academicJournals

Vol. 12(24), pp. 3878-3883, 12 June, 2013
DOI: 10.5897/AJB12.2730
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Anti-poliovirus activity of medicinal plants selected from the Nigerian ethno-medicine

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Accepted 10 May, 2013

This study was carried out to validate acclaimed anti-poliovirus effect of crude methanol extracts from 14 medicinal plants used by traditional healers in Southwest Nigeria. Plant samples were powdered and extracted by cold maceration into absolute methanol and maximum non toxic concentration (MNTC) of each plant extract to rhabdomyosarcoma (RD) cells was determined in tissue culture. Using serial two-fold dilution of the MNTC (specific for each extract), ability of extract to inhibit viral-induced cell death (CPE) in tissue culture was evaluated three days post-infection by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assays. 50% inhibitory concentration (IC_{50}) and 50% cytotoxic concentration (CC_{50}) was determined by statistical analysis. Selective index was calculated as ratio of CC_{50} to IC_{50}. Out of the 14 plant extracts evaluated for anti-poliovirus activity, *Senna siamea* (Lamk.) Irwin et Barneby (bark extract) and *Zephyranthes candida* Lindl (whole plant) demonstrated significant *in vitro* activity with IC_{50} of 0.0019 and 0.121 µg/mL, respectively. Bioassay-guided fractionation of extracts indicated that activities were retained in chloroform fraction of *Z. candida*, and also in hexane and chloroform fractions of *S. siamea*, but none of the polar fractions were active. These results support the traditional use of *S. siamea* and *Z. candida* as antiviral agents and suggest that they could provide a possible source for anti-poliovirus drug discovery and development.

Key words: Anti-poliovirus activity, traditional medicine, MTT colorimetric assay.

INTRODUCTION

From the early times, plants have always been the basis of African traditional medicine. High reliance on medicinal plants and concurrent use of orthodox medicine for the treatment of infectious diseases is prevalent in Nigeria and in most African countries. The use of traditional medicine in Africa is widespread with an estimated 80% of the population consulting traditional healers. In addition to being accessible, the traditional healthcare system offers a cheaper, individualized and culturally acceptable alternative to the costly allopathic system (Eastman, 2005). Infectious viral diseases still constitute a major threat to public health. This remains an important global challenge due to the fact that viruses have resisted prophylaxis or therapy longer than any other form of life (Vanden Berghe et al., 1986). Viral resistance and viral latency leading to recurrent infections in immunocompromised patients have been some of the reasons for continuous search for newer antiviral drugs (Field and Biron, 1994; Severson et al., 1999; Khan et al., 2005). A wide range of ethnomedicinal plants showed strong antiviral activities either by inhibiting replication, or genome synthesis of many viruses (Semple et al., 2001; Kott et al., 1999; Mohamed et al., 2010). Hence, development of new antivirals from natural source is a strong alternate

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Table 1. Maximum non toxic concentration of crude plant extracts[a]

Plant extracts (part use[b])	Family	MNTC (µg/ml)
Crysophyllum albidum (s)	Sapotaceae	10
Khaya senegalensis (l)	Meliaceae	1
Khaya senegalensis (sb)	Meliaceae	1
Lippia multiflora (l)	Verbenaceae	1
Parquetina nigresence (rb)	Periplocaceae	10
Poga oleosa (f)	Rhizophoraceae	10
Senna siamea (sb)	Fabaceae, Caesalpinioideae	1
Sida acuta (w)	Malvaceae	10
Spondias mombin (sb)	Anacardiaceae	1
Spondias mombin (l)	Anacardiaceae	1
Tetrapleura teraptera (s)	Mimosaceae	0.1
Thoningia sanguinea (f)	Balanophoraceae	10
Uvaria chamae (sb)	Annonaceae	10
Zephyranthes candida (w)	Amaryllidaceae	0.1

l = leaf; s = seed; f = fruit; sb = stem bark; rb = root bark.

(Chattopadhyay, 2006).

