CO_2 . The extract was thereafter removed after incubation to prevent any interaction with the viruses when the virus inoculum was added. The wells were washed with PBS, then 100 μL of 100 TCID₅₀ of each virus dilution in 1% MEM medium was added to the wells. This was incubated and the presentation of CPE was investigated daily for 7 days using an inverted microscope (Inverskop 40C) and scored (Hierholzer and Killington, 1996; Omilabu *et al.*, 2010).

Post - infection inhibition test

About 100 μL of vero cell line was added to each of 96-well of a microtiter plate and incubated for 24 hr at 37°C. The medium was aspirated and discarded afterwards. Then 100 μL of 100 TCID₅₀ of each virus dilution in 1% MEM medium was added to the wells. The plate was incubated in a 5% CO_2 incubator for 2 hr. Thereafter media and unbound virus were washed off with PBS and cells were refreshed with 1% MEM medium containing different extract concentrations and incubated at 37°C in a 5% CO_2 incubator and the presentation of CPE was investigated daily for 7 days using an inverted microscope (Inverskop 40C) and scored

according to the method of Hierholzer and Killington (Hierholzer and Killington, 1996).

Results

This study has shown that poliovirus is susceptible to *Curcuma longa*. Table 1 shows the various phytochemical profile of turmeric as in the extract. There was no inhibition in wells inoculated with measles virus only and extract (control) only, at the same concentration of 0.031 $\mu\text{g } \mu\text{L}^{-1}$. Phytochemical analysis of the extract showed presence of terpenes, alkaloids, flavonoids, saponins, tannins, cardiac glycosides and phenol as the composite bioactive phytochemicals in the extract as shown in Table 1. The virucidal activity of the extracts on test viruses was evaluated using the maximum non-toxic concentration of the extract, which was shown to be 0.031 $\mu\text{g } \mu\text{L}^{-1}$. The extract was observed to have virucidal activity against 100 TCID₅₀ of the viruses as shown in Table II. Table III shows the result of the mechanism of action of the methanol extract on the test viruses, while Tables IV shows the Inhibitory Concentrations (IC₅₀) and Selectivity Index (SI) of the extracts on the test viruses. Figure 1 shows the toxicity profile of *Curcuma longa*. MV was observed to have minimal resistance to the extract at all the concentrations. The highest level of viral inhibition was observed in PV (where cells remained intact), when compared as shown in Fig II. Plates 1 and II show the different observations made of the cultured plates, inoculated and control plates for each virus and at different concentrations.

Table 1: Phytochemical composition of *Curcuma longa* extract

Extract	Constituents										
	Alkaloids		Cardiac glycosides		Flavonoids		Saponins	Tannins	Terpenes	Phenol	Steroid
	Wagner's	Dragendorff's	Kedde's	Kellar Killani's	Ferric chloride	Lead acetate					
<i>C. longa</i>	++	++	+	+	++	++	+	+	+	++	-

Key: ++ = abundant + = Present - = absent

Table 2: Virucidal activity of *Curcuma longa* extract ($\mu\text{g } \mu\text{L}^{-1}$) against 100 TCID₅₀ of the virus

Extract	Extract concentration ($\mu\text{g } \mu\text{L}^{-1}$)									
	1	0.5	0.25	0.125	0.063	0.031	0.016	0.008	0.004	0.002
Measles						2 ⁺	3 ⁺	4 ⁺		
Polio						0	2 ⁺	3 ⁺		

Key: 4⁺: Complete (100%) cytopathic effect (CPE), 3⁺: 75% CPE, 2⁺: 50% CPE, 1⁺: 25% CPE, 0: No CPE

Table 3: Pre and Post infection antiviral activity of methanol extract of *C. longa* on 100 TCID₅₀ of the test viruses

Extract	Pre infection Antiviral Activity						Post Infection Antiviral Activity					
	Extract concentration ($\mu\text{g } \mu\text{L}^{-1}$)											
	0.063	0.031	0.016	0.008	0.004	0.002	0.063	0.031	0.016	0.008	0.004	0.002
<i>C. longa</i>	Measles virus											
		3 ⁺	3 ⁺	4 ⁺				3 ⁺	4 ⁺	4 ⁺		
	Polio virus											
		0	2 ⁺	3 ⁺				2 ⁺	3 ⁺	3 ⁺		

