The anti-respiratory syncytial virus activity of methanol leaf extract of 
*Aspilia africana*

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**ABSTRACT**

Respiratory syncytial virus (RSV), to date, is still the most frequent cause of infantile and childhood viral lower respiratory tract illness on a global scale, and at the same time, causing significant impact in the elderly. Presently, there is no licensed vaccine or safe and specific antiviral agent that could be used against the virus. Medicinal plants are known to be a promising source for the discovery of utilizable anti-RSV treatments. In this present study, we screened the anti-RSV and cell cytotoxicity activities of methanol extract of *A. Africana* by virus plaque reduction and corresponding cell viability methods in Hep-2 and Vero cells of human and mammalian origin respectively. The corresponding set-up for analyses of *A. Africana* extract on cell viability were performed by the reduction of thiazolyl blue tetrazolium bromide (MTT) in the Hep-2 cells following the established method. The results of the evaluation of the extract of *A. africana* showed anti-RSV activities with inhibition of RSV at IC$_{50}$, 42.5 ± 8.89, while the cell cytotoxic effect in Hep2 cells recorded was TC$_{50}$, 121.4 ± 7.21. Therefore, our results show that the methanol extract of AA exhibited anti-RSV activities and has a moderate effect on the cell viability of the recipient host cell, indicating that target molecular substances against RSV can be developed from the extract.

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**INTRODUCTION**

Respiratory syncytial virus (RSV), till date, remains the most frequent etiology of viral lower respiratory tract illness (LRTI) in infants and children on a global scale (Onah et al., 2017). RSV, an RNA virus with a single-stranded genome and belonging to the genus *pneumovirus*, is documented to be responsible for the hospitalization of over 3 million people and over 200,000 deaths globally (Nair et al., 2010). In less developed parts of the world, about 17% of child hospital admission cases due to acute respiratory infections were linked to RSV (Nwankwo et al., 1994; Weber et al., 1998; Peltola and Ruuskanen, 2008). RSV worldwide prevalence is also on the increase, and it is well known that by 2 years, practically every child gets an RSV infection (Jorquera and Tripp, 2017). Apart from childhood infections, RSV could also significantly affect the aged, thus making it a clear public health concern requiring medical solutions. Despite this, there is yet no credible, safe, and certified vaccine available due to several inadequately understood problems (Graham, 2011; Borchers et al., 2013; Wright et al., 2000). The availability of an approved, safe, and effective antiviral therapeutic agent is equally not existent (Simões et al., 2015). Ribavirin, a broad-spectrum antiviral drug is the only antiviral therapeutic agent usu-
ally utilized as adjunct treatment for critical patients and is also saddled with some challenges which ranges from relative inefficacy, safety problems, high cost, and difficulty in administration, thus leading to its disuse in most hospitals (Kneyber et al., 2000; Odimegwu et al., 2018), hence the need for more searches for a suitable antiviral inhibitor of RSV. Generally, medicinal plants are known to be a useful reservoir of anti-viral agents (Semple et al., 1998; Kott et al., 1999; Sindambiwé et al., 1999). Therefore exploration of the rich reserves of medicinal plants can yield the needed anti-RSV agents for use globally and especially in resource-poor countries (Ali and., 2016; Onah et al., 2017). Hence, our focus in the current study was to examine Aspilia africana based on presently existing folklore and scientific information about their broad-spectrum biological effects. Aspilia africana (AA) is a herb commonly known in Nigeria as “Orangila” in Igbo, “Yunyun” in Yoruba, and “Tsozalin” in Hausa (Ajeigbe et al., 2013), is widespread in Africa (Oyedapo et al., 1997). It belongs to the family Compositae and is about 1m tall and have been morphologically described (Kuiate et al., 1999; Nwafor et al., 2018). The ethnomedicinal uses of this plant includes its use in the treatment of wound (Okoli et al., 2007), rheumatic pains (Dimo et al., 2002), bacterial infections (Macfoy and Cline, 1990), bee and scorpion stings, and eye treatments and procedure (Oyesola et al., 2010). The phytochemical analyses of AA reveals the presence of sesquiterpenes, monoterpenes (Etiosa et al., 2018), saponins, and tannins (Obadoni and Ochuko, 2002) in the leaves.

MATERIALS AND METHODS

Collection of medicinal plant and extraction
Leaves of AA were sourced from Nsukka, identified by Mr. Ozioko A.O of the Bioresources Development and Conservation Programme (BDCP), Nsukka, Enugu State, Nigeria. The leaves were rinsed, dried, and using cold maceration were extracted with 90% v/v aqueous methanol, concentrated in a rotary evaporator to the methanol extract.

Reagents
Dulbecco’s Modified Eagle’s medium (DMEM): Life Technologies, UK; Dimethyl sulfoxide (DMSO): J.T Baker; Thiazolyl blue tetrazolium bromide (MTT): Applichem; Dimethyl formamide (DMF): Fischer Scientific; purity 99.96%, Sodium dodecylsulphate (SDS): Applichem; purity 99.5%.

