Antiviral activity of Salidroside from the leaves of Nigerian mistletoe (*Loranthus micranthus* Linn) parasitic on *Hevea brasiliensis* against respiratory syncytial virus

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Abstract: Isolated Salidroside from the leaves of Nigerian mistletoe (*Loranthus micranthus* Linn) parasitic on *Hevea brasiliensis* was evaluated for its antiviral activity against respiratory syncytial virus. Semi- preparative HPLC separation of the ethyl acetate fraction of the leave extract of *Loranthus micranthus* Linn parasitic on *Hevea brasiliensis* led to the isolation of a polyphenol. Using spectroscopic methods (1D and 2D NMR and mass spectroscopic data) as well as by comparison with literature data the structure of the compound was determined as 6-O-galloyl salidroside. The antiviral activity of the isolated compound was evaluated against the respiratory syncytial virus. The isolated Salidroside showed potent inhibition towards a recombinant straining respiratory syncytial virus with Inhibitory Concentration (IC_{50}) value of 10.3±1.50 µg/mL. The result indicates that Salidroside is an efficient antiviral agent against RSV infection and might be useful for the management of RSV pathogenesis.

Keywords: *h*RSV, *Loranthus micranthus* Linn, Salidroside, structure elucidation, anti-*h*RSV activity.

INTRODUCTION

Human Respiratory Syncytial Virus (*h*RSV) is a negative-sense, single-stranded RNA virus of the family *Paramyxoviridae*, which includes common respiratory viruses such as those causing measles and mumps (Akhter and Al Johani, 2011). *h*RSV is a major cause of lower respiratory tract infections (LRTIs) in infants and young children (Akhter and Al Johani, 2011; Lai *et al*., 2013). This is especially true for high-risk groups, including infants with congenital heart disease and immunosuppressed patients, where infection by hRSV causes severe mortality (Shin *et al*., 2013). Respiratory illness caused by RSV such as bronchiolitis or pneumonia usually lasts about a week, but some cases may last several weeks. RSV was on average, responsible for 17% of acute respiratory infections in children admitted to hospital in the developing countries (Odimegwu *et al*., 2011). It has been estimated that acute lower respiratory infection by *h*RSV caused approximately 66,000-199,000 deaths of children under the age of five worldwide in 2005 (Falsey *et al*., 2005). In Nigeria it is reported that RSV infections occur all year round with a peak during the rainy season (Odimegwu *et al*., 2011). Treatment with adrenaline, bronchodilators, steroids, antibiotics, and ribavirin confer no real benefit (Shruti *et al*., 2013). Several natural compounds, including amentoflavone and cimicifugin inhibiting *h*RSV have been reported (Shin *et al*., 2013). Therefore, the search for novel anti-viral inhibitors of RSV from plant origin is of high importance (Esimone *et al*., 2008). Mistletoes are hemi parasitic plants growing on different host trees. They depend on their host plant for water and mineral nutrition, even though they produce their own carbohydrates through photosynthesis (Agbo *et al*., 2013). Mistletoes leaves are traditionally used in the management of diarrhoea, diabetics, and microbial invasions (Osadebe and Ukwueze, 2002; Osadebe *et al*., 2004; Osadebe *et al*., 2012). The chemical constituents of mistletoes sourced from different hosts included steroids and triterpenoids (Omeje *et al*., 2011); catechin derivatives and quercetin glycosides (Agbo *et al*., 2013). The present work deals with the isolation, structure elucidation and identification of polyphenol from the methanol extract of leaves of mistletoe parasitic on *Hevea brasilensis*.

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MATERIALS AND METHODS

General

The optical rotation of the isolated compound was recorded on a Perkin-Elmer 241 MC polarimeter. 1D (^{1}H, ^{13}C, DEPT 135) and 2D (COSY, HSQC, HMBC) NMR spectra of the isolated compound was recorded on Bruker ARX (500 MHz). LC-MS measurement was performed on a Thermofinnigan LCQ DECA mass spectrometer. Analytical HPLC analysis was performed with a HPLC system (Dionex, Munich, Germany). Routine detection was set at 235,254, 280 and 340nm (Xu *et al*., 2007).

Semi-preparative HPLC was performed on a C18 column (300×8 mm, Eurospher 100-10) on a MERCK HITACHI system equipped with a UV Detector. Vacuum liquid chromatography (VLC) was performed on silica gel (230-400 mesh, Merck) using a glass column (i.d. 3×30cm). Gel permeation column chromatography (CC) was performed on Sephadex LH-20 (Merck, Germany) using a glass column (i.d. 3×110cm).