Poliomyelitis caused by the Poliovirus is a major cause of morbidity and mortality among children in developing countries. Polio eradication by vaccination of children in Nigeria has been largely unsuccessful due to the characteristic problems of accessibility, limited supervision, cultural hindrances and occasional vaccine-associated paralytic poliomyelitis. The need to consider alternative ways of managing the infection becomes imperative.

Ethnobotany has been described as a useful tool in selection of plants containing compounds active against viruses that cause human disease (Vlietinck and Vanden Berghe, 1991; Ajaiyeoba and Ogbole, 2005). This informed our interest in carrying out an ethnobotanical survey in 4 local government areas in South-western Nigeria, which led to the identification of plants with potential antiviral activities (Ajaiyeoba et al., 2006). From the survey, 14 medicinal plants were selected for preliminary evaluation of anti-poliovirus activity. These were *Sida acuta* Burm.f. (Malvaceae) leaf, *Thonningia sanguinea Vahl* bulb, *Chrysophyllum albidum* G.Don seed, *Senna siamea* (Lamk.) Irwin et Barneby leaf and stem bark, *Lippia multiflora* (Moldenke) leaf, *Zephyranthes candida* Lindl whole plant, *Parquetina nigrescens* (Afzel. Bullock) root bark, *Tetrapleura tetraptera* (Schumach & Thonn) seed, *Uvaria chamae* (Beauv) bark, *Poga oleosa*. (Pierre) seed, *Khaya senegalensis* stem and bark, *Spondias mombin* (Linn) stem and bark (Ajaiyeoba et al., 2006).

MATERIALS AND METHODS

Cells and viruses

Poliovirus Type-PV1 (Sabin strain) was obtained from stool isolate at the WHO Polio Laboratory, Department of Virology, University of Ibadan, Nigeria. Human Rhabdomysarcoma (RD) cells (CDC, Atlanta, Georgia) was used for both cytotoxic and antiviral studies. Cells were grown in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml of penicillin, 100 µg/ml of streptomycin, 2 mM L-glutamine, 0.07% $NaHCO_3$, and 1% non-essential amino acids and vitamin solution. The test medium used for cytotoxic assays and antiviral assays contained only 2 and 5% of fetal bovine serum. Virus titers were determined by cytopathic effect in RD cell and were expressed as 50% tissue culture infective concentration ($TCID_{50}$) per ml. All viruses were stored at −70°C until use.

Plant materials collection, authentication and extraction

Plants were selected based on frequency mentioned in the survey and availability. Stem bark and root bark as well as leaves were collected by staff of Forestry Research Institute of Nigeria (FRIN); seeds were bought from Oje market, Ibadan, Oyo State, Nigeria. *Z. candida* was collected from the grounds of the University College Hospital (UCH), Ibadan. Voucher specimens were identified by Mr. Seun Osiyemi at the herbarium of Forestry Research Institute of Nigeria (FRIN). Samples were deposited at the herbarium under various FHI numbers. The plants were sun-dried and ground to powdery forms. Each plant (400 g) was extracted by maceration in redistilled methanol for 72 h at room temperature (RT). Extracts were filtered and the solvents were evaporated using a rotary evaporator at 40°C. The dried extracts were stored in the refrigerator at 4°C until needed for bioassay. The crude methanol extract of active plants was fractionated, using liquid-liquid extraction into hexane, chloroform, ethyl acetate and methanol.

Preparation of extracts stock

Crude extracts, 5 mg each were dissolved in dimethlysulfoxide (DMSO) and filtered through a sterile syringe filter (0.2 µm pore diameter) to give a concentration of 1 mg/ml stock; the stock was

diluted to obtain a final concentration of 100 µg/ml (0.1% DMSO), designated as "neat".

