



***In-vitro* Evaluation of Antiviral Activity of *Moringa oleifera* Extracts against Polio virus**

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Authors' contributions

This work was carried out in collaboration among all authors. Author HMA designed the study and carried out the collection of Moringa plant parts, the polio virus samples/strains, and performed all the laboratory analysis. Author MYI managed the literature searches, wrote the protocol and draft of the manuscript. Author MNY prepare/tabulates the results and performed the statistical analysis. Authors ABS and AFU respectively, managed and supervised entire work. All authors read and approved the final manuscript.

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ABSTRACT

Background: *Moringa oleifera* plant parts extract have been utilized tremendously in traditional medicine, having various pharmaceutical activities such as antifungal, antibacterial and antiviral properties.

Aim: This study was carried out to evaluate antiviral activity of aqueous extract of *Moringa* leaves, seeds and flowers against Polio virus isolates (Vaccine Strains P1&P3), MDG-17-04852; MDG-17-04881.

Study Design: This is a baseline study carried out to determine the efficacy of *Moringa oleifera* in the treatment of poliomyelitis.

Place and Duration of Study: This study was carried out at the University of Maiduguri Teaching Hospital Maiduguri and Abubakar Tafawa Balewa University, Bauchi from September 2018 to October, 2019.

Methods: Phytochemical substances was extracted and screened from the *Moringa* using standard laboratory techniques. Continuous cell line L20B cells was used to isolate polio virus. Polio virus stock was prepared and titration was carried out to determine TCID₅₀ by Kerber's formula, L-d(S - 0.5). Cytotoxicity of the extracts was evaluated using the end-point cytopathic effect assay. Antiviral assay and Polio virus Intratypic Differentiation (ITD) was performed using Real-Time PCR.

Results: The results revealed present of Tannins in leaves and flowers, Alkaloids and Saponins in all the extracts, Glycosides and Steroids in leaves and Flavonoids in flowers only. Cytotoxicity of the extracts shows 50% effects in 100mg and 50mg concentrations, while 33.3% and 16.7% was observed in 25mg and 12.5mg (P>.05). The titration of the tissue culture infective dose 50% (TCID₅₀) shows that Polio virus type 1 and 3 had a titre value of 10^{6.5} and 10^{6.25} respectively. The ITD results observed presence of SI₁ and SI₃ from the extracts.

Conclusion: This study found that the *Moringa* extracts did not neutralized the Polio virus strains studied, as the phytochemicals show no antiviral activity. Therefore further study is needed in this area to ascertain its antiviral potentials against poliomyelitis.

Keywords: *Moringa oleifera*; phytochemical constituents; polio virus; cytotoxicity; cytopathic effect.

1. INTRODUCTION

Antiviral agent is a substance other than a virus-containing vaccine or specific antibody which can produce either a protective or therapeutic effect to the clear detectable advantage of the virus infected host [1]. All over the world, herbal medicines are considered to be one of the most important areas of interest in traditional medicine systems [2]. Man entirely depends on plants and plant products directly for his basic needs as food, clothing, and shelter and indirectly for their beneficial influence on climate and maintenance of his immediate and remote environment and this makes plants vital for his survival and the basis for his continued existence [3].

In 1978, WHO has emphasized on the importance of scientific research into herbal medicine and since then the developing countries of the world has started research programs to clinically prove the therapeutic value of their native medicinal plant in order to get them registered as possible addition to the WHO's list of essential drugs [4]. In recent times, medicinal plants occupy an important position for being the paramount sources of drug discovery, irrespective of its categorized groups- herbs, shrubs or tree [5]. Nowadays, the use of traditional medicines for their therapeutic properties is not restricted to the developing countries [3].

