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ANTI-MEASLES VIRUS ACTIVITY OF SELECTED MEDICINAL PLANTS OF NIGERIA

ABSTRACT

This study was carried out as a preliminary investigation into selected medicinal plants of Nigeria with the mind of discovering and developing a drug with anti-measles virus activity as an alternative to vaccination. Ten plant parts were dried, powdered and extracted by cold maceration using absolute methanol, and maximum non-toxic dose (MNTD) of each extract to Vero cell was determined. The cytotoxic activity and ability of extracts to inhibit viral-induced cytopathic effect (CPE) in tissue culture were evaluated three days post-inoculation and incubation, by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. Cytotoxic concentration at 50% (CC_{50}) and inhibitory concentration at 50% (IC_{50}) were determined using graphpad prism, and selective index (SI) was calculated as ratio of CC_{50} to IC_{50}. Out of the ten plant extracts screened, *Xylopia aethiopica* leaf extract with IC_{50} of 1.248µg/ml, *Uvaria chamae* root and stem bark extracts with IC_{50} 1.216µg/ml and 3.281µg/ml, respectively demonstrated significant in vitro anti-measles virus activity. Bioassay-guided fractionation and further screening of active extracts showed activity to reside in the hexane and dichloromethane fractions of *X. aethiopica* leaf and *U. chamae* root and stem barks. These results suggest that these two plants could possibly lead to anti-measles virus drug discovery and development.

Keywords: Medicinal plant, anti-measles activity, cytotoxicity, cytopathic effect,

1. INTRODUCTION

Measles is a highly contagious disease caused by measles virus. Before widespread vaccination in 1980, measles caused an estimated 2.6 million deaths each year (1). Despite the availability of a safe and cost-effective vaccine, measles remains one of the leading causes of death among young children globally. Approximately 122,000 (mostly children under age five) died from measles in 2012 (1). Despite the availability of effective live vaccines, measles is still responsible for 4% of deaths in children younger than 5 years of age worldwide (2). Measles virus (MV) is transmitted via aerosol droplets. Measles virus, a member of the genus *Morbillivirus* in the family *Paramyxoviridae*, is an enveloped virus with a non-segmented, negative-strand RNA genome (3). Measles virus causes a common, acute

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infectious disease characterized by fever, cough, conjunctivitis and a generalized maculopapular rash (3,4).

Medicinal plants contain substances that could be used for therapeutic purposes or which are precursors for the synthesis of useful drugs. Medicinal uses of plants range from the administration of the roots, barks, stems, leaves and seeds to the use of extracts and decoctions from the plants (5,6). Many plants are constantly being screened for their anti-inflammatory, diabetic, antibacterial, anticancer, and antiviral properties. Scientific research is also being carried out on them to ascertain, validate, and verify their medicinal potentials, and to isolate the bioactive agents and the active compounds (7). Medicinal plant products have been used as folk remedies for different kinds of ailments including viral diseases. Infectious viral diseases still constitute a major threat to public health, therefore there is an increasing need for search of new compounds with antiviral activity as the treatment of viral infections with the available antiviral drugs is often unsatisfactory. The problem of viral resistance and latency leads to conflicting efficacy in recurrent infections in immune-compromised patients. In this study, selected plants in Nigerian ethnomedicine reported to have antimicrobial activities were screened for anti-measles virus activity.

2. MATERIALS AND METHODS

2.1 Plant materials, collection, authentication and extraction

The plant materials used were selected based on their reported anti-microbial activities. Fresh plant samples were collected from medicinal herb sellers at Bode market, and Forestry Research Institute of Nigeria (FRIN), both in Ibadan, South-west Nigeria. *Uvaria chamae* plant parts (leaf, root and stem bark) were collected from Eruwa, and *Xylopia aethiopica* plant parts (leaf and stem bark) from Ibadan, Oyo-state. Plant collections were authenticated at the herbarium unit of FRIN where voucher specimens were deposited for identification. The various plant parts were ground with a hammer mill and air-dried. Known weight (100gm) of each plant part was extracted by maceration at room temperature in methanol (redistilled) for 72h. Each extract was filtered and solvent removed using a rotary evaporator at 40^0C. Dried extracts were stored in the refrigerator (4^0C) until assayed. Ten extracts from medicinal plant parts from Nigerian ethno-medicine namely: *Diospyros barteri* leaf, *Xylopia aethiopica* leaf and stem bark, *Picralima nitida* stem, *Cajanus cajan, Argemone Mexicana, Morinda lucida, Uvaria chamae* leaf, stem and root bark, were used. The methanol extract of active plants were fractionated using liquid-liquid extraction into hexane, dichloromethane, ethylacetate and methanol.