Maximum non toxic concentration test of extracts

This test was carried out to determine the maximum non toxic concentration test (MNTC) of crude extract to rhabdomyosarcoma cell in tissue culture. Serial ten-fold dilutions (100 to 0.001 µg/ml) of the extracts were made with maintenance medium. 96-wells plates previously seeded with monolayers of RD cells were treated with various concentrations of each extract. Plates were incubated at 37°C in 5% CO_2 humidified incubator for 72 h. Plates were then observed under the microscope for cell death/cytophatic effect (CPE). The minimum dilution of extracts with no toxic effect on the cells was referred to as maximum non toxic concentration. The maximum non-toxic concentration (MNTC) for an extract was the dilution of extract at which the microscopic examination, cells showed normal morphology and cell density in presence of extracts when compared to control cells grown without extract, and showed at least 95% of the optical density of the untreated cells as measured by a spectrophotometer at 540 nm (Multiscan 347, MTX lab) in MTT assay.

Cytotoxicity assay

The MTT colorimetric assay

A tetrazolium yellow colorimetric assay was used to evaluate the reduction of viability of cell cultures with or without the extracts. The basic assay involved infection of cells culture with the virus in the presence of test compounds. The ability of the compounds to inhibit cell killing is measured post-infection using MTT dye; mitochondrial enzymes of viable cells convert MTT to a soluble, colored formazan product. The assay was carried out as earlier described by Mossmann (1983).

Determination of cytotoxicity of extracts by MTT colorimetric assay

After treatment with serial ten-fold dilutions (100 to 0.001 µg/ml) of each extract as described earlier, plates were incubated for 48 h at 37°C. The medium was removed, and then 25 µl of MTT solution (5 mg/ml) was added to each well and incubated for 2 h at 37°C. MTT solution was removed from the wells and 120 µl dimethylsulfoxide (DMSO) was added to dissolve insoluble formazan crystal. Optical density was measured spectrophotometrically (Multiscan 347, MTX lab) at 540 nm. Data obtained from quadruplicate wells was used to determine CC_{50}. The percentage cytotoxicity was calculated as:

$$\% \text{ cytotoxicity} = \frac{A - B}{A} \times 100$$

Where, A is the mean optical density of untreated wells and B is the optical density of wells with plant extracts.

Anti-poliovirus activity assay

Inhibition of cytophatic effect (CPE): Neutralization assay

Serial two-fold dilutions were made from MNTC of extracts-specific for each extract (Table 1). Viral dilution were made with 2% maintenance medium to obtain 100 $TCID_{50}$ per drop of 50 µl; to confluent cell monolayers in a 96-well plate, serial two-fold dilutions of crude extracts were added and allowed to adsorb for 60 min at 37°C, and 100 $TCID_{50}$ (50% tissue culture-infective concentration) virus suspension was then added. For positive control, cells were infected with the same concentration of virus but without the addition of extract untreated cells (virus control), and as a negative or cell control, only maintenance medium was added to the cells (uninfected, untreated cell). The plates were incubated at 37°C in 5% CO_2 humidified incubator for 72 h. For the preliminary assay, plates were observed under the microscope for CPE. Scoring of wells was from 1+ to 4+ (25% to 100% of CPE). The concentration 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}).

Extracts and fractions from the two most active plants were subjected to MTT colorimetric assays (Mosmann, 1983).

Data analysis

Selective index, CC50 and IC_{50}

The 50% cytotoxic concentration (CC_{50}) and the 50% inhibitory concentration (IC_{50}) for each extract were calculated from concentration-effect-curves after linear regression analysis. The selective index is defined as CC_{50} over IC_{50}. It is the ratio given by the cytotoxic concentration divided by the inhibitory concentration. The selectivity index is a comparison of the amount of a test compound that causes the inhibitory effect to the amount that causes death.

RESULTS

Preliminary antiviral screening

Extracts *of Z. candida* and *T. teraptera* had the lowest maximum non-toxic concentration to RD cells in tissue culture medium with both of them having a concentration of 0.1 µg/ml. Result of the preliminary antiviral screening showed that only 3 out of the 14 crude plant extracts tested were active on poliovirus, inhibiting the cytophatic effect of the virus in tissue culture. Crude extract of *Z. candida* had 100% inhibition at 0.1 µg/ml, 75% at 0.05 and 0.025 µg/ml. Extract of *S. siamea* also inhibited viral growth of 100% at 1 µg/ml, with 75% at 0.5 µg/ml and 25% at 0.25 µg/ml. *L. multiflora* was active at its MNTC (1 µg/ml) with 75% inhibition, slightly active at 0.5 µg/ml with 25% inhibition. The result of the preliminary antiviral properties of plant extracts are presented in Tables 2.

