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Original Article

In vitro evaluation of the antiviral activity of extracts from the lichen *Parmelia perlata* (*L.*) Ach. against three RNA viruses

Charles O. Esimone,[1] Kenneth C. Ofokansi,[1] Michael U. Adikwu,[1] Emmanuel C. Ibezim,[1] Dominic O. Abonyi,[1] Georgina N. Odaibo,[2] David O. Olaleye.[2]

[1]Division of Pharmaceutical Microbiology, Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, Nigeria; [2]Department of Virology, University College Hospital, University of Ibadan, Nigeria.

Abstract

Background: Substances extracted from lichens have previously been reported to possess antimicrobial activities against various groups of bacteria, fungi and viruses. Due to the high abundance of *Parmelia perlata* in the Eastern parts of Nigeria, we decided to explore whether it possesses antiviral activity against some common animal and human viruses.

Methodology: The dried and powdered lichen was extracted with acetone, water and 4% (v/v) NaOH (to yield a crude polysaccharide fraction) using standard methods. The cytotoxicity of the extracts was investigated on HEP-2, Vero and L_{20} cell lines. The antiviral properties were determined against yellow fever, poliomyelitis and infectious bursal disease virus of chickens using the end-point cytopathic effect assay. Phytochemical evaluations of the extracts were also carried out.

Results: Phytochemical tests showed the presence of flavonoids, saponins, tannins, glycosides, steroidal aglycone, carbohydrates and also the presence, in trace amounts, of some oligodynamic elements. Cytotoxicity tests revealed that while L_{20} was susceptible to the extracts at a concentration of 50 µg/ml, the extracts were generally toxic to the cell lines at concentrations above 500 µg/ml. The order of sensitivity of the cell lines was L_{20} > HEP-2 > Vero. The water and acetone extracts showed no activity against the viruses when tested at concentrations below the cytotoxic level while the crude polysaccharide fraction showed activity against yellow fever virus with an IC_{50} of 15 µg/ml. The time of addition of the test extracts to the infected cells did not have significant effect on cytopathic effect inhibition.

Conclusions: The results showed that the crude polysaccharide fraction from *Parmelia perlata* possesses specific antiviral activity against yellow fever virus. It is postulated that a major mechanism of inhibition of yellow fever infection by the crude polysaccharide fraction of the lichen could be by attack on the viral envelope.

Key Words: *Parmelia perlata*, Lichen, Yellow fever, infectious bursal disease virus, Polio virus, Cytotoxic activity, Antiviral activity.

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Introduction

There are fewer substances available for the treatment of viral infections when compared with the large amount of available antibiotics for the treatment of bacterial infections. At the same time, the frequencies of viral resistance to the relatively few antiviral drugs are increasing [1]. This, coupled with the challenges posed by the human immunodeficiency virus (HIV) and its related diseases, has renewed interest in the search for a new antiviral agent in recent times [2]. However, progress in the development of useful new antivirals has been painstakingly slow. Hence the increasingly urgent need to find effective therapeutics for viral infections justifies a broad and accelerated search for antiviral.

Plants and plant products present some hope to scientists, serving as an alternative avenue to discovery from the current mainstream approach of attempting to design narrow spectrum drugs for specific molecular targets [2-4]. While numerous reports exist on the antimicrobial effect of lichens against various groups of bacteria and fungi [5-8], only very few reports exist on the antiviral activity of these plants/products [9]. Recently, we have demonstrated that extracts from the lichen *Ramalina farinacea* inhibited the growth of two different viral classes (HIV-1 and Adenoviruses) [10].