Moringa oleifera also known as horseradish tree, drumstick tree and mother's best friend (English), Nebedy (Senegal), Marun (Thailand), Malunggay (Philippine) and Benzolive tree (Haiti) [6]. In Nigeria, it is called Zogale, Zoggale-

Gandi(Hausa), Ewe Igbe and Idagbo-Monoye (Yoruba) and Ikwa Oyibo (Igbo). *Moringa* is a highly valued plant belonging to the family Moringaceae, distributed in many countries of the tropics and sub-tropics, it is cultivated almost all over Nigeria and its leaves and fruits are used as vegetables[7]. The tree grows as high as 9 meter with a soft and white wood and corky and gummy bark. Each compound leaf contains 3-9 very thin leaflets dispersed on a compound (3 times pinnate) stalk. Flowers are white and fragrant, producing long, pendulous, 9-ribbed pods, with 3-angled winged seeds [7].

Moringa various parts have been utilized tremendously in traditional medicine practices [8]. Many bioactive phyto-constituents have been reported in different parts of the plant, such as beta- carotene, proteins, vitamins and a variety of Phenolics [9]. The plant is rich in zeatin, Quercetin, beta-sitosterol, caffeoylquinic acid and kaempferol, a rare combination of important bioactive compounds. Various parts of this plant such as the leaves, roots, seed, Moreover, almost all parts of the plant have been utilized in traditional medicine practices [10], antifungal, antispasmodic, anti-inflammatory and diuretic activities [11]. The leaves and young buds of the plant are used as vegetable and can be rubbed on the temples for relieving headache while the root and root bark are regarded as antiscorbic and can be used externally as counter irritants [12].

Furthermore, bark, fruit, flowers and immature pods have been reported to act as cardiac and circulatory stimulants; a natural antibiotic, detoxifier, outstanding immune builder

and is used in many countries to treat malnutrition and malaria [11]. The seeds are also used in water purification and therefore helps in reducing the incidence of water-borne diseases [11].

Polio virus has an RNA genome that is a member of the Enterovirus subgroup of the family Picornaviridae. Enteroviruses are transient inhabitants of the gastrointestinal tract, and are stable in an acidic environment. There are three polio virus serotypes (P1, P2 and P3) with minimal heterotypic immunity between the three serotypes, i.e, immunity to one serotype does not produce significant immunity to the other serotypes [13]. The polio virus is inactivated by heat, formaldehyde, chlorine and ultraviolet light [14]. Polioviruses and the Enteroviruses are distinguished from the other picornaviruses on the basis of physical properties such as buoyant density in caesium chloride and stability in weak acid. The three poliovirus serotypes are distinguished from the other enteroviruses by neutralization with serotype-specific antisera and the propensity to cause paralytic illness. The Mahoney strain of type 1 poliovirus is the prototype for the polioviruses, the genus Enterovirus, and the family Picornaviridae. It is among the most-studied and best-characterized agents of human disease [15].

Infection with the polio virus occurs via the faecal-oral route; meaning that one ingests the virus is shed in the stool of an infected individual. Up to 95% of all polio infections are asymptomatic and leads to the development of minor symptoms such as fever, headache and sore throat. Approximately 8% of polio infection consist of a minor, nonspecific illness without clinical or laboratory evidence of central nervous system (CNS) invasion and known as abortive poliomyelitis, characterised complete recovery in less than a week. Less than 1% of all polio infections result in flaccid paralysis. An acute flaccid paralysis (AFP) case is defined as any child under the age of 15 years with sudden onset of flaccid/floppy paralysis (muscular weakness) or any person of any age in whom polio is suspected. In many aspects this neurological phase of infection is thought to be an accidental diversion of the normal gastrointestinal infection [16].

Individuals who are exposed to the virus, either through infection or by immunization with polio vaccine, develop immunity. In immune individuals, IgA antibodies against polio virus are

present in tonsils and gastrointestinal tracts and are able to block virus replication; IgG and IgM antibodies against polio virus can prevent the spread of virus to motor neurones of the central nervous system (CNS) [13]. Factors that increase the risk of polio virus infection or affect the severity of the disease include immune deficiency [17], malnutrition, tonsillectomy [18], physical activity immediately following onset of paralysis [19], skeletal muscle injury due to injection of vaccines or therapeutic agents and pregnancy [20] (Evans, 1960). Although the virus can cross the placenta during pregnancy, the foetus does not appear to be affected by either maternal infection or polio vaccination. Maternal antibodies also cross the placenta, providing passive immunity that protects the infant from polio infection during the first few weeks of life [21].