Cytotoxic activity

Data analysis with statistical program GraphPad Prism showed that the crude extract of *Z. candida* had CC_{50} value of 0.2931 µg/ml, while its chloroform fraction from *Z. candida* had CC_{50} value of 0.1884 µg/ml; hexane, ethyl acetate and methanol fractions had very similar cytotoxic activity pattern with CC_{50} values of 27.5, 29.73 and 21.5

Table 2. Preliminary activity of active plant extracts[a]

Plant extract	Concentration (µg/mL)	Percent inhibition
Senna siamea bark	1	100
	0.5	75
	0.25	25
	0.125	0
Lippia multiflora	1.0	75
	0.5	25
	0.25	0
	0.125	0
Zephyranthes candida	0.1	100
	0.05	75
	0.025	75
	0.0125	0

anti-poliovirus activity were determined by observation of CPE under microscope.

Table 3. Cytotoxic and anti-poliovirus activity of crude extract and fractions of *Senna siamea*[a].

***S. siamea* extract/fraction**	**MNTC (µg)**	**CC_{50} (µg/ml)**	**IC_{50} (µg/ml)**	**SI**
Crude	1	2.96	0.121	24.4
Chloroform	10	39.24	0.227	172.8
Ethyl acetate	10	13.49	3.41	3.90
Hexane	10	12.52	0.507	24.5
Methanol	100	172.1	17.94	9.60

Cytotoxic and anti-poliovirus activity were determined by MTT assay. MNTC = maximum non toxic concentration; CC_{50} = 50% cytotoxic concentration; IC_{50} = 50% inhibitory concentration; SI = selective index.

Table 4. Cytotoxic and anti-poliovirus activity of crude extract and fractions of *Zephyranthes candida*[a].

***Z. candida* extract/ fraction**	**MNTC (µg)**	**CC_{50} (µg/ml)**	**IC_{50} (µg/ml)**	**SI**
Crude	0.1	0.293	0.0019	157.80
Chloroform	0.1	0.188 [1]	0.0012	157.60
Ethyl acetate	10	27.50	3.78	7.30
Hexane	10	29.73	1.62	18.30
Methanol	10	21.50	6.36	3.38

Cytotoxic and anti-poliovirus activity were determined by MTT assay. MNTC = maximum non toxic concentration; CC_{50} = 50% cytotoxic concentration; IC_{50} = 50% inhibitory concentration; SI = selective index.

µg/ml, respectively.

The CC_{50} value of crude extract of *S. siamea* was 2.958 µg/ml, while its fractions showed a wide range of cytotoxic activity; hexane fraction and ethyl acetate fraction had CC_{50} value of 12.52 and 13.49 µg/ml, respectively; chloroform 39.24 µg/ml; methanol fraction is the least toxic with 172.1 µg/ml.

Anti-poliovirus activity

Results obtained indicate that crude extract of *Z. candida* had IC_{50} value of 0.0019µg/ml while the crude extract of *S. siamea* had IC_{50} value of 0.21 µg/ml as shown in Tables 3 and 4. The chloroform fraction of *Z. candida* was most active with of all the fractions with IC_{50} value of

0.0012 µg/ml; it was also more active compared to the crude extract. Chloroform fractions of *S. siamea* was the most active fraction with IC_{50} value of 0.227 µg/ml, and the hexane fraction was also active with IC_{50} value of 0.507 µg/ml. The methanol fraction was not very active compared to the other fraction. All extracts/fraction showed agreeable correlation coefficient (R^2) values (a correlation coefficient R^2 of +1 indicates that two variables are perfectly related in a positive linear sense, in this case it means that the activity of extracts/fraction perfectly relate to concentration in a positive way). Selective indices varied widely between 3.38 and 172.8 (Tables 3 and 4).