Key: 4⁺: Complete (100%) cytopathic effect (CPE), 3⁺: 75% CPE, 2⁺: 50% CPE, 1⁺: 25% CPE, 0: No CPE

Table 4: Inhibitory concentration (IC₅₀) of 50% and selectivity index (SI) of extracts that inhibited the test viruses

Extract	Vero cell	Virus Replication	SI
	IC ₅₀ (µg µL ⁻¹)	IC ₅₀ (µg µL ⁻¹)	
VIRUCIDAL ACTIVITY			
Polio Virus			
<i>C. longa</i>	0.067	0.031	2.16
PRE -INFECTION ANTIVIRAL ACTIVITY			
Polio virus			
<i>C. longa</i>	0.067	0.031	2.16

Key: IC₅₀ = 0.031; SI= 2.16

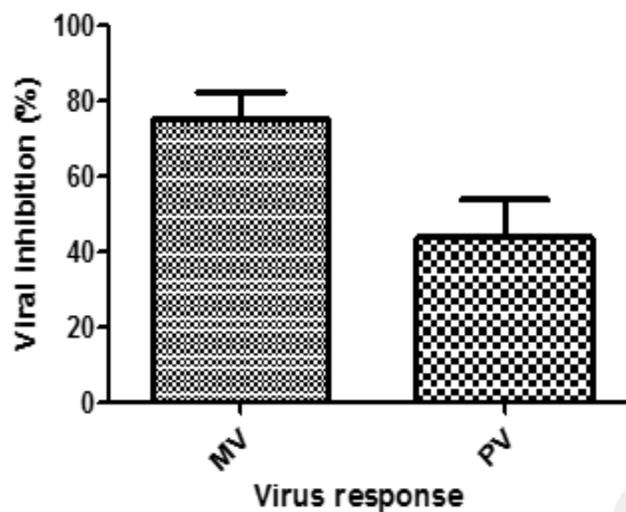
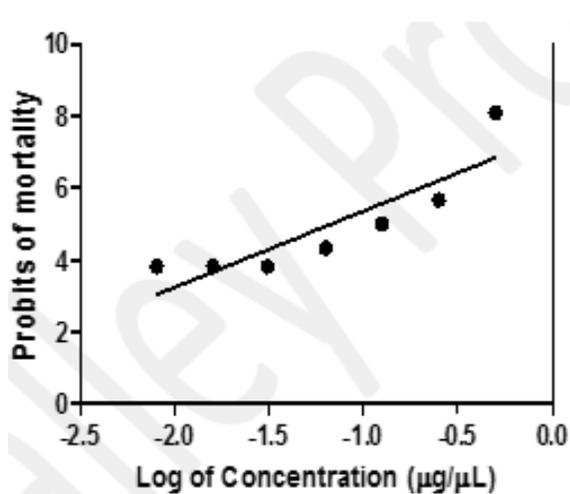


Fig 1: Toxicity profile of *Curcuma longa* (Turmeric) **Fig 2:** Comparison between measles virus and polio virus response to *C. longa*

