In vitro Antiviral Activity of *Nauclea latifolia* Root Bark Extract Against the Respiratory Syncytial Virus

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Authors' contributions

This work was carried out in collaboration between both authors. Authors DCO and COE both designed the study and wrote the protocol. Author DCO performed the experiments, statistical analysis and wrote the first draft of the manuscript. Both authors read and approved the final manuscript.

Article Information

Original Research Article

ABSTRACT

Extract of *Nauclea latifolia* (NL) root bark collected from the Nigerian flora was examined for anti-RSV activity. Preliminary data showed anti-RSV activities with IC_{50} =75.62 µg/ml when tested against the recombinant strain rgRSV expressing the green fluorescent protein. Corresponding assays for the cytotoxic effect of the extract against utilized cell lines gave TC_{50} = 333.82 µg/ml. Further screening of against the circulating RSV A2 strain established their promising anti-RSV utility. Time of additional studies for the elucidation of the possible mechanism of action gave 74.38, 69.42, and 71.90% reduction of RSV plaque forming units at the respective 0, 2, and 4 hours post-infection addition times.

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1. INTRODUCTION

The respiratory syncytial virus (RSV), a member of the *Paramyxoviridae* family, is a major cause of acute upper and lower respiratory tract infections in very young human population and adults [1,2]. Since the immune response to RSV infection is not protective, RSV infections reoccur throughout adulthood. RSV has also gained recognition as a significant cause of morbidity and mortality in the elderly and severely immunocompromised individuals.

Currently, there is no vaccine to prevent RSV infection, and the available therapeutic agents are of limited utility. Ribavirin is the only commercially available agent used to treat RSV infection. The utilization of ribavirin is limited due to efficacy and toxicity concerns as well as the very long treatment regimen required for its delivery by aerosol inhalation [3-6]. Some small-molecule inhibitors of RSV have been identified, but to date, none are clinically approved [7-9]. Potent antiviral agents have previously been harnessed in medicinal plants. Since medicinal plants have consistently served as suitable lead sources for potent anti-viral agents, efforts are to be continually made towards developing anti-RSV compounds from phytomedicine. This is the primary goal of this present study.

Nauclea latifolia (NL) Smith (Rubiaceae) is a small tree (or shrub) which grows to about 4 m high, and it is quite abundant in several regions of Africa. The roots and stem bark of the plant are used in Nigerian communities for the treatment of jaundice, fever, diarrhoea, malaria, dysentery and diabetes and hypertension [10]. Various biological screening investigations have revealed antibacterial [10], antidiabetic [11] and antiplasmodial [12] activities. NL is reported to contain the following phytochemical components; indole alkaloids, triterpenes, steroids and saponins [13-15]. There has not been any report of antiviral anti-respiratory syncytial virus activity for NL. Our present investigation embarked on present the result of our study to screen NL for possible antiviral anti-respiratory syncytial virus activity.

2. MATERIALS AND METHODS

2.1 Plant Material Preparation

NL root bark parts were collected in Nsukka, Enugu State, Nigeria between 2008 and 2009.

Extracts were prepared accordingly as indicated in Table 1 and stable preserved at -20ºC until used. The root bark was properly crushed and extracted with water. The plant was authenticated by the Dept of Pharmacognosy, the University of Nigeria, Nsukka and a voucher specimen deposited accordingly.

2.2 Human Cell Lines and Human Respiratory Syncytial Virus (HRSV)

Human larynx epidermoid (HEp-2) and Vero cell lines (Vero cells) were used to culture HRSV (Long strain: ATCC VR-26). Cells were propagated in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin antimicrobials.

HRSV was propagated in DMEM with 0.5% FCS and antimicrobials 1% penicillin/streptomycin. Viral titer was determined and expressed as plaque forming units per ml (pfu/ml) [16]. A virus was stored at -80ºC until use.