Plant material

Loranthus micranthus leaves parasitic on *H. brasiliensis* were collected from Enugu-Ezike in Enugu State, Nigeria in the month of January 2012. The leaves were identified by Mr. A. O. Ozioko of the Bioresources Conservation and Development Program (BDCP), Nsukka. A voucher specimen (LM1610) was deposited at the herbarium of the Institute.

Extraction and isolation

Five hundred gram (500g) of the air-dried leaves were milled into powder and extracted with 3.0L of methanol at room temperature for 48h by cold maceration. The extract was evaporated *in vacuum* at 40^{o}C. The methanol extract (50g) was suspended on 400mL of 10% methanol-water mixture and successively portioned with *n*-hexane, ethyl acetate and *n*-butanol to yield *n*-hexane, ethyl acetate *n*-BuOH and water soluble fractions, respectively. Part of the ethyl acetate soluble fraction (5g) was purified further by vacuum liquid chromatography using silica gel (500g) as the stationary phase. The column was then eluted with a gradient of *n*-hexane-ethyl acetate (10:0 → 0:10, each 500mL) and dichloromethane-methanol (9:1→1:9, each 1000mL) to afford EFA-EFJ sub-fractions. Fraction EFI (600.0mg) was purified using Sephadex LH-20 column chromatography (100% MeOH) to afford EF_1-EF_9 sub-fractions. Fraction EF_2 (77.30mg) was separated by semi-preparative HPLC with methanol-water as mobile phase to give Salidroside (11.20mg). The structure of the isolated compound was identified based on comparisons of physical and spectroscopic data with published values.

Bioassay study

Cell and virus

The human larynx carcinoma cell line HEp-2 (Invitrogen, Germany) was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum (FCS) and penicillin/streptomycin (100U/mL) as previously described (Corse and Machamer, 2002). Virus was propagated by infecting HEp-2 cell monolayer with a previously prepared small-scale isolate stock at a multiplicity of infection (MOI) of 0.01 (Lai *et al*., 2006).

Cytotoxicity assay

Cell viability was determined by a MTT cell viability assay. HEp-2 cells (Human Larynx carcinoma cell line) seeded in a 96-multiwell plate and cultured in DMEM containing increasing concentrations (12.5, 6.25, 2.125 μg/mL) of salidroside were incubated at 37^{o}C in 5% CO_2 for 48h. The culture medium was replaced with fresh medium containing 50µL of MTT (5mg/mL) and incubated further for 1h to allow the formazan production. After that the MTT containing medium was aspirated and 200μL of DMSO was added to lyse the cells and solubilize the water insoluble formazone (Shruti *et al*., 2013). The optical density of lysates were determined at 550nm using a multiwell microplate reader and the concentration of 50% cellular toxicity (TC_{50}) determined by simple regression analysis.

Anti-respiratory syncytial virus assay

The antiviral activity of the isolated compound was assayed by the use of a recombinant strain rgRSV expressing the green fluorescent protein (rg) as a reporter gene as previously described (Ternette *et al*., 2007; Jan *et al*., 2011). HEp-2 cells were plated in triplicates into 96-well plates at 6000 cells/well and incubated for 24h. The prepared compound was added to the well, followed by the virus with multiplicity of infection (MOI) of 0.01 and incubated for 48h at 37°C+5% CO_2. Control wells containing virus and the same MOI in D-5 (containing 0.5% DMSO) but without drugs were also set up.

After 48 h incubation, the number of plaque forming units (pfu) in drug-treated and untreated HEp-2 cells was determined by fluorescence microscopy where localized green cells harboring rgRSV were counted as viral plaques. Percentage viral plaques reduction were then calculated, and IC_{50} determined by simple regression analysis.

STATISTICAL ANALYSIS

Results were presented as mean ± standard error of the mean (SEM) of at least triplicate determinations (n=3). To demonstrate statistical significance of data, a One-way Analysis of Variance (ANOVA) using Graph Pad Prism 5 software (GraphPad Software, Inc., San Diego, CA) was performed followed by Dunnett's *post hoc* test. Generally, differences between test and control treatments are considered significant at $p<0.05$.