Virus and cell lines
Zhang et al. (2002) have described the construction of rgRSV, which is a recombinant RSV strain with the green fluorescent protein (gfp), enabling easy visualization under the fluorescent microscope. We made use of the HEp-2 (Human larynx epidermoid) to culture rgRSV strain. HEp-2 and Vero cells (African green monkey kidney cell) were grown in DMEM that is supplemented with 10% fetal calf serum, 1% penicillin and streptomycin while RSV was grown in DMEM that is supplemented with 0.5% Fetal calf serum, 1% penicillin and streptomycin respectively. Following virus propagation and experimentation, titres were determined by enumeration of virus plaques and reported as plaque-forming units per ml (pfu/ml) (Esimone et al., 2008; Lai et al., 2013; Ternet et al., 2007). Virus stocks were preserved at 80°C for future use after purification procedures.

Assay for cytotoxicity
The thiazolyl blue tetrazolium bromide (MTT) assay for the determination of cytotoxicity is previously outlined (Esimone et al., 2008; Lai et al., 2013). Thus, HEp-2 cells and Vero cells were seeded in a population density of 6000 cells/well in a 96-well plate containing 200 μl/well of 5% FCS DMEM in triplicate wells. Serial concentrations 100, 50, 25, 12.5 and 6.25 μg/ml of AA extract were solubilized in the 5% FCS DMEM containing Dimethyl sulfoxide (DMSO) such that the final concentration of DMSO in the cell culture does not exceed 0.6%. A ‘no drug’ control (5% FCS DMEM containing 0.6% DMSO) was used as the set-up for control. The entire experimental set-up was incubated at 37°C + 5% CO2 for 2 days. Following the exhaustion of the incubation time, 50 μl of 5mg/ml MTT solution (dissolved in Phosphate buffered saline, PBS) was introduced into each well and incubated (37°C + 5% CO2 for 1 hour) to permit reduction and formation of formazan. After medium removal, 200 μl (20% sodium dodecyl sulphate, SDS solution in water/dimethylformamide (1:1), pH 4.7) was added to dissolve the crystals of formazan formed and trapped with the Hep-2 and Vero cells, and the set-up was further incubated overnight. Thereafter, the optical densities were read at 550nm in a multi-well microtitre plate reader (Tecan, Austria). Optical densities values from triplicate wells of drug-treated and no-drug-treated (control) set-up were employed for the calculation of the mean and standard error of the percent values for each drug concentration. Finally, the 50% cellular toxicity concentration (TC50) of the AA extract was determined according to simple regression analyses.

Antiviral activity
Evaluation of anti-respiratory syncytial activity followed the described method (Esimone et al., 2008; Lai et al., 2013). A 6000 cells/well population of
HEp-2 cells were seeded in 200 μl of 5% FCS DMEM in 96 well plate and incubated overnight. Next day, AA extract (100, 50, 25, 12.5, and 6.25 μg/ml) and virus MOI of 0.01 (MOI: multiplicity of infection) were added sequentially to the relevant wells and incubated for 48 hours at 37°C + 5% CO₂. Cell culture wells containing the virus, but no extract served as no-drug treated control. After the incubation, virus plaques which appear green under fluorescence microscope were enumerated as plaque-forming units (pfu) in both drug-treated and no-drug-treat (control) experimental set-up. Percent virus plaques reduction was calculated, and simple regression analysis was used to establish the standard 50% inhibitory concentration IC₅₀.

**Statistical analysis**

Anti-respiratory syncytial virus activity were determined based on the standard error of the mean (±SEM) resulting from percent values of triplicate measurements. All statistical data were obtained using GraphPad Prism software version 6 (GraphPad Software Inc., La Jolla, CA).

**RESULTS AND DISCUSSION**

**RSV Inhibition assays**

Before infecting Hep-2 cells to achieve a multiplicity of infection of 0.01. Different concentrations of the extracts were pre-incubated with cells at 37°C for 30 minutes, followed by recombinant rgRSV. Results are presented as percentage virus infectivity (plaque-forming units) in Hep2 cells of drug-treated wells relative to no-drug-treated wells (control). The mean and standard error of mean (SEM) of triplicate readings are presented.

**Cell Viability Assays**

Hep-2 cells were incubated with different concentrations of *Aspilia africana* extract. After 48 hours of incubation alongside the infectivity assay, MTT solution was introduced into each well, incubated for 1 hour, after which medium were aspirated, and 200 μl (20% sodium dodecyl sulphate, SDS solution in water/dimethylformamide (1:1), pH 4.7) added to each well and allowed to dissolved the formed formazan overnight. The next day, absorbance was measured at 550 nm. Results are presented as percentage cell viability (Optical density, OD) in Hep2 cells of drug-treated wells relative to no-drug-treated wells (control).