2.3 Cytotoxicity

The cytotoxicity of the extracts was evaluated using the MTT assay [7]. HEp-2 cells were seeded onto a 96-well plate at a concentration of 6000 cells/well and a volume of 200µl of D-5 (5% FCS DMEM medium) per well. The extracts were solubilized in 0% FCS DMEM medium containing DMSO to give 100, 50, 25, 12.5, 6.25 µg/ml extract such that the final DMSO concentration in cell culture did not exceed 0.6%. D-5 containing 0.1% DMSO was used as the "no drug" control. After incubation at 37ºC under 5% CO_2 for two days, a solution of MTT (5 mg/ml, 50µl per well) was added to each well and further incubated at 37ºC + 5% CO2 for one h to allow for formazan production. After this time, medium was removed, and 200µl of 10% SDS solution (pH 4.7) was used to dissolve the resulting blue formazan crystals in living cells overnight. The optical density was determined at 550 nm using a multi-well microtiter plate reader (Tecan, Austria). Every single value of the triplicates was expressed as percent of the mean of triplicates of the "no drug" control cultures, and the mean and standard error of the percent values were calculated for each triplicate. The concentration of 50% cellular toxicity (TC_{50}) of the test compounds was calculated by simple regression analysis.

2.4 Antiviral Activity Testing of Extracts

The antiviral activities of all extracts were assessed by the use of a recombinant strain rgRSV expressing the green fluorescent protein (rg) as a reporter gene [17]. HEp-2 cells were plated in triplicates into 96-well plates at 6000 cells/well and incubated overnight. The prepared extract as above was added to the well first followed by the virus (MOI of 0.01) and incubated for 48 h at 37ºC + 5% CO_2. Control wells containing virus alone at the same MOI in D-5 (containing 0.5% DMSO) but without drugs were equally be set up. After a 48 h incubation, the number of plaque forming units (pfu) in drug-treated and untreated HEp-2 cells was determined by fluorescent microscopy where localized green cells harbouring rgRSV were counted as viral plaques. Percentage viral plaques reduction were then calculated, and IC_{50} determined by simple regression analysis.

2.5 Determination of Antiviral Activity against RSV A2 Strain

The antiviral activities of all extracts against RSV A2 strain were also assessed employing an immunocytochemical technique for plaque reduction determination [7,17]. Again, set-up was done as above, and after 48 h incubation, the numbers of plaque forming units (pfu) in drug-treated and untreated HEp-2 cells were determined. After the 48 h incubation, the number of plaque forming units (pfu) in drug-treated and untreated HEp-2 cells was determined by immunocytochemical staining with a monoclonal antibody to the RSV-P protein (3C4) as described previously [18]. After removal of the supernatant, cells were fixed for 10 min with 80% ethanol, allowed to dry in the air before re-hydrating with PBS-T (phosphate-buffered saline: 10 mM sodium phosphate, 150 mM NaCl, 0.05% v/v Tween 20, pH 7.2) for 5 min. After that, cells were incubated with 1: 250 dilution in PBS-T of 3C4 antibody for 45–60 min at 37ºC. After washing three times with PBS-T, cells were similarly incubated with the secondary antibody (peroxidase-conjugated rabbit anti-mouse IgG, P0260 (Dako, Germany) diluted 1: 400 in PBS-T. Cells were again washed three times with PBS-T followed by incubation with the red-colour staining AEC substrate (10 ml phosphate citrate pH 5.0, 200 ml AEC and 10 ml hydrogen peroxide) for 30 min at 37ºC. Supernatants were discarded, and 100 ml of water was added to each well. Reddish- brown plaques showing

fused cells were enumerated microscopically. Every single value of the triplicates was expressed as percent of the mean of triplicates of control cultures (infected with same MOI of virus in the absence of the drugs) and the mean, and standard deviation of the percent values was calculated for each triplicate.