RESULTS

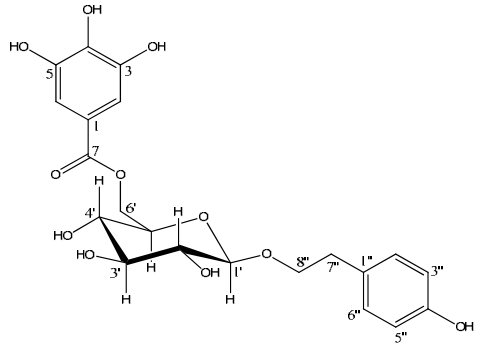
Isolation and Structure Elucidation

The methanol extract of the leaves was partitioned with *n*-hexane, ethyl acetate and *n*-butanol. The ethyl acetate fraction was purified to yield the Salidroside (fig. 1). By means of spectroscopic analysis, they were identified as 6-O-galloyl Salidroside (table 1). The optical rotation of the compound was determined as -40.7(*c* 0.10, MeOH). This secondary metabolite was obtained from mistletoe parasitic on *H. brasiliensis* for the first time.

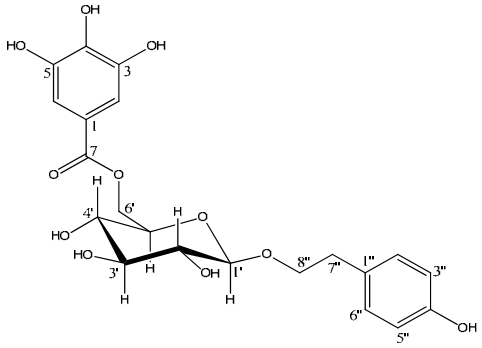
Cell viability assay

To determine the possibility that cytotoxicity of salidroside could affect *h*RSV replication, we carried out

the cytotoxicity of salidroside by MTT assay. Salidroside did not show any significant cytotoxicity at the concentrations used for antiviral assay (fig. 2). The 50% cytotoxic concentration of salidroside was 9.12±0.90 μg/mL. Thus, salidroside does not induce severe cell cytotoxicity and the antiviral activity of salidroside is not due to the non-specific cell cytotoxicity of salidroside.



(A)



(B)

Fig. 1: A= Chemical structure of Salidroside. B=Key ^{1}H-^{1}H COSY (bold line) of Salidroside

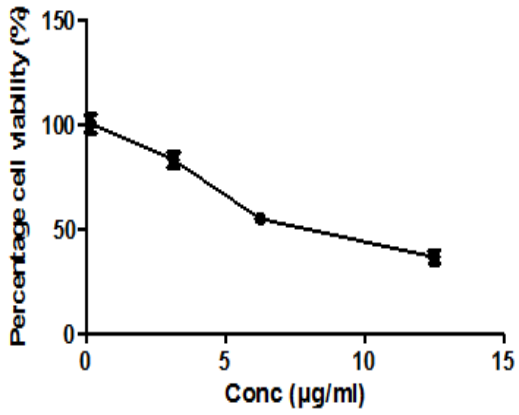


Fig. 2: Effects of Salidroside on cell viability. Hep-2 cells were treated with increased concentration of Salidroside for 48h and subject to a MTT assay. Data represent Means ± SEM. All the experiments were performed in triplicate and data represent the percentage of viable cells (means ± SEM).

Anti-RSV activity

The isolated compound exhibited strong anti-RSV activity in a dose dependent manner with Inhibitory Concentration (IC_{50}) value of 10.3±1.50μg/mL (fig.3)

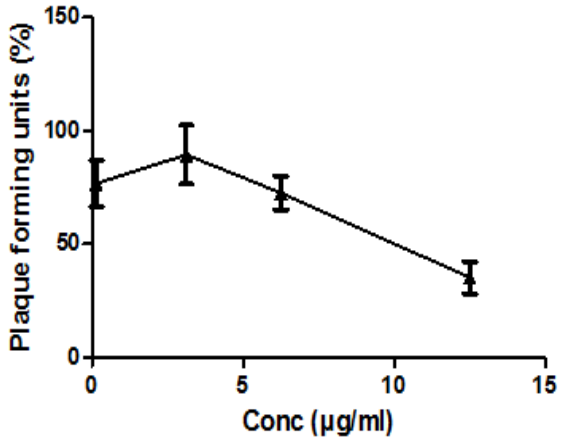


Fig. 3: Inhibition of RSV infectivity by salidroside. Anti-*h*RSV effect of the salidroside was assessed by recombinant strain rgRSV assay. Data represent means ± SEM.

HPLC analysis of the isolated compound

6-O-galloyl Salidroside was an off-white solid. The retention time (t_R) of the isolated compound is 15.42 mins. The UV spectrum in MeOH showed λ_{max} 216.6 and 275