In this report, the results of the determination of antiviral activity of crude extracts of the lichen *Parmelia perlata* are presented. *Parmelia perlata* grows on trees and rocks especially in the West of Britain and also in the tropics [11]. It forms large light-grey patches with rounded lobes, which often have black hairs at the edges. Clusters of greyish white powdery reproductive structures (soredia) grow on the upturned tips of some of the lobes and large spore-producing apothecia with dark-brown discs and prominent margins sometimes develop in the centre. In recent times, there has been a renewed interest in the search for antivirals from the plant kingdom [4,12]. This interest provided the necessary stimulus to undertake this present study, which aims to evaluate the *in vitro* cytotoxic and antiviral properties of extracts from the lichen *P. perlata*.

Materials and Methods

Collection of the plant material

The lichen was collected from oil bean tree (*Pentaclethra macrophylla* Benth) trunks in Umuowulla forest, Ogbozalla Opi, Nsukka Local Government Area of Enugu State, Nigeria. Mr. J.M.C. Ekekwe of the botanical garden, University of Nigeria, Nsukka, made botanical identification and Voucher specimen has been deposited in the University Herbarium.

Cell lines and viruses

The continuous cell lines used—laryngeal epidemoid carcinoma (Hep2), monkey kidney (Vero) and L_{20} cells—were propagated using RPMI (Rolswell Park Memorial Institute) medium (Gibco, Germany) supplemented with 2% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were obtained from the Department of Virology, University College Hospital, Ibadan, Nigeria.

Stock culture of poliomyelitis virus and infectious bursal disease virus (IBDV) of chickens were obtained from the virology laboratory, University College Hospital (UCH) Ibadan, Nigeria. Yellow fever virus was obtained through a passage of yellow fever vaccine (log No. 5708, STAMARIL PASTEUR) kindly supplied by the Public Health Department (National Programme on Immunization Unit), Ministry of Health, Enugu State, Nigeria.

Extraction

Sufficient samples of the lichen were oven-dried for 24 hours at 40^{o}C and ground to a powdered form using a hand mill. To obtain the crude polysaccharide fraction (CPF), a 1 g sample of the powder was weighed into a conical flask containing 20 ml of 40% w/v sodium hydroxide solution and 1% w/v sodium borohydride solution. These were shaken for 24 hours in a Gallenkamp orbital shaker at 150 rpm at 28^{o}C. The homogenate was neutralized using glacial acetic acid, centrifuged at 300 rpm for 5 minutes at 28^{o}C and the supernatant collected. The extract was precipitated and partially purified with 98 % ethanol.

For the water extract (WTE), a 200 g sample of the powdered lichen was weighed into a 1,000 ml capacity conical flask containing 400ml of distilled water and extracted for 48 hours in an oven at 40^{o}C. This was then filtered with a Whatman filter paper and the filtrate evaporated to dryness in an oven at 55^{o}C. A similar procedure to that of the water extract was adopted for the acetone extract (ACE) except that the extraction was done at room temperature (28^{o}C).

Phytochemical analysis

A small quantity of the powdered lichen was subjected to phytochemical tests using established standard procedures for the determination of alkaloids, tannins, saponins, glycosides, flavonoids, carbohydrates, fats and oil [13-15].

Preparation of Virus Stock (Inoculum Build-up)

Yellow fever virus

A yellow fever virus (vaccine) suspension (0.5 ml) was used to infect a confluent monolayer of Vero cells in 250 ml tissue culture flask and adsorbed for 1hour to allow the viruses to adhere onto the cells. Non-adherent particles were washed off using 2% RPMI (Rolswell Park Memorial Institute) medium and the infected cells overlaid with 20 ml of 2% RPMI (maintenance medium) and incubated until full cytopathic effect was observed in 5 to 6 days. This was further re-passed twice and the harvested virus stored at –20^{o}C until used.

Poliomyelitis virus and IBDV (Gumboro virus)

A 0.2 ml of stock suspensions each of infectious bursal disease virus (IBDV) and

poliomyelitis virus was measured out using a micropipette and used to infect a confluent monolayer of Vero and L_{20} cells respectively in a 50 ml tissue culture flask and processed as described for yellow fever virus. Cytopathic effects were observed after 2 days and 6 days respectively for poliomyelitis and Gumboro virus (IBDV).

