In vitro antiviral activity of peptide-rich extracts from seven Nigerian plants against three non-polio enterovirus species C serotypes

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Abstract

Background: As frequent viral outbreaks continue to pose threat to public health, the unavailability of antiviral drugs and challenges associated with vaccine development underscore the need for antiviral drugs discovery in emergent moments (endemic or pandemic). Plants in response to microbial and pest attacks are able to produce defence molecules such as antimicrobial peptides as components of their innate immunity, which can be explored for viral therapeutics.

Methods: In this study, partially purified peptide-rich fraction (P-PPf) were obtained from aqueous extracts of seven plants by reverse-phase solid-phase extraction and cysteine-rich peptides detected by a modified TLC method. The peptide-enriched fractions and the aqueous (crude polar) were screened for antiviral effect against three non-polio enterovirus species C members using cytopathic effect reduction assay.

Results: In this study, peptide fraction obtained from Euphorbia hirta leaf showed most potent antiviral effect against Coxsackievirus A13, Coxsackievirus A20, and Enterovirus C99 (EV-C99) with IC50 < 2.0 µg/mL and selective index \(\geq 81\). EV-C99 was susceptible to all partially purified peptide fractions except Allamanda blanchetii leaf.

Conclusion: These findings establish the antiviral potentials of plants antimicrobial peptides and provides evidence for the anti-infective use of E. hirta in ethnomedicine. This study provides basis for further scientific investigation geared towards the isolation, characterization and mechanistic pharmacological study of the detected cysteine-rich peptides.

Keywords: Antiviral, Circular peptides, Enteroviruses, Euphorbia hirta, CPE reduction assay
individuals [28]. These have highlighted the need for antiviral drug discovery.

Enteroviruses are non-enveloped icosahedra virion with single-stranded positive sense RNA genome of 7.5 kb size. They belong to 13 species of genus Enterovirus in the picornaviridae family, four (EV-A to D) of which have been found to constantly infect humans [9]. Clinical manifestations include aseptic meningitis, neonatal sepsis, myocarditis, type 1 diabetes, hand-foot-and-mouth disease, and acute flaccid paralysis. Poliovirus, the aetiological agent of poliomyelitis is a typical member of Enterovirus species C alongside Coxsackievirus A13 (CV-A13), CV-A20, Enterovirus C99 (EV-C99) and others [7, 20].

In Nigeria, circulating vaccine-derived polioviruses (cVDPVs) have been implicated to result from recombination of non-polio enterovirus species C (NPESC) members particularly CV-A13, CV-A20, CV-A11, and CV-A17 with oral polio vaccine (OPV) [1]. The International Health Regulations (IHR) classified Nigeria as a state infected with cVDPVs with potential risk of international spread [12]. Yet, there is currently no available antiviral drugs approved for enterovirus infections.

Peptides, for therapeutic considerations have been faced with concern and limitations such as poor pharmacokinetic properties, and high molecular weight (immunogenicity) [17, 23, 24, 31, 45]. Some techniques such as cyclization, incorporation of unnatural amino acids, recombinant techniques have been employed to enhance properties of target peptides [17]. Diverse peptides are produced by plants for various metabolic purposes including defence against attacks from microbes, herbivores and pests [8]. As plants continue to be a veritable source for drug discovery, the presence of cysteine-rich peptides including the circular peptide-rich) was each dissolved in 2 mL dimethylsulfoxide (DMSO) to obtain stock solutions (10 mg/mL). For antiviral screening, 20 mg of fractions (crude and partially-purified peptide fraction (P-PPF) by eluting with 20% and 80% solvent B, respectively. The P-PPFs were freeze-dried and stored in the refrigerator at 4 °C until used for bioassay.

Thin layer chromatography (TLC) chemical detection of peptides

A modified method previously described by Wen-Yan et al. [48] and Attah et al. [2] was adopted for the TLC chemical detection. Pre-coated TLC plates (G254 MERCK, Germany) and solvent system n-butanol:acetic acid:water (3:1:1) were used. Each solvent-dissolved peptide extract was spotted on the TLC plate and developed in the solvent system above. Plates were allowed to dry, viewed under UV at 254 and 365 nm. Dried plates (TLC chromatograms) were swiftly sprayed or dipped in freshly prepared G-250 modified stain or ninhydrin, respectively.

Preparation of extract stock

For antiviral screening, 20 mg of fractions (crude and peptide-rich) was each dissolved in 2 mL dimethylsulfoxide (DMSO) to obtain stock solutions (10 mg/mL).