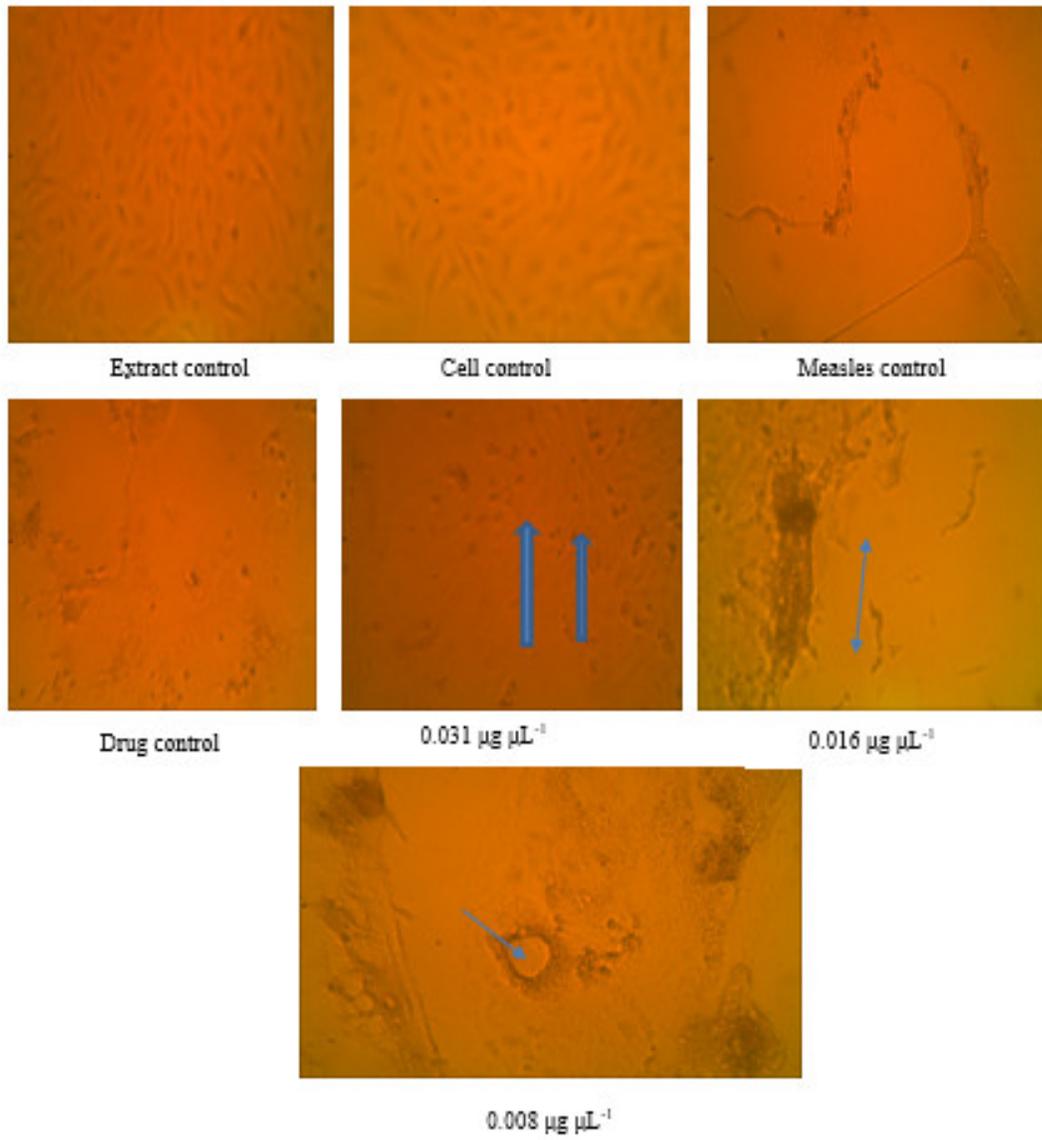


Plate I: Virucidal activity of methanol extract of *C. longa* on 100 TCID₅₀ of measles virus