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69 **2.2 Preparation of extract stock**

70 Crude extracts (5mg each) were dissolved in dimethylsulfoxide (DMSO) and filtered using a
71 sterile membrane filter of 0.2µm pore size, to give a concentration of 1mg/mL as stock. This
72 was diluted ten-fold to obtain a final working concentration of 100µg/mL designated as
73 "neat".

74

75 **2.3 Virus and cell line**

76 Measles virus was propagated from live attenuated (freeze-dried) measles vaccine which was
77 obtained from the Public Health Centralized Immunization Clinic of the University College
78 Hospital (UCH), Ibadan, Nigeria. It was manufactured by Serum Institute of India Ltd., with
79 batch number ZA131-XB. Vero cell used for virus propagation, cytotoxicity and antiviral
80 studies was obtained from the WHO Reference Polio laboratory in the Department of
81 Virology, UCH, Ibadan, Nigeria. The cells were grown in Eagle's Minimum Essential
82 Medium (MEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml of
83 penicillin, 100µg/ml of streptomycin, 2mM L-glutamine, 0.07% $NaHCo_{3,}$ and 1% non-
84 essential amino-acids and vitamin solution. Maintenance medium used for the assays
85 contained only 2% fetal bovine serum. Virus titre was determined by cytopathic effect in
86 Vero cells and expressed as 50% Tissue Culture Infective Concentration ($TCID_{50}$) per mL.
87 The virus solution was dispensed in 1ml cryovials and stored at -80^{0}C until use.

88

89 **2.4 Maximum non-toxic dose (MNTD) of extracts**

90 The maximum non-toxic dose (MNTD) for an extract or fraction was the dilution of extract at
91 which, by microscopic examination cells showed normal morphology and cell density in the
92 presence of extracts when compared to control cells grown without extract, and showed at
93 least 95% of the optical density of the untreated cells as measured by a spectrophotometer (
94 multiscan 347, MTX lab) at 540nm in MTT assay. Each of the crude extracts was re-
95 dissolved in dimethylsulfoxide (DMSO) to give a concentration of 1 mg/mL from which 0.1
96 mL (100 µg) was added to 0.9 mL of maintenance medium to obtain a dilution of 100 µg/mL,
97 designated as "neat". Serial ten-fold dilution of the extracts was made from "neat" using
98 maintenance medium as diluents to obtain different concentrations (10^{-1} to 10^{-6}). Using a
99 microtitre dispenser, 50 µL of extract from the various dilutions made, was dispensed in
100 triplicate to 96-well microtitre plates previously seeded with monolayers of Vero cells. The
101 plates were incubated at 37^{0}C in 5% carbon-dioxide humidified incubator for 72 h observed
102 under the microscope for cytopathic effect and scored accordingly. Scoring of wells was from

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1+, 2+, 3+ to 4+ corresponding to 25%, 50%, 75% and 100% CPE. The minimum dilution of extracts with no toxic effect on the cells ie: where the cell morphology was 100% was referred to as maximum non-toxic dose (MNTD).

2.5 Cytotoxicity Assay

2.5.1 The MTT colorimetric assay

The MTT (3-(4,5-dimethyl thiazole-2-yl)-2,5-diphenyl-tetrazolium bromide) colorimetric assay (8) was used to evaluate the reduction of viability of cell cultures in the presence and absence of the extracts. The basic assay involved infecting the cell culture with the virus in the presence of test agents (plant extracts/fractions). The ability of the extracts to inhibit viral induced cell-killing was measured post-infection using the tetrazolium dye (MTT), which was metabolized by mitochondrial enzymes of viable (surviving) cells to an insoluble, coloured formazan product. The level of metabolism that occured in the individual well of the 96-well microtitre plate was dependent on the number of healthy viable cells present, which was inversely proportional to the level of CPE and cell destruction caused by the virus.