2.6 Protection from Viral-induced Cytopathic Assay

Hep-2 cell cultures were prepared in 96-well plastic plates (8 mm diameter; Falcon Plastics, Oxnard, Ca.) [19]. After 24hrs of incubations at 37ºC in a CO_2 incubator, when the cell cultures became confluent, the culture medium was removed from the monolayer cells. To confluent monolayers of Hep-2 cells in 96-well plates, virus suspension (containing virus at MOI of 3 together with 100 µl of D5 medium containing the appropriate concentrations of the test compounds were added. As the virus control, similar virus suspensions in medium without compounds were added. The plates were incubated at 37ºC in a humidified CO_2 atmosphere (5% CO2), for 5-6 days. After that, cytopathic effect (CPE) was observed. The virus-induced CPE was determined by MTT assay as described above in cytotoxicity assay. The reduction of virus multiplication was calculated as % of virus control:

Percentage (%) CPE= (OD Mock – OD herb+virus / OD Mock – OD virus control) × 100

The concentration reducing CPE by 50% in respect to virus control was estimated from graphic plots using the regression statistic, and was defined as 50% inhibited or protective concentration (EC_{50})

2.7 Time Course Assay (Time-of-addition Studies)

The antiviral time-of-addition activity of the extract was examined at different time points before and after viral inoculation by plaque reduction assay [7,17]. HEp-2 cells were plated in triplicates into 96-well plates at 6000 cells/well and incubated overnight. The prepared extract as above was added to the well first followed by the virus (MOI of 0.01) and incubated for 48 h at 37ºC + 5% CO_2. Control wells containing virus alone at the same MOI in D-5 (containing 0.5% DMSO) but without drugs were equally be set up. Additionally, triplicate wells were also inoculated with same MOI of the virus and incubated for two h (time two h.p.i) or 4 h (time four h.p.i) at 37ºC þ

5% CO_2. After the designated incubation period, wells were washed with PBS and replaced with D-5 medium containing extract (10 µg/ml in each case) or Ribavirin (5 µg/ml). Control wells were replaced with 0.5% DMSO in D- 5 medium (without drugs). After a 48 h incubation, the number of plaque forming units (pfu) in drug-treated and untreated HEp-2 cells was determined by fluorescent microscopy where localized green cells harboring rgRSV were counted as viral plaques. Percentage viral plaques reduction were then calculated, and IC50 determined by simple regression analysis.

3. RESULTS

3.1 Activity Against rgRSV

Aqueous extract from NL was evaluated for antiviral activity against RSV. The TC_{50}, IC_{50} values and selective index (SI) of preliminary screening investigations are shown in Table 1. Moreover, Fig. 1 and two show plots display of the activity and cell viability ranges. *Nauclea latifolia* (NL) showed substantial anti-RSV activity *in vitro*. Additionally, NL demonstrated promising activity (IC_{50}= 58.15µg/ml, TC_{50}=187.35µg/ml, SI=3.22) and thus making it worthy of further future investigations especially if we take into consideration of the need for a large selective index (ratio of cytotoxicity (TC_{50}) to antiviral activity (IC_{50}) (SI= TC_{50}/IC_{50}).

Table 1. Result of anti-RSV parameters

Plant (Herb)	IC_{50}	TC_{50}	SI index
Nauclea latifolia (NL)	58.15	187.35	3.22
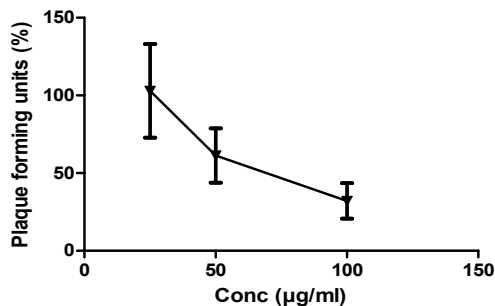
Inhibition of RSV infectivity in Hep 2 cellline