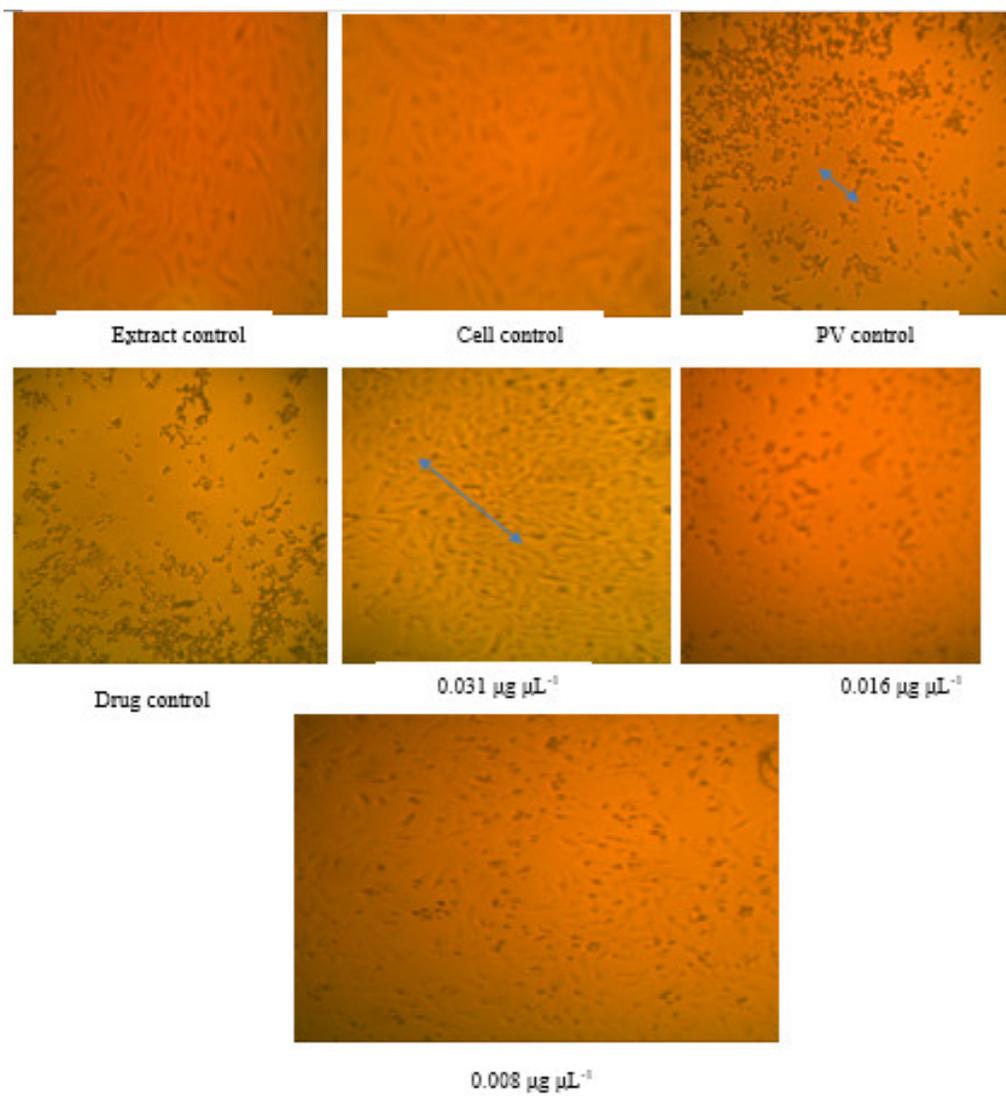


Plate 2: Virucidal activity of methanol extract of *C. longa* on 100 TCID₅₀ of polio virus

Discussion

Polio and Measles viruses are seriously implicated in the morbidity and mortality of children in developing countries (WHO, 2020). Despite availability of effective vaccines against these viral agents, they continue to pose a huge challenge in numerous communities, hence the urgent need for more effective natural means of combating these infections. This study therefore, was carried out to assess the susceptibility of polio and measles virus to extracts of indigenous *Curcuma longa* plant.

Following toxicity evaluation of the extracts on Vero cells (Fig. I), it was observed that, concentration of $0.067 \mu\text{g } \mu\text{L}^{-1}$ at 50% Inhibitory Concentrations (IC_{50}), was higher than that of the maximum non-toxic concentration ($0.031 \mu\text{g } \mu\text{L}^{-1}$). This means that the extract, although still toxic and would require further purification before it could be used as a pharmaceutical raw material for drug production, was safe to be applied to mammalian cells, as this study has shown. In addition, the potential of this extract as a pharmaceutical raw material was shown in the fact that IC_{50} value of $0.067 \mu\text{g } \mu\text{L}^{-1}$ exhibited by *C. longa* was less than the limit of $<20 \mu\text{g mL}^{-1}$ recommended by the National Cancer Institute (USA), for crude plant extracts (Abdel-Hameed *et al.*, 2012).