2.5.2 Determination of Cytotoxicity of extracts using MTT colorimetric assay

This was done to determine the cytotoxic concentration of extracts/ fractions at 50% (CC_{50}). After treatment with ten-fold serial dilution (100 to 0.001µg/ml) of each extract as described earlier, the plates were incubated at 37^0C in a carbon-dioxide environment for 72 h. The medium was removed, and 25µl of MTT solution (5mg/ml in DMSO) was added to each well and incubated for 2h at 37^0C. MTT solution was removed from the wells and 125µl DMSO was added to dissolve insoluble formazan crystals formed. The plates were shaken gently for 15mins, and optical density was measured using a multiscan 347 spectrophotometer at 540nm. Data obtained from quadruplicate wells were used to determine the $CC_{50.}$ The percentage cytotoxicity was calculated as:

% Cytotoxicity = (A-B) /A x 100

Where, A = the mean optical density of untreated cells.

B = the optical density of cells treated with plant extracts/ fractions.

2.6 Anti-measles virus activity assay

This was done using inhibition of cytopathic effect (neutralization assay). Serial two-fold dilutions were made from MNTD of each extract/fraction (Table 1). Viral dilutions were done with 2% maintenance medium to obtain 100 $TCID_{50}$ per drop of 50µl. The serial two-

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fold dilutions (50µl) of the extracts/fractions were added to confluent monolayer of cells in a 96-well plate in quadruplicates, and allowed to adsorb for 60mins, after which 50µl of 50% tissue culture infective dose ($TCID_{50}$) virus suspension was added. For cell control, a row of cells was not infected with virus and not treated with extracts while, for virus control, a row of cells was infected with the same concentration of virus but without addition of extracts/fractions. Maintenance medium was added to all the wells to make up to same volume. The plates were incubated at 37^0C in 5% CO_2 humidified incubator for 72h. Plates were observed under the microscope and wells were scored from 1+, 2+, 3+ to 4+ representing 25%, 50%, 75% and 100% CPE, respectively (Table 2). The concentration of extract/fraction that reduced 50% of CPE with respect to the virus control was estimated from the statistical plots of the data and was defined as the 50% inhibitory concentration (IC_{50}). Fractions from the two most active plants were subjected to MTT colorimetric assay (8).

2.7 Statistical analysis

Data obtained were analyzed using non-linear regression statistical programme (graphpad prism) to determine the 50% cytotoxic concentration (CC_{50}) and the 50% inhibitory concentration (IC_{50}) for each extract/fraction. The selective index is a comparison of the amount of a test agent that causes the inhibitory effect to that amount that causes death.

3. RESULTS

3.1 Preliminary antiviral screening

Of the twelve medicinal plant extracts screened, three extracts namely, *Xylopia aethiopica* leaf, *Uvaria chamae* root and stem bark were found to exhibit anti-measles activity at 1.248µg/ml, 1.216µg/ml and 3.281µg/ml respectively, concentrations non-toxic to the cell line used. Extracts of *Diospyros barteri*, *Cajanus cajan*, and *Argemone mexicana* had the lowest MNTD on Vero cell in tissue culture with concentration of 1 µg/ml each. The other plant extracts had the same MNTD with concentration of 10 µg/ml each as shown in Table 1. Results obtained from the preliminary antiviral screening showed that only three of the ten crude plant extracts tested showed activity on measles virus, inhibiting the cytopathic effect of the virus in tissue culture. Crude extract of *Xylopia aethiopica* leaf inhibited viral growth with 100% inhibition at 5 and 10µg/ml, 50% inhibition at 2.5µg/ml and 25% inhibition at 1.25µg/ml. Extract of *Uvaria chamae* root bark also showed 100% inhibition at 5 and 10µg/ml, 50% inhibition at 2.5µg/ml, and 25% inhibition at 1.25µg/ml. *Uvaria chamae* stem

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bark extract inhibited viral growth by 100% at 10µg/ml, 75% inhibition at 5µg/ml and 50% inhibition at 2.5µg/ml, however, there was no activity at 1.25µg/ml as shown in Table 2.