Fig. 1. Inhibition of rgRSV infectivity by herbs

Various concentration of extract from *Nauclea latifolia* (NL) was preincubated with recombinant rgRSV for 30 min at 37ºC before infecting Hep2 cells to achieve an MOI of 0.01. The results are expressed as a percentage of the activity (plaque

forming units for the antiviral evaluation) of cells cultured in the absence of the compounds. The mean and the standard error of at least triplicate determinations are given.
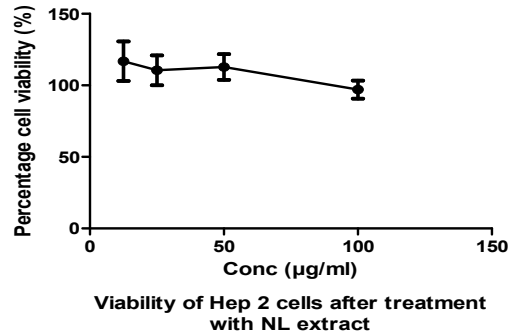

Viability of Hep 2 cells after treatment with NL extract

Fig. 2. Effect on Hep2 cell viability by herbs

Hep2 cells were incubated with various concentrations of extract from *Nauclea latifolia* (NL). After corresponding 48 hrs of incubation alongside the infectivity assay, MTT was added for 1hr, and formazan crystal dissolved overnight, and absorbance measured at 550 nm. The results are expressed as a percentage of the activity (cell viability) of cells cultured in the absence of the compounds. The mean and the standard error of at least triplicate determinations are given.
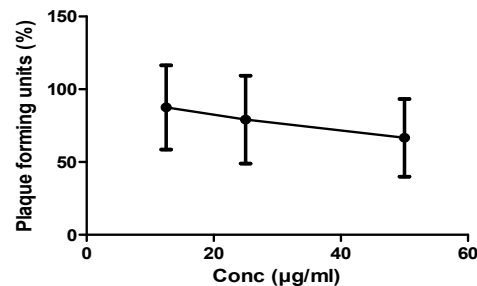
Inhibition of RSV A$_2$ strain infectivity by NL

Fig. 3. Inhibition of RSV A2 strain infectivity by herbs

3.2 Antiviral Activities against RSV A2 Strain

Activities against RSV A2 strain is shown in Table 1 and Fig. 2. The results showed that NL retained its anti-RSV status following incubation in cell culture infected with RSV A2 strain.

Various concentrations of *Nauclea latifolia (NL)* were preincubated with recombinant RSV A2 strain for 30 min at 37ºC before infecting Hep2 cells to achieve an MOI of 0.01. The anti-RSV activities of the compounds (~) were determined

48 h latter via an immunocytochemical technique for RSV-induced plaque detection. The results are expressed as a percentage of the activity (plaque forming units for the antiviral evaluation) of cells cultured in the absence of the compounds. The mean and the standard deviation of at least triplicate observations are given.

3.3 Herb-mediated Antiviral Cell Protection Assay

The evaluation for herb-induced protection from viral-induced cell cytopathic effects assay was done. The results showed cell protection capacities with EC_{50} values of 5.92 µg/ml for *Nauclea latifolia* (NL) respectively. This is a striking outcome compared to the classical infectivity inhibition outcome.
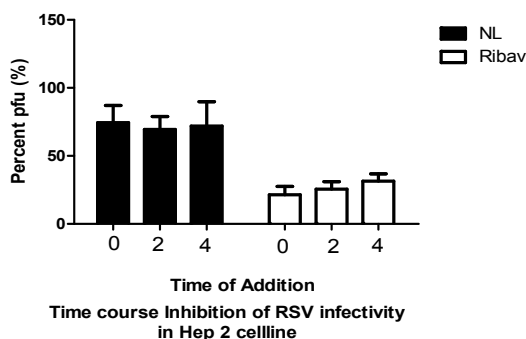

Fig. 4. Time of addition assay

3.4 Time of Addition Assay

In order to determine the stage in the RSV replication cycle that is targeted by the herbs. Infectivity time assays were carried out. The result shown in Figure 2 reveals various Time interference points for the herbs. NL shows interference at 0, 2, and 4hrs post-infection addition of the herbs. Moreover, NL showed a better inhibition at 2hrs time post-infection addition.

In order to determine the stage in the RSV replication cycle that is targeted by the extract infectivity time assays were carried out. Shown are various time-interference points for the NL and control Ribavirin (Ribav). NL recorded reasonable viral inhibition at 2 and four h time post-infection addition of the extract.