DISCUSSION

Despite the popular use of medicinal plants preparations in several parts of the world, individually or in combination, report about their interactions on the living system is scarce; ethnobotanical studies have been shown to be a useful guide to the selection of plants containing compounds active against viruses that cause human disease (Vlietinck and Vanden Berghe, 1991). Out of 14 medicinal plants screened, three, namely; *Z. candida*, *S. siamea* and *L. multiflora* were active on poliovirus. The two most active plant; *Z. candida* and *S. siamea* were selected for further screening while *L. multiflora* was not fractionated since it was only active in one subsequent dilution of the maximum non-toxic concentration.

Vanden Berghe et al. (1993) suggested that for a plant extract to be considered active; the antiviral activity of crude plant extracts should be detectable 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. *Z. candida* belongs to the Amaryllidaceae family, a family of bulbous plant, which has long been known for their medicinal and toxic properties (Bastida et al., 1998).

Some plants of the Amaryllidaceae have been used in the primitive treatment of human cancer, and a variety of tumor inhibiting alkaloids has been isolated from *Zephyranthes* species, (Kojima et al., 1998). Its antitumor activity in this study might be due to the presence of some tumor inhibiting alkaloids widely distributed in the *Zephyranthes* species. It has been shown that, it is possible for the crude extract to exhibit more biological activity than the fractions; this is usually attributable to synergistic activity of compounds present in the plant (Amoros et al., 1992).

The cytotoxic activity of crude extract of *S. siamea* was observed to be greater than its fractions (Table 3); thus suggesting the possibility of synergistic effect of the extracts. All extracts/fractions of the two plants were significantly active on poliovirus and results obtained were concentration dependent.

The chloroform fraction of *Z. candida* with IC50 value of 0.0012 µg/ml, chloroform and hexane fraction of *S. siamea* with IC50 value of 0.227 and 0.507 µg/ml, respectively demonstrated the highest degree of anti-poliovirus activity, which led to the conclusion that the antiviral property of both plants resides in their medium to low polarity fractions. The cytotoxicity evaluation of the extracts and fractions corresponds directly to their antiviral activities. Ethanol extracts of *K. senegalensis* was earlier reported to have anti-poliovirus activity (Kudi and Myint, 1999); this is at variance with the findings in this study.

This may be attributed to the different cell line used. Hudson (1990) reported that variation in activity is directly related to cell types used. In contrast to earlier results reported by Ananil et al. (2000), *L. multiflora* demonstrated slight anti-poliovirus activity. This is probably due to low levels of antiviral compounds in the extract in a non-cytotoxic dilution. The cell cytotoxic concentrations (CC_{50}) of crude extract, hexane and chloroform fractions of *S. siamea* were several magnitudes higher than the effective concentrations inhibiting CPE by 50% (IC_{50}), indicating that this extracts were relatively non-toxic (SI, between 24.4 and 172.8), while the ethyl acetate and methanol fractions are highly toxic (low safety margin) and are not considered to be good fractions for antiviral use. Only the crude extracts and chloroform fraction of *Z. candida* showed acceptable therapeutic margin (≥1, the higher the therapeutic margin the safer the drug).

Kudi and Myint (1999) reported the antipoliovirus activity of some Nigerian medicinal plants which includes; *Bauhinia thonningii*, *Cassia goratensis*, *Anacardium occidentale*, *Butyrospermum parkii*, *Boswelia dalzielii*, *K. senegalensis*, while there has been no previous report on the anti-poliovirus activity of *S. siamea* and *Z. candida* from Nigerian ethnomedicine.

Conclusions

Ethnomedicine and ethnobotany have presented themselves as viable tools for drug discovery, this is more evident in this study as the two active plants, *Z. candida* and *S. siamea* were selected from an earlier ethnobotanical survey. Work is presently being undertaken to isolate and identify the antiviral components of these active extracts. It is possible that the elucidation of the active constituents in these plants may provide useful leads in the development of antiviral therapeutics.

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