Poliomyelitis, often called Polio or infantile paralysis, is an acute viral infectious disease of tremendous public health concern [22]. Over the years, 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 [23]. Therefore, there is an urgent need of developing safe and effective drug for poliomyelitis and medicinal plants seems to present suitable alternative sources of antiviral drugs [22]. Poliomyelitis is highly contagious and spreads easily by human-to-human contact. In endemic areas, wild polioviruses can infect virtually the entire human population. In fact, poliomyelitis has been a public health concern and there is no drug for its treatment but prevention has been by the use of vaccines.

Polio virus infection continues to pose a threat to public health despite the many campaigns towards its eradication. The infection is highly communicable, mostly in children. Infection leads to acute flaccid paralysis (AFP). Among those paralysed, 5% to 10% die when their breathing muscles become immobilized [22]. Poor hygiene standards amongst other things in our people could be attributed to the high rate of infection and possess a challenge in the eradication of Polio virus. Diseases and condition caused by the three serotypes of Poliovirus are permanent, irreversible and irreparable, therefore there is urgent need to proffer appropriate treatment due to its devastating effect on human nature. Lack of appropriate treatment is a limiting factor which cannot be overlooked. There is therefore a need

for an antiviral agent that will be used for the treatment of this disease. This study aimed to evaluate antiviral activity of aqueous extract of *Moringa oleifera* leaves, seeds and flowers against Polio virus isolates using virus-induced cytopathic effect assay.

2. MATERIALS AND METHODS

2.1 Collection and Identification of *Moringa oleifera* Plant Samples

The *Moringa oleifera* leaves, flowers, and seeds were obtained from the University of Maiduguri Agricultural farm yard. They were transported to the Department of Crop Production and identified by a Professor of Horticulture; according to description of Herbarium of Orman Botanical Garden, Ministry of Agriculture, Giza, Egypt [24, 25].

2.2 Extraction of Active Components of *Moringa* Leaves, Flowers and Seeds

Air-dried (at room temperature/20-25°C) leaves, flowers, and seeds of *Moringa* were grounded. Exactly 150g of the powdered samples were used for the extraction in 1.5 L of distilled water for 72hrs at 25°C ±2°C. The mixture was filtered and concentrated on a water bath to dryness. The aqueous extracts was stored at 4°C in freeze-dried form and used for the study [26]. The physicochemical properties of the extracts examined and noted.

2.3 Cell Line Used for Isolation of Polio Virus

The continuous cell line used was L20B cells (a genetically engineered mouse cell line expressing the human poliovirus receptor CD155) propagated using Eagles Minimum Essential Medium (EMEM) (Gibco, Germany) supplemented with 10% foetal calf serum, maintained with 2% heat-inactivated foetal calf serum (FCS), 100µl/ml penicillin and 100µg/ml streptomycin. The L20B cells were obtained from the WHO National Polio laboratory/ITD, University of Maiduguri Teaching Hospital, Maiduguri, Nigeria. Stock suspensions of the two serotypes of poliomyelitis virus, namely P1 (SL1), and P3 (SL3), were isolated from Acute Flaccid Paralysis (AFP) cases from samples submitted to the laboratory. The L20B cell line was propagated as described by [27].

2.4 Preparation and Titration of Polio Virus Stock

The Polio virus stock was prepared and titration was carried out as described by [27].

2.5 Determination of TCID₅₀ of Polio Virus

TCID₅₀ was determined by Kerber's formula $L - d(S - 0.5)$

Where,

L = Log of the reciprocal of the lowest dilution

d = Log of the dilution factor

S = No. of wells with CPE

0.5 = Correction factor

2.6 Cytotoxicity Assay of the Extracts

Cytotoxicity of the extracts was evaluated using the end-point cytopathic effect assay on L20B cell lines. The Poliovirus (AFP Isolates (Vaccine Strains P1&P3) MDG-17-04852; MDG-17-04881; WHO National Polio Lab. University of Maiduguri Teaching Hospital, Maiduguri, Nigeria). In this assay, L20B cells were seeded onto a 96-well plate at a concentration of 10⁴ cells/ml and a volume of 100 µl per well. A volume of 100µl of the different concentrations of test extracts (100mg/ml, 50mg/ml, 25mg/ml and 12.5mg/ml) were added to culture wells in quadruplicate. Culture medium without any drug was used as the cell control. The plates were incubated at 36°C in a standard incubator and observed daily under the inverted microscope for cytopathic effect for 7 to 10 days before termination [22]. In addition, a slight modification was made as this concentration was toxic to the cell therefore, 10 fold serial dilution of each extracts at the same concentration was performed and 10⁻⁴ has been more appropriate.