3.2 Cytotoxicity Activity

The CC_{50} determined using a non-linear regression statistical programme, showed that the crude extract of *Uvaria chamae* root bark was most toxic among the three plant extracts with CC_{50} of 15.90 µg/ml, while *Uvaria chamae* stem bark was least toxic with CC_{50} of 38.92 µg/ml. Methanol fraction from *Xylopia aethiopica* leaf was more toxic than its crude extract with CC_{50} of 10.47 µg/ml and most toxic among all the fractions of the other plant extracts. The methanol fraction from *Uvaria chamae* stem bark was more toxic than its other fractions with CC_{50} value of 23.01 µg/ml, while hexane, dichloromethane, and ethylacetate fractions and the crude extract have similar cytotoxic activity pattern with CC_{50} values of 39.10 µg/ml, 38.95 µg/ml, 38.15 µg/ml and 38.92 µg/ml respectively (Table 3).

3.3 Anti-measles virus Activity

Results obtained were concentration- dependent and showed that the crude extracts of *U. chamae* root bark and *X. aethiopica* leaf had similar inhibitory activity on measles virus in culture medium with IC_{50} of 1.216 µg/ml and 1.248 µg/ml respectively (Table 3) . The crude extract of *Uvaria chamae* stem bark had the least inhibitory activity with IC_{50} of 3.281 µg/ml. However, the dichloromethane fraction of *U.chamae* stem bark showed the highest anti-measles virus activity among all the fractions tested with IC_{50} of 1.8×10^{-1} µg/ml while the dichloromethane fraction of *X. aethiopica* leaf followed closely with IC_{50} of 6.9×10^{-1} µg/ml. The hexane of *X. aethiopica* leaf followed with IC_{50} of 1.25 µg/ml. The methanol and ethylacetate fractions were not very active except for the ethylacetate fraction of *X. aethiopica* leaf that showed fair activity with IC_{50} of 2.0 µg/ml. All extracts/fractions showed agreeable correlation coefficient (R^2) values.

4. DISCUSSION

Recently interest in plant extracts exhibiting antimicrobial activity and isolation of their active principles have been on the increase. Medicinal plants have been used for different kinds of ailments and infectious diseases including viral diseases; for example, plant species such as Lemon balm (*Melissa officinalis*), garlic (*Allium sativum*), and tea tree (*Melalenca alternifolia*) have broad spectrum antimicrobial property. Also, medicinal mushroom (*Lepista nuda*) is reported to possess anticancer, antimicrobial, antiviral, anti-inflammatory, and diabetic activities (9), while Indian gooseberry (*Phyllanthus emblica*) is reported to have

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antimicrobial, antiviral, and anticancer properties (10). A plant extract is considered active when the antiviral activity of crude plant extract is detected in at least two subsequent dilutions of the maximum non-toxic concentration to ensure that the activity is not directly correlated with the toxicity of the extract (11). The most active plants: *Xylopia aethiopica* leaf and *Uvaria chamae* stem and root bark were selected for fractionation and further antiviral screening. *Xylopia aethiopica* and *Uvaria chamae* both belong to the family Annonaceae. *Xylopia aethiopica* is an aromatic plant whose fruits are commonly used as spice in the Western part of Africa. Traditionally, a combination of various parts of the plant have been employed in different therapeutic preparations (12), such as skin infections, gastrointestinal infections like cholera and dysentery. Plants of the genus *Xylopia* have been reported to yield products such as acetogenins, alkaloids, flavonoids, and terpenoids (13). Even though it has been reported to have broad spectrum antimicrobial activity (14), there is no report of its use in the treatment of viral infections. *Xylopia* species are characterized by high contents of alkaloids and flavonoids which have been confirmed to be responsible for their observed biological activities (15). It was reported that some alkaloids isolated from *X. championii* stem and stem bark showed high scavenging activity (16) while, two alkaloids isolated from the same plant exhibited exceptionally high antioxidant activity at a concentration of 0.5mg/mL compared to the standard antioxidant DL-α-tocopherol in the DPPH assay (17)

Uvaria chamae is a medicinal plant used widely to treat fevers and has antibiotic properties (18). It has been reported to be used against diseases such as cancer, jaundice, typhoid, syphilis, and gonorrhea (19). A flavanone compound, uvarinol isolated from *U. chamae* was reported to have cytotoxic activity. Phytochemical studies of the plant revealed the presence of bioactive components comprising flavonoids, alkaloids, tannins, saponins, and phenols which have been reported to be responsible for the medicinal properties of *U. chamae*, which form the basis of its use in herbal medicine in Nigeria. Okwu and Omodamiro (20) reported that its particularly high flavonoid content gives it its protective activity against allergies, microbes, platelet aggregation, ulcers, hepatoxins, viruses and tumors.