Cytotoxicity tests of the extract

A 1 g sample each of the three extracts (CPF, WTE and ACE) was weighed and suspended in 9 ml each of sterile distilled water (for CPF and WTE) and 9 ml of 0.5% dimethylsulphoxide (DMSO) for ACE. The suspensions were agitated for 3 minutes to homogeneity using the vortex mixer type 16700 (Bamstead/Termolyne) before preserving at 4^{o}C. Serial dilutions of the extracts were then made to give 1%, 0.1% and 0.01% concentrations of the extracts in 2% RPMI. Thereafter, the growth medium (2 % RPMI) from the previously incubated confluent monolayers of Vero, L_{20} and HEP-2 cells in 96–well plates were aspirated and 100 µl aliquots of the diluted extracts seeded into the wells in quadruplicates. They were then overlaid with 100 µl of 2% RPMI to bring to a final volume of 200 µl in each well. Control wells were also set up in parallel with the inoculated wells. The plates were sealed and incubated at 37^{o}C and observed daily for toxicity using an inverted microscope for 7 to 10 days before termination.

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

Stepwise 10-fold (1/10) dilutions of the virus suspensions were made up to 10^{-8} in tissue culture tubes using 2% RPMI. The 100 µl aliquots of each dilution step were inoculated into the wells of a 96-well tissue culture plate containing confluent Vero cells for yellow fever virus and Infectious Bursal Disease (Gumboro) virus and L_{20} cells for poliomyelitis virus and 100 µl of 2 % RPMI bringing the overall volume to 200 µl. Each dilution step was seeded into four separate (quadruplicate) wells. The entire arrangements were incubated at 37^{o}C and scored for cytopathic effect daily for 7 to 14 days before terminating the readings. The end point titres were calculated using the method of Kinchinton *et al.* [16].

Determination of antiviral activity

Various concentrations of the extracts were mixed in equal volumes (100 µl) with 100 $TCID_{50}$ of the viruses, all in 2% RPMI. These were incubated for 1 hour before aliquots of 200 µl of each mixture were used to infect a confluent monolayer of the respective cells in a 96-well tissue culture plate. The virus and cell controls were also set alongside these. They were later incubated at 37^{o}C and scored daily using an inverted microscope for cytopathic effect for 10 to 14 days.

Results

The result of the phytochemical analysis (Table 1) shows that the lichen *P. perlata* contains flavonoids, saponins, tannins, glycosides (cardiac, cynogenetic but not the anthracene type), steroidal aglycone and carbohydrates but does not contain alkaloids.

Table 1. Result of Phytochemical Tests on *Parmelia perlata*.

Test	Inference
Alkaloids	-
Cyanogenetic Glycosides	+
Cardiac Glycosides	+
Anthracene Glycosides	-
Steroidal Glycosides	+
Saponins	+
Tannins	+
Flavonoids	+
Proteins	+
Carbohydrates	+
Reducing sugar	+

Key: + indicates presence of phytochemical secondary metabolite
- indicates absence of phytochemical secondary metabolite.

The results from phytochemical analysis have been observed to be consistent with findings in several lichen species except that the anthracene glycosides which constitute an important class of lichen substances [15] were absent; so also are the alkaloids. Other components found are abundant in several species of lichens [15, 17]. Glycosides are found commonly in lichens and include the galactose-arabitol glycoside (umbilicin) and the galactose-mannitol (peltigeroside) both of which abound in different lichen genera [18]. The

result of the elemental analysis (Table 2) shows that zinc and lead are present in larger quantities (0.632 and 0.716 mg/100g sample respectively) than cadmium, silver and chromium which were 0.038, 0.216 and 0.036 mg/100 g respectively while mercury, argon and cobalt were not present in detectable quantities. Low quantities of zinc and copper had earlier been reported in the lichen *Ramalina farinacea* [17] which was attributed to the natural habitat of the lichen (tree trunks) where these elements exist in minute quantities.