2.7 Antiviral Activity Assay

Various concentrations of the extracts (100mg/ml, 50mg/ml, 25mg/ml and 12.5mg/ml) were mixed in equal volumes (100 µl) with 100 TCID₅₀ of the virus, all in 2% EMEM. These were incubated for 2 hours 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 36°C and scored daily using an inverted microscope for cytopathic effect for 10 days as described by [27].

2.8 Polio Virus Intratypic Differentiation (ITD)

This was performed using Real-Time Polymerase Chain Reaction (ABI 7500 version 2.3), according WHO ITD SOP protocol [23].

2.9 Data Analysis

In this study, Null Hypothesis State that the pharmacological properties of aqueous *Moringa* extracts has no antiviral effect on Acute Flaccid Paralysis isolates of Polio virus serotypes. The cell survival percentage (CSP) was calculated for cytotoxicity and antiviral activity of the aqueous extracts as described by [28]. The results were analyzed by one-way Analysis of Variance (ANOVA), using Statistical Packages for Social Sciences (SPSS) – 19. Cut-off value was considered as $P < 0.05$.

3. RESULTS

The *Moringa* extracts from this study appeared brownish, sticky, and consistent, with characteristic odour. Yield value is 11.35%; highly soluble in aqueous solvent; with Relative Density of 1.0486; pH (10%) 4.554; and has no Rancidity, with Melting point of 28°C.

The phytochemical constituents of the extracts (Table 1), revealed present of Tannins in leaves and flowers, Alkaloids and Saponins in all the

extracts. Glycosides and Steroids was found in leaves and Flavonoids in flowers only. However, phytochemicals not detected in all the extracts are Phlobatanins and Reducing Sugars, but Free Anthraquinones were found in leaves and seeds.

Cytotoxicity of *Moringa* extracts on L20B cells (Table 2) shows 50% effects in 100mg and 50mg concentrations, while 33.3 and 16.7 percent was observed in 25mg and 12.5mg, which statistically shows no significant difference between the extracts ($P > 0.05$). The titration of the tissue culture infective dose 50% (TCID₅₀) of Polio virus serotypes (Table 3), shows that Polio virus type 1 and 3 had a titre value of 10^{6.5} and 10^{6.25} respectively. This signified that the viral cells are adequate enough for this study.

Antiviral activities of the extracts on L20B cells concentrations of the extracts (100mg, 50mg, 25mg, and 12.5mg/ml) and 100TCID₅₀ of the virus were used to determine the viral inhibitory effect. The extracts has no antiviral activity on the Polio virus serotypes used in this study.

The Intratypic Differentiation (ITD) of antiviral activity of *Moringa* Leaves, Seeds, and Flowers extracts on Polio virus serotypes 1 and 3 was presented in Table 5. The ITD results observed the presence of SI₁ and SI₃ from all the extracts. These further confirmed that the aqueous *Moringa* extracts did not neutralized the virus

Table 1. Phytochemical constituents of *Moringa oleifera* extracts used in this study

Phytochemicals	Moringa extracts and test inference		
	Leaves	Seeds	Flowers
Tannins	+	–	+
Alkaloids	+	+	+
Saponins	+	+	+
Glycosides	+	–	–
Steroids	+	–	–
Flavonoids	–	–	+
Phlobatanins	–	–	–
Reducing sugars	–	–	+
Free Anthraquinones	+	+	–

Key: + = Present, – = Absent

Table 2. Cytotoxicity assay of *Moringa* extracts on L20B cells used in this study