Vero cells were incubated with different concentrations of *Aspilia africana* extract. Results are presented as percentage cell viability (Optical density, OD) in Vero cells of drug-treated wells relative to no-drug-treated wells (control).

In RSV research, the focus has been to discover therapeutic agent with superior efficiency and safety than the already available ribavirin. As a result, several plants has been screened with potential anti-respiratory syncytial virus components discovered in various studies; though some were weak candidates for further development into clinically useful substances when their cytotoxicity profiles are considered ([Baker et al., 1995; Golankiewicz et al., 1995; Odimegwu et al., 2011]). Thus the quest for more ef-
cacious and less cytotoxic substances is sought for. This led us to screen AA for possible anti-RSV effect because of its reported folkloric and ethnomedicinal properties. The methanol extract was studied, and our results show that the extract of AA possesses notable anti-RSV activities given the recorded cytotoxicity concentration (TC_{50}), inhibitory concentrations (IC_{50}), and Selective index (SI) (Table 1, Figure 1 and Figure 2). The derived IC_{50} value (42.5 μg/ml) of AA shows its notable ability to inhibit the growth of RSV (rgRSV) by 50% in a culture of HEp-2 cells, which is also amenable to natural infection by RSV. The inhibited lgRSV strain harbours the recombinant green fluorescent protein which allows the enumeration of viable virus under the microscope which definitely improves the accuracy of data obtained in real-time during the life cycle of virus infectivity evaluation, and differs markedly from results normally obtained if unviable alcohol-fixed viruses were to be stained (Esimone et al., 2008). Thus, in calculating the 50% inhibition of RSV infectivity, only infected HEp-2 cells showing fluorescent green when observed under the fluorescent microscope were enumerated, thus eliminating error that could result due to possible loss of dead cells through the old conventional methods involving alcohol cell fixation and corresponding cell death. During RSV infection, syncytial formation mediated principally by RSV fusion protein-cell membrane interaction and its associated cell cytopathy represents a major feature of RSV disease infection. Inhibition of infectivity is definitely a key way of addressing this problem, and thus protect cells from the lethal effect of virus infection. Therefore, the capability of any substance (including medicinal plants extract) to reduce the infectivity of a virus such as RSV would be most desirable (Wang et al., 2008).

The cytotoxic effect (TC_{50}) recorded for the extract of AA (121.4 μg/ml) (Table 1, Figure 2) appears relatively promising, especially when it is juxtaposed with the relativity parameter of Selective index (SI) which gave approximately 3. This value sometimes is typical of extracts where moderate values may normally be encountered owing to the fact that they are a product of a cocktail of different phytochemical substances of natural origin contained within the medicinal plant, and this is also true of the cytotoxicity outcome (Figure 3) in Vero cells which is monkey-derived cell line. Therefore, their predictability, inhibitory, and cytotoxicity profiles are expected to escalate positively (for some constituents) and negatively (for other constituents) when they are further chemically purified and isolated. Hence, further purification and isolation processing of AA extract would be warranted to properly characterize the plethora of anti-RSV agents present within it.

Although there have been reported studies describing the antimalarial and antimicrobial activities of AA (Okoli et al., 2007; Ukwueze et al., 2013; Etiosa et al., 2018), however, this current study is apparently the only concise antiviral assay, and clearly the first report of the anti-respiratory syncytial virus activity of the plant. The evaluation of medicinal plants for suitable anti-RSV drug candidates has persisted, and progressively, a number of potential anti-respiratory syncytial virus substances have been reported from several studies, however, with many being too cytotoxic (Baker et al., 1995; Golankiewicz et al., 1995; Odimegwu et al., 2011). Thus, improved cytotoxicity value will represent a desirable feature in an ideal anti-RSV substance, and this can particularly be improved upon for AA and its potential constituents. Thus, the plant extract could still be considered for further development of anti-RSV drugs.

Prospectively, the development of anti-RSV drugs from AA seems necessary and would begin with the isolation of defined chemical moieties that would serve as lead sources for the development of more potent and safer anti-RSV compounds. Specific goals would be to isolate, characterize, and evaluate available compounds from AA, and where necessary, to generate chemical derivatives towards improving/increasing the selectivity index. This is a definite and realizable outlook for the further development of AA as a source of anti-RSV molecular therapeutics.

**CONCLUSIONS**

From our study, *A. Africana* methanol extract was evaluated against the RSV wherein it notably reduced virus infectivity, and have thus proven to be a potential source of future antiviral agents against the RSV.

| Plant (Herb) | IC_{50} | TC_{50} | SI Index |
|-------------|---------|---------|----------|
| Aspilia africana | 42.5±8.89 | 121.4±7.21 | 2.86 |

**Table 1: Anti-RSV Screening of Aspilia Africana in Hep2 cells**
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