Cell and virus

Human breast adenocarcinoma cancer cell line (MCF-7) obtained from WHO national Polio Lab, Ibadan, Nigeria was used for both cytotoxic and antiviral studies. Cells were grown in Eagle’s minimum essential medium (MEM) supplemented with 10% foetal bovine serum (FBS), 100 units/mL of penicillin, 100 μg/mL of streptomycin, 2 mM L-glutamine, 0.07% NaHCO3, 1% non-essential amino acids and vitamin solution at 37 °C in a humidify incubator (85–95% humidity). Three species C enterovirus members, including two serotypes of coxsackie virus A (CV-A13 and CV-A20) and a numberer Enterovirus C serotype (EV-C99) were obtained from stool isolates [9] by the Enterovirus research group, Department of Virology.
The test medium used for cytotoxic assays and antiviral assays contained only 2% FBS.

Preparation of viral stocks
To increase the quantity of virion stocks, virus suspension (200 µL) was inoculated into the T25 flask of cultured MCF-7 cells, and incubated at 37 °C for about 72 h for 100% cytopathic effect. Afterwards, medium was centrifuged and aliquots of supernatant were made into cryovials. All viral stocks were stored at −70 °C until use.

Virus titre was determined by virus-induced cytopathic effect (vCPE) in MCF-7 cell and were expressed as 50% tissue culture infective concentration (TCID\textsubscript{50}) per mL. Briefly, 100 µL MCF-7 cell suspension (1 × 10\textsuperscript{5} cells/mL) was seeded into a 96-well microtitre plate and incubated for 24 h to form monolayer. Afterward, virus suspension (100 µL) was inoculated into the eight wells (as replicates) of each column 1–10 with varying (ten-fold seri -ally diluted- 10\textsuperscript{−1} to 10\textsuperscript{−10}) concentration per column. Column 11 and 12 served as the cell control. Plate was incubated at 37 °C, and daily CPE scoring was done for about 7 days when cell control wells started dying off. The TCID\textsubscript{50} values were determined using Spearman–Karber’s method and 100 TCID\textsubscript{50} was used for the antiviral assay.

Cytotoxicity assay
The maximum nontoxic concentration (MNTC) test of crude fractions to MCF-7 cells in culture was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Aldrich®) assay, a colorimetric assay that reliably measures cell viability. Previously described method by Mossmann [32] was adopted. Briefly, previously seeded monolayers of MCF-7 cells in a 96-well microtitre plate was treated with six serial ten-fold dilutions (1000 to 0.01 µg/mL) of stock solutions of crude and peptide-rich fractions in maintenance medium (2% MEM) for 72 h. Afterwards, plates were observed for MNTC on the cells under an inverted microscope (OLYMPUS CKX31). Afterward, old medium was removed and 25 µL of prepared MTT reagent in phosphate buffer saline (PBS) (2 mg/mL) was added to each well, including controls and plate returned to the incubator for 2 h. Then, DMSO (75 µL) was added to solubilize the formazan crystals formed. Optical density values were obtained by spectrophotometry (Multiscan 347, MTX lab) at 490 nm. Data obtained was used to determine 50% cytotoxic concentration (CC\textsubscript{50}).

Data analysis
Selective index, CC\textsubscript{50} and IC\textsubscript{50}
The 50% cytotoxic concentration (CC\textsubscript{50}) and the 50% inhibitory concentration (IC\textsubscript{50}) for each extract was calculated from non-linear regression analysis using GraphPad prism5. The selective index, which is the index of safety margin is defined as CC\textsubscript{50} over IC\textsubscript{50}.

Results
Thin layer chromatography (TLC) chemical detection of cysteine-rich peptides
The bound P-PPf was eluted from the aqueous-rich fraction by reverse-phase solid-phase extraction (RP-SPE) using C\textsubscript{18} columns (Phenomenex, Aschaffenburg, Germany). On spraying with freshly prepared G-250 modified stain, all partially purified peptide fraction spotted on TLC pre-coated plates produced a bright blue colouration indicating the presence of cysteine-rich peptides which may be circular in their configuration (Fig. 1a). Furthermore, on spraying with ninhydrin (which characterizes presence of amino acids, amines and linear peptide by colour change from purple to red) Ninhydrin presented colour changes indicative of the presence of peptides, likely a combination of linear and circular peptides if present (Fig. 1b).
Tissue culture infective dose (TCID$_{50}$)
As determined by Spearman–Karber’s method, the virus titre for CV-A13 and EV-C99 gave the value of $10^{-4}$ with 100 TCID$_{50}$ calculated as $10^{-2}$, while CV-A20 a virus titre value of $10^{-3}$ with 100 TCID$_{50}$ calculated to be $10^{-1}$.