Table 2. Elemental Analysis of the Lichen, *Parmelia perlata*.

Element	Concentration in lichen (mg/100g of lichen)
Zn	0.632
Pb	0.716
Cd	0.038
Ag	0.216
Cr	0.036
Hg	ND
Co	ND

ND = Not detectable.

A summary of the cytotoxicity tests of the extracts on the cell lines (Hep-2, Vero and L_{20} cells) is clearly depicted in Table 3.

Table 3. Toxicity of the extracts on Hep-2 Vero and L20 cells.

		Concentration of Extract (µg/ml)					
Cells	Extracts	10^5	10^4	10^3	10^2	10	1
Hep-2	CPF	+	+	-	-	-	-
	ACE	+	+	-	-	-	-
	WTE	+	-	-	-	-	-
Vero	CPF	+	-	-	-	-	-
	ACE	+	-	-	-	-	-
	WTE	+	-	-	-	-	-
L_{20}	CPF	+	+	+	-	-	-
	ACE	+	+	+	-	-	-
	WTE	+	+	+	-	-	-

It is evident that the alkali extract (CPF) was toxic to Hep-2 cells up to10^4 µg/ml, whereas toxicity was observed for Vero and L_{20} cells at 10^5 µg/ml and 10^3 µg/ml respectively. A similar trend was observed for the acetone extract (ACE). The water extract (WTE) exhibited little toxicity to both Hep-2 and Vero cell (10^5µg/ml) but showed toxicity up to 10^4 µg/ml in L_{20} cells. Evaluation of the TC_{50} (Tables 4-6) further revealed that with the exception of L_{20}, all the extracts were generally not cytotoxic to the cells at concentrations below 500 µg/ml. Above 500 µg/ml, cytotoxicity was observed in all the cell lines in the order: L_{20}>Hep2>Vero.

Table 4. Results of preliminary screening tests of the extracts on polio virus using L_{20} cell lines

Extract	IC_{50} (µg/ml)	TC_{50} (µg/ml)	S.I.
CPF	150	50	0.33
ACE	>10,000	50	<0.005
WTE	200	50	0.25

Key: CPF=crude polysaccharide fraction; ACE=Acetone extract; WTE=Water extract; IC50=Concentration of the extract that inhibits viral infectivity (cytopathic effect) by 50%; TC50=Concentration of extract that is cytotoxic to 50% of cells; S.I.=Selectivity Index = TC50/ IC50. The mean values of quadruplicate experiments are shown.

Table 5. Results of preliminary screening tests of the extracts on IBDV using Hep-2 cells

Extract	IC_{50} (µg/ml)	TC_{50} (µg/ml)	S.I.
CPF	>10,000	500	<0.05
ACE	>10,000	500	<0.05
WTE	>1000	5000	<5.00

Key: CPF=crude polysaccharide fraction; ACE=Acetone extract; WTE=Water extract; IC50=Concentration of the extract that inhibits viral infectivity (cytopathic effect) by 50%; TC50=Concentration of extract that is cytotoxic to 50% of cells; S.I.=Selectivity Index = TC50/ IC50. The mean values of quadruplicate experiments are shown.

Table 6. Results of preliminary screening tests on yellow fever virus using vero cells

Extract	IC_{50} (µg/ml)	TC_{50} (µg/ml)	S.I.
CPF	15	500	33.33
ACE	>10,00	500	<0.50
WTE	500	1000	2.00

Key: CPF=crude polysaccharide fraction; ACE=Acetone extract; WTE=Water extract; IC50=Concentration of the extract that inhibits viral infectivity (cytopathic effect) by 50%; TC50=Concentration of extract that is cytotoxic to 50% of cells; S.I.=Selectivity Index = TC50/ IC50. The mean values of quadruplicate experiments are shown.