Conc. (mg/ml)	Toxicity level (percentage toxicity) per extract/tube		
	Leaves	Seeds	Flower
100	03 (50)	03 (50)	03 (50)
50	03 (50)	03 (50)	03 (50)
25	02 (33.3)	02 (33.3)	02 (33.3)
12.5	01 (16.7)	01 (16.7)	01 (16.7)

Table 3. Titration of the Tcid₅₀ of polio virus serotypes used in the study

Virus dilution	Polio type 1				Polio type 3			
	Microtitre wells				Microtitre wells			
	1	2	3	4	1	2	3	4
10 ⁻¹	+	+	+	+	+	+	+	+
10 ⁻²	+	+	+	+	+	+	+	+
10 ⁻³	+	+	+	+	+	+	+	+
10 ⁻⁴	+	+	+	+	+	+	+	+
10 ⁻⁵	+	+	+	+	+	+	+	+
10 ⁻⁶	+	+	+	+	+	+	+	+
10 ⁻⁷	+	+	+	+	+	+	-	+
10 ⁻⁸	-	-	-	-	-	-	-	-
10 ⁻⁹	-	-	-	-	-	-	-	-

Table 4. Antiviral activity of aqueous *Moringa oleifera* extracts on polio virus serotypes used in the study

Extracts conc. (mg/ml)	P ₁			P ₃		
	L	S	F	L	S	F
100	+	+	+	+	+	+
50	+	+	+	+	+	+
25	+	+	+	+	+	+
12.5	+	+	+	+	+	+
VC	+	+	+	+	+	+
CC	-	-	-	-	-	-

Key: VC = Virus control, CC = Cell Control; P₁ = Polio type 1, P₃ = Polio type 3
 L = Leave S = Seed F = Flower
 + = Cytopathic Effect (CPE) Present
 - = No Cytopathic Effect (CPE) Absent

Table 5. Intratypic differentiation of antiviral activity of *Moringa* leaves, seeds and flowers extracts on polio virus serotypes

Moringa/PV mixtures (mg)	Primers/probes and P ₁					Primers/probes and P ₃					ITD
	PanEV	S ₁	S ₂	S ₃	PanPV	PanEV	S ₁	S ₂	S ₃	PanPV	
NTC	-	-	-	-	-	-	-	-	-	-	Valid
PC	+	+	+	+	+	+	+	+	+	+	Valid
100	+	+	-	-	+	+	-	-	+	+	SI ₁ SI ₃
50	+	+	-	-	+	+	-	-	+	+	SI ₁ SI ₃
25	+	+	-	-	+	+	-	-	+	+	SI ₁ SI ₃
12.5	+	+	-	-	+	+	-	-	+	+	SI ₁ SI ₃

Key: PC = Positive Control, NTC = Negative Test Control, PV = *Moringa* and Polio virus Mixtures, PanEV = Enterovirus family, PanPV = Polio Virus family, S₁, S₃ = Sabin -Like (Vaccine strain), P₁ = Polio type 1, P₃ = Polio type 3

studied, hence the extracts phytochemical constituents show no antiviral effect on Polio virus type 1 and 3 respectively.

4. DISCUSSION

Moringa preparations were reported as having antimicrobial, anti-trypanosomal, hypotensive, antispasmodic, antiulcer, anti-inflammatory, hypo-cholesterolemic, and hypoglycaemic activities, as well as having considerable efficacy

in water purification by flocculation, sedimentation, antibiosis and even reduction of Schistosome cercariae titre [6]. This study revealed the presence of pharmacologically useful substances such as tannins, flavonoids, saponins in the leaf, seed, and flower of *Moringa oleifera* confirmed the diverse claims and application of parts of the plant in treatment of ailments [29, 30, 31]. Anwar and Bhangar [32] found that *Moringa* leaves contain flavonoids, glycosides, steroids, alkaloids, tannins and

saponins. Seeds contained alkaloids saponins, free Anthraquinones; whereas flowers has alkaloids, tannins, flavonoids and saponins.