The CC_{50} values of hexane, dichloromethane fractions of *X. aethiopica* leaf, and dichloromethane fraction of *U. chamae* stem bark were much higher than the IC_{50} values, which indicate that they are non-toxic and suitable for anti viral use; as compared with the methanol fraction of *X. aethiopica* leaf which was highly toxic with CC_{50} and IC_{50} values of 10.47µg/ml and 19.78µg/ml respectively. The possibility of crude extract showing more biological activity than its fractions can be attributed to synergistic activity of compounds

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present in the plant (21). This probably explains the higher anti-measles virus activity exhibited by the crude extract of *U. chamae* root bark with IC_{50} of 1.22µg/ml, than the fractions (Table 3). The IC_{50} values of the dichloromethane fraction of *U. chamae* stem bark, and the dichloromethane and hexane fractions of *X. aethiopica* leaf as shown in table 3, is an indication that the anti-measles virus activity of both plants resides in their non-polar fractions. Even though the anti-oxidant and antitumor activities of these two plants have been associated with their high contents of acetogenins, alkaloids, and flavonoids (13, 17), there is a dearth of report of their antiviral activity.

5. CONCLUSION

The two active plants found in this study *Xylopia aethiopica* and *Uvaria chamae* belong to the family Annonaceae. Three fractions showed significant anti-measles virus activity. Further studies are on-going to isolate and identify the anti-measles virus constituents of these active fractions. This may provide useful guide in antiviral drug discovery and development.

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Table 1: MNTD of crude plant extracts on Vero cells

Plant extracts	Family	MNTD(µg/ml)
*Diospyros barteri (*leaf)	Ebeneceae	1
Xylopia aethiopica (leaf)	Annonaceae	10
Xylopia aethiopica (stem bark)	Annonaceae	10
Picralima nitida (stem)	Apocynaceae	10
Cajanus cajan (whole plant)	Fabaceae	1
Argemone mexicana (whole plant)	Papaveraceae	1
Morinda lucida (whole plant)	Rubiaceae	10
Uvaria chamae (leaf)	Annonaceae	10

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Uvaria chamae (stem bark)	Annonaceae	10
Uvaria chamae (root bark)	Annonaceae	10

MNTD=Maximun non-toxic dose

Table 2: Preliminary Anti-measles activity of active plant extracts

Plant extract	Concentration (µ/ml)	%Inhibition
Xylopia aethiopica leaf	10	100
	5	100
	2.5	50
	1.25	25
Uvaria chamae root bark	10	100
	5	100
	2.5	50
	1.25	25
Uvaria chamae stem bark	10	100
	5	75
	2.5	50
	1.25	-

Anti-measles virus activity as determined by observation of CPE under microscope

Table 3: Cytotoxic and anti-measles virus activity of crude extracts and fractions of active plants

Extracts/fractions	MNTD(µg)	CC_{50} (µg/ml)	IC_{50} (µg/ml)	SI(CC_{50}/IC_{50}
Xylopia aethiopica leaf				
Crude	10	29.25	1.248	23.4
Hexane	10	35.72	1.246	28.7
Dichloromethane	10	38.78	0.691	56.1
Ethylacetate	10	22.19	2.020	11.0
Methanol	1	10.47	19.78	0.5
Uvaria chamae root bark				
Crude	10	15.90	1.216	13.1
Hexane	10	26.37	4.663	5.7
Dichloromethane	10	26.38	4.619	5.7

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Ethylacetate	10	25.77	30.89	0.8
Methanol	10	45.52	6.255	7.3
Uvaria chamae stem bark				
Crude	10	38.92	3.281	11.9
Hexane	10	39.10	3.284	11.9
Dichloromethane	10	38.95	0.182	214.0
Ethylacetate	10	38.15	8.880	4.3
Methanol	1	23.01	100.4	0.2

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