Table 7 shows the results of the virus titration and each result represents quadruplicate cultures. The 50% tissue culture infective dose ($TC1D_{50}$) shown against each virus was calculated according to the method of Kinchington *et al*. [16].

One emerging fact from the titration of the viruses/determination of the $TCID_{50}$ is that the poliomyelitis virus had the highest titre followed by yellow fever and Gumboro viruses, which had similar titres (Table 7). These findings show the relative infectivity and the amount of virus particles per specimen.

Table 7. Results of the titration/determination of the $TCID_{50}$ of the viruses.

Virus strain	Virus dilution						
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	$TCID_{50}$
Yellow fever	+	+	+	?	-	-	$10^{3.5}$
Poliomyelitis	+	+	+	+	+	-	$10^{5.5}$
IBDV (Gumboro)	+	+	+	?	-	-	$10^{3.5}$

Key: + =cytopathic effect; - =no cytopathic effect; ? =query (indeterminate).

The results of the antiviral activity of the lichen extracts against poliomyelitis, IBDV and yellow fever viruses are presented in Tables 4 to 6 respectively. From the results, CPF prevented cytopathic effects caused by yellow fever virus on the Vero cells at concentrations 30-fold lower than its corresponding toxic concentration. This translates to a selectivity index of about 33.3. All the other extracts did not exert any noticeable activity against the test viruses at concentrations below the cytotoxic levels. The potent activity of CPF against the yellow fever virus (IC_{50} of 15 µg/ml) is plausible, especially considering that a previous report shows a major limitation to the detection of antiviral effects of compounds in *in vitro* assays is the extremely low concentration of extracts tolerated by cells in the artificial systems [2].

Discussion

Anthraquinones, especially the polyphenolic and/or polysulphonate substituted types, have been shown to exhibit potent antiviral properties [19,20]. The lichen *Parmelia perlata* possesses glycosides other than the anthracene type, the parent precursor of the anthraquinones. Cohen *et al.* [9] also isolated anthraquinones, bianthrones and hyperacin derivatives from lichens whose antiviral activities were positively correlated with increasing substitution of chlorine in the anthraquinone structure. Therefore, it is plausible to suggest that similar manipulations can improve the antiviral effects of the nascent compounds in the crude lichen extracts. Plant polysaccharides have also been shown to exhibit potent antiviral activities, especially against enveloped viruses [21,22]. This corroborates the observed effect of CPF against the yellow fever virus, which is an enveloped RNA virus. One can conclude that the activity of CPF against yellow fever virus (an enveloped positive-sense RNA virus) as against poliomyelitis and IBDV (which are non-enveloped RNA viruses) is suggestive that the viral envelope could be the target for the antiviral action of the extract. However, an empirical conclusion to this effect could only be substantiated after further screening of CPF against several other enveloped viruses and after detailed molecular elucidation studies.

Varying the time of addition of the extracts to the incubated cell lines pre- or post-infection with

the viruses did not have any marked effect on the efficacy of the extract.

In our previous study [10], we showed that out of about fifteen indigenous Nigerian medicinal plants screened for antiviral activity by a vector-based assay technique, only extracts from the lichen *Ramalina farinacea* exhibited potent antiviral activity against HIV-1 (an enveloped RNA virus) and adenovirus type 5 (a non-enveloped DNA virus). Here we show that another tropical lichen from Nigeria also possesses antiviral activity, although against another viral family. Taken together, these studies demonstrate that lichens might represent a unique repertoire of novel antiviral phytomedicinal agents. In the current search for novel antiviral agents against current and emerging viral diseases, it might indeed be worthwhile to conduct pilot screening studies of diverse lichen species that are scattered around the globe.

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Corresponding Author: C.O. Esimone, Department of Pharmaceutics, University of Nigeria, Nsukka 410001, Enugu State, Nigeria, Tel: +234 80 638 584 70, e-mail: coesimone@yahoo.com.

Conflict of interest: No conflict of interest is declared.