The presence of key elements in *Moringa* leaves, seeds and flowers as revealed by elemental analysis is no doubt responsible for its recommendation and adoption as a nutritional supplement⁶. Parts of *Moringa* trees have been used to combat malnutrition, especially among infants and nursing mothers. Three non-governmental organizations in particular (Trees for Life, Church World Service and Educational Concerns for Hunger Organization) have advocated *Moringa* as “natural nutrition for the tropics.” Leaves can be eaten fresh, cooked, or stored as dried powder for many months without refrigeration, and reportedly without loss of nutritional value. *Moringa* is especially promising as a food source in the tropics because the tree is in full leaf at the end of the dry season when other foods are typically scarce [6].

The cytotoxicity of *Moringa* extracts on L20B cells, showed 50% cytotoxic effects in 100mg/ml and 50mg/ml concentrations while 33.3% and 16.7% was observed in 25mg/ml and 12.5mg/ml respectively. Similar cytotoxicity level was detected by [33] and [22] who also used the same plant. The titres of tissue culture infective dose 50% (TCID₅₀) on Polio virus serotypes 1 and 3 ($10^{6.5}$ and $10^{6.25}$) is consistent with the findings of [22].

Unlike in this study, antiviral assay of *Moringa* by [33], confirmed that the seed had antiviral properties against Newcastle Disease virus. This was revealed by the total inhibition of virus growth in ovo at 100, 200 and 250 mg/ml. Okoye et al.[22] also reported that Poliomyelitis viral infectivity was inhibited by *Moringa oleifera* Lam leaf extracts giving a range of specificity indices of 2.47 to >125. This shows that the extracts selectively inhibited the virus and 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. However, despite its diverse claim as an excellent source for antiviral drugs due to fewer side-effects, low cost, more bioavailability, and easy availability along with less potential to cause resistance; *Moringa* was reported to possess antiviral activity against DNA and RNA viruses [34].

The results of our study, indicated the plant extracts did not exhibit any antiviral potential on

polio virus serotype 1 and 3 used. These could be due the fact that [21] used the National Institute of Biological Stock Control (NIBSC) strains of the virus, which is a positive control we usually used to confirmed positive cases. They also used both aqueous and ethanolic extracts which showed inhibition. But the present study used polio virus isolated from acute flaccid paralysis cases generated from the WHO National Polio laboratory, University of Maiduguri Teaching Hospital, Nigeria. This could be considered as vaccine strain slightly mutated due to host physiological changes and ecological variations as the virus was known to mutate to what is now called Vaccine derived polio virus (VDPV) that causes paralysis [35] (WHO, 2007).

This study found that the non-inhibitory effect of the plant observed was not just due to cells cytotoxicity or deterioration but actual inability of the extracts to exert its pharmacological effects on the virus using Real-Time Polymerase Chain Reaction (RT-PCR). The extracts-virus mixtures were used to determine the Intratypic Differentiation (ITD) of antiviral activity of *Moringa* Leaves, Seeds, and Flowers extracts on Polio virus serotypes 1 and 3. However, the results revealed the presence of SI₁ and SI₃ from all the extracts. These further confirmed that the extracts did not neutralized the virus strains. Therefore the extracts phytochemical constituents has no antiviral effect on Polio virus type 1 and 3 respectively. In view of the above therefore, *Moringa oleifera* extracts has no antiviral potential as potent therapeutic drug for management of poliomyelitis.

5. CONCLUSION

In this study, there was 50% cytotoxic effect observed on L20B cell line at 100mg and 50mg/ml concentrations of aqueous extracts of *Moringa* leaves, seeds and flowers used. The titration of TCID₅₀ of Polio virus stock produced the titre of $10^{6.5}$ and $10^{6.25}$ for serotypes 1 and 3 respectively. The phytochemical screening of aqueous the extracts confirmed earlier results of the presence of saponins, alkaloids, glycosides, tannins, steroids, flavonoids, and free Anthraquinones. Aqueous extracts evaluated showed no antiviral activity on Polio virus isolated from acute flaccid paralysis (AFP) cases used in this study, even after exposure time of 10 days. RT-PCR analysis confirmed that the aqueous phytochemical constituents could not neutralize the poliovirus serotypes used in the study.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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