C. longa contains a range of phytochemicals that may have contributed to its strong antiviral activity against polio virus as shown in table 1. According to Gupta *et al.* (2015), polyphenol is responsible for the bright orange-yellow color of *C. longa*, while alkaloids and flavonoids contributed to its antimicrobial potentials. These bioactive phytochemicals, which have variously been reported as being antimicrobials may have accounted for the antiviral activities of this plant as observed in the study (Cowan, 1999).

The result shown in Table II and Figure II confirmed *Curcuma longa* as a potential antiviral

agent against poliovirus. The extract inhibited PV at the concentration of $0.031 \mu\text{g } \mu\text{L}^{-1}$, as the integrity of the cells remain same even after incubation with test virus. The result in Figure 1 revealed that MV was resistant to the extract at all the concentrations tested. Polio virus is non-enveloped and may have become easily susceptible to the extract, probably, due to its naked nature. This study has shown that *C. longa* is not only antibacterial as reported in previous studies [Gupta *et al.*, 2015], but also antiviral. Antiviral activity of *C. longa* was previously reported by Kim *et al.* (2010), the team investigated the antiviral activity of the bioactive curcumin found in *C. longa* in Huh7 replicon cells expressing hepatitis C virus (HCV) and reported that this bioactive component inhibited hepatitis C virus replication through suppression of the Akt-SREBP-1 pathway, by decreasing HCV gene expression (Kim *et al.*, 2010). There is a significant difference between the responses of measles and polio to *C. longa*, with MV showing 75% resistance, while PV showed only 43.5% as shown in Figure II. Measles virus is a negative sense, single-stranded RNA genome. The inhibitory activity of *C. longa* has been similarly reported against Coxsackievirus (Lee *et al.*, 2005), HCV and HIV-1 and 2 (Sui *et al.*, 1993), which are all RNA viruses.

The study finding is in agreement with result of *in vitro* antiviral assessment of *C. longa* and its derivative against herpes simplex virus type 1 (HSV-1) in cell culture, where a remarkable antiviral activity was found. In the study, Curcumin reduced early gene expression and infectivity of HSV-1 in cell culture assays (Zandi *et al.*, 2010). Other studies have also reported the antiviral activities of *C. longa* against different classes of viruses (Chen *et al.*, 2010; Moghadamtousi *et al.*, 2014).

Table III showed that *C. longa* could become a veritable source of an entry/attachment inhibitor. Inhibition of viral entry or attachment to cell

receptors translates to usefulness of the inhibiting compound as a prophylactic agent, which can be of great value as an immune booster or in drug development, for prevention of infection by the virus. In a similar study, curcumin inhibited entry of all HCV genotypes in primary human hepatocytes, by affecting the membrane fluidity leading to impairment of viral binding and fusion (Anggakusuma *et al.*, 2014).

Potential antiviral agents must distinguish host and viral roles with a high level of specificity, thus, inhibiting one or more stages of the viral lifecycle without unpleasant side effects (De Clercq and Fields, 2006). The clinical value of the extract (Table 4), was determined by its Selectivity Index (SI). The extract had SI values which exceeded its IC₅₀ values. As reported earlier, a compound with a low IC₅₀ and a high SI is most likely to have value as an antiviral drug (De Clercq, 2005). As a result, the extract of *C. longa* which showed inhibitory activities against PV could become the basis for antiviral drug development and could safely be administered for the prevention and treatment of infections caused by the virus and possibly, other single-stranded RNA viruses. The methanol extract of *C. longa* used in this study showed considerable inhibitory activity against poliovirus infection. As a non-enveloped RNA virus, susceptibility to *C. longa* at the pre-infection stage reveals the potential usefulness of *C. longa* in polio prophylaxis.

Conclusions

This study has shown that *Curcuma longa* has inhibitory antiviral activity. As a result, this important plant (spice) can be harnessed as a potent antiviral or immune booster compound against viruses. There is need for nations to endeavor to keep wild polio virus permanently eradicated, by continuous surveillance and vaccination, boosting the immune status of children, and active research directed at polio and measles, two important childhood diseases in the globe.

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Competing interests: None

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