4. DISCUSSION

New anti-RSV substances better desirable activity and tolerability in comparison to the standard ribavirin has remained a continued strong focus of research in the antiviral infectious diseases discipline. In the past, potent and useful anti-viral agents have been developed from medicinal plants [20-22]. Presently several potential anti-respiratory syncytial virus (anti-RSV) substances remain at the various stage of developments, and the list keeps growing [9,23-25]. Consequently, continued investigations for the discovery of newer anti-RSV candidates are ongoing. There have been several reports regarding the wide and varied biological effects and utility of extractives from NL. Interestingly, these reports included some antimicrobial activities against some bacterial and viral isolates. However, our search in the literature suggest that no report has been made concerning any anti-RSV screening. Therefore, this could be the very first time that *Nauclea latifolia* is being reported for anti-RSV activity. The study carried out here centred largely on an evaluation of the extract for antiviral activity. Here, we report its evaluation against two subtypes of RSV and the possible mechanistic explanation of its activities by time-point of inhibition behaviour.Our study thus shows that NL inhibited the rgRSV notably (Figs. 1 and 2). Although with a slightly elevated the IC_{50} when assessed by the resultant ratio IS index the antiviral utility is not in question. The aqueous extract clearly demonstrated above-board tolerability which in some other settings has been a drawback for some previously discovered potent antiviral drugs. Consequently, the quest to further validate the emerging data by employing clinical isolate of RSV equally showed that the earlier observed anti-RSV activity could fairly be replicated (Fig 3), and this is comparable to previously reported findings [18]. Thus, NL also conveniently inhibited the replication of clinical RSV A2 strain in cultured Hep2 cells with a derived IC_{50} value of 5.64 µg/ml. This represents about 80% proportion of infectivity reduction observed with rgRSV strain.

As earlier pointed out, the average-rated IC_{50} value notwithstanding the overall utility of NL as an antiviral anti-RSV substances is further underscored given the robust outcome from the cell protection assay value obtained (EC_{50}: 5.92µg/ml). It implies that even at small-to-moderate concentration amounts, NL could effectively diminish RSV infectivity in a cell population. This portends quite hopeful application for RSV universal therapy, and this property helpful [26]. The exact mechanism through which this relatively efficient

management outcome occurs is not fully understood. However, it is possible that a combination of direct antiviral activity and cellular modulatory effects may interplay. While the latter function may require several cellular and immune or other platforms to monitor, the former function could relatively be elucidated on our infectivity platform. Therefore, we proceed to uncover this antiviral axis through the time-course (time of addition assay). The time of addition assay allows us to establish the likely cellular events or viral structures/function that the antiviral substance is possibly interacting with since viral metabolism and course of events occur in stages which are usually time-bound. While most early infection and some replication-related events may occur upstream of 2 hr time point, all other replication events would occur downstream of the same time-point. From our observation, NL was to some extent active at all indicated time-points (0, 2, and 4 hrs), although more activity was recorded after the 2hrs time of addition of NL. This suggests that NL could be inhibitory to several early and late phase events, and so could be that multiple targets do exist. While, it is possible that a single chemical moiety could have several biological interaction partners in the virus or cellular compartment, another explanation may be that several possible compound present in the NL extract may well be making their antiviral contributions on different target which consequently adds up to give the final picture/scenario, a case that has been extensively reviewed [27].

So, given all of these, our thinking is that NL holds potential for utility as an anti-RSV intervention after further evaluations and development. The way to go in our further investigation is to isolate the several possible compounds present in NL and to further screen them for anti-RSV properties. Furthermore, we could include other cellular and immunological platforms to additional characterize their mode of activity [19,28].

5. CONCLUSION

In concluding, the outcome of this screening exercise NL has demonstrated anti-RSV activity. Moreover, cellular protection from RSV-induced cell pathology represents another desirable property of NL. Our study also suggests that NL may to interfere with several viral targets. Therefore, further developing NL may hold promise for future antiviral anti-RSV application.

ETHICAL APPROVAL AND CONSENT

These are not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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