Cytotoxic activities of crude and pre-purified peptide fractions
The aqueous crude and P-PPf of each plant both had equal MNTC value in MCF-7 cells. All the tested fractions had a common MNTC value of 10 µg/mL, except for Allamanda blanchetii and Euphorbia humifusa (100 µg/mL) (Table 1). As shown by the CC$_{50}$ values (Table 2), the peptide fraction of Ixora coccinea (ICp) relatively had the highest cytotoxicity (19.7 µg/mL) followed by the peptide of Allamanda cathartica (20.5 µg/mL), while the peptide fraction of Euphorbia humifusa (EHu) had the lowest (169.0 µg/mL).

Antiviral screening of crude and peptide fractions
All tested fractions showed considerable antiviral activity variably on the three viruses (Table 2). Also, all P-PPfs showed antiviral activity across the three NPESC

| S/N | Family         | Name                          | Code | Voucher (FHI) No | MNTC (µg/mL) |
|-----|----------------|-------------------------------|------|-----------------|--------------|
| 1   | Apocynaceae    | Allamanda blanchetii A.DC    | AB   | 112880          | 100          |
| 2   | Apocynaceae    | Allamanda cathartica L       | AC   | 112881          | 10           |
| 3   | Euphorbiaceae  | Euphorbia graminea Jacq     | EG   | 112894          | 10           |
| 4   | Euphorbiaceae  | Euphorbia hirta L            | EHi  | 112893          | 10           |
| 5   | Euphorbiaceae  | Euphorbia humifusa Wild      | EHu  | 112948          | 100          |
| 6   | Phyllantaceae  | Phyllanthus amarus Schumach. & Thonn | PA   | 112892          | 10           |
| 7   | Rubiaceae      | Ixora coccinea L             | IC   | 112882          | 10           |
members except for *Allamanda blanchetii*, *Allamanda cathartica*, *Phyllanthus amarus*, and *Ixora coccinea*. In general, the antiviral activity of crude and peptide fractions of each plant is consistent, with enhanced effect observed with the peptide fractions.

**Discussion**

Historically, medicinal plants have been a valuable source for drug discovery. Plant peptides are gaining attention for drug discovery exploration especially, cysteine-rich circular peptides due to their stability [3, 11, 50]. Antimicrobial function of plant peptides in plant innate immunity can be explored for antiviral drug discovery [3, 16]. Though poliovirus infection is on the edge of eradication, there is need to search for antivirals against nonpolio enteroviruses that can substitute the niche as the leading cause of paralysis in children [5].

In this study, all tested pre-purified peptide fractions from the *Euphorbia* species notably showed antiviral effect across all the NPESC serotypes. *Euphorbia hirta* evidently showed best activity with IC₅₀ (< 2 µg/mL) and high index of safety margins (SI ≥ 81). Members of Euphorbiaceae family especially, *Euphorbia* species extract have been demonstrated for in vitro antiviral activity against RNA and DNA viruses [10, 13, 21, 22, 25, 37, 38, 40, 42, 44, 51]. Also, various in vitro antiviral activities against hepatitis B, herpes simplex virus, influenza viruses, rhinovirus, and enterovirus [4, 6, 30, 33, 43, 46] have been displayed by some small molecules from *Euphorbia*. Thus, this finding is consistent with reports on antiviral potentials of *Euphorbia* species. Among the three *Euphorbia* species tested, *E. hirta* was observed to show best antiviral activity across the three NPESC serotypes with its p-PPf exerting highly selective antiviral activity, more enhanced than its crude fraction; which is further evident in the relatively higher selective index values of P-PPf of *E. hirta* (Table 2). *E. hirta* has been documented in ethnomedicine use against infections including viral infections in Philippines, India, Pakistan and Sri Lanka [41]. Similar peptides with varying proportion or varying peptide constituents in the tested *Euphorbia* species could be responsible for their unequal antiviral activity. Ongoing process of isolation and characterization of the peptides will reveal this clearly.

Table 2  Antiviral Activity of crude aqueous fraction and partially purified peptide fractions on three NPESC members

| Extract | CC₅₀ (µg/mL) | CV-A13 | SI | CV-A20 | SI | EV-C99 | SI |
|---------|-------------|--------|----|--------|----|--------|----|
|         | IC₅₀ (µg/mL) |        |    | IC₅₀ (µg/mL) |    | IC₅₀ (µg/mL) |    |
| ABp     | 167.2       | 33.31  | 5.0| NA     | NA | NA     | NA |
| ACc     | 24.3        | NA     | NA | 2.67   | 9.1 | 1.64   | 14.8|
| ACp     | 20.5        | NA     | NA | 1.09   | 18.8| 1.18   | 17.3|
| ICc     | 109.8       | NA     | NA | 1.75   | 62.9| 1.85   | 59.5|
| ICp     | 19.7        | NA     | NA | 0.54   | 36.4| 0.54   | 36.8|
| EHic    | 81.6        | 2.81   | 29.0| 1.65   | 49.3| 0.80   | 102.5|
| EHlp    | 159.0       | 0.94   | 169.0| 1.96   | 81.0| 0.53   | 301.7|
| EHuc    | 167.5       | 17.28  | 9.7 | 19.57  | 8.6 | 9.45   | 17.7|
| EHuP    | 169.0       | 20.94  | 8.1 | 5.47   | 30.9| 5.30   | 31.9|
| EGc     | 33.2        | 1.969  | 16.8| 16.63  | 23.5| 0.531  | 73.6|
| EGp     | 39.1        | 3.203  | 12.2| 1.11   | 61.5| 0.59   | 116.3|
| PAP     | 68.0        | NA     | NA | NA     | 0.59| 116.3  |    |

C—crude aqueous fraction; p—partially purified peptide fraction; NA—not active; SI—selective index; CC₅₀—50% cytotoxic concentration; IC₅₀—50% inhibitory concentration; CV-A13—Coxsackievirus A13; CV-A20—Coxsackievirus A20; EV-C99—Enterovirus C99

Partially purified peptide fractions from *Allamanda blanchetii* showed moderate antiviral effect only on CV-A13 while *Allamanda cathartica* lacked antiviral effect only on CV-A13. This varying antiviral effects of the two *Allamanda* species observed across the three NPESC serotypes could suggest disparate peptide constituents in the two species. Nguyen and his group reported the presence of allotides, proline-rich cystine knot α-amylase inhibitors from *Allamanda cathartica*; the extremely stable disulphide-rich peptides with alpha amylase activity and poor antimicrobial activity [36]. The antiviral assay design was prophylactic and not therapeutic. Thus, possible mechanism of antiviral action could be the prevention of virus attachment/entry into susceptible MCF-7 cell line used or inhibition of a replication stage that is downstream of entry or direct effect on virion (virucidal). CV-A13 and CV-A20 use cell surface receptor intercellular adhesion molecule 1 (ICAM-1) for entry into susceptible cells [19], thus binding of peptides to the glycoprotein ICAM-1 is a possible antiviral target. However, alternate cell entry have been documented for CV-A20 other than ICAM-1 [34], indicating the differing
results for some partially purified peptides exhibiting antiviral activity on CV-A13 and not on CV-A20. Plant-derived cysteine knot peptides include alpha amylase inhibitors, cyclotides, thionins, and defensins whose bioactivities lead to blocking of viral infection by clustering the viral particles and blocking receptor binding [35, 47]. These disulphide stabilised peptides mediate in the inhibition of viral entry, viral particle disruption, interference with essential cell signalling or viral gene expression [26], or by other poorly-understood mechanisms. In addition to the antiviral activities, cysteine-rich peptides such as defensins modulate adaptive immune responses via mobilization of dendritic cells, induction of their maturation, enhancement of antigen uptake, and mobilization of T Lymphocytes (CD4+ and CD8+ effector T cells) to sites of infection, due to the T cell-chemoattracting effect of defensins [47, 49].

Conclusion

Semi-purified cysteine-rich peptides in the tested Euphorbia species displayed notable antiviral activity against non-polio enterovirus species C; CV-A13, CV-A20 and EV-C99 in MCF-7 cell culture system. To the best of our knowledge, this is the first antiviral report on semi-purified peptides from the tested plant species and therefore provides scientific rationale for a more extensive study of the individual peptides, molecular targets, safety and efficacy as potential peptide-based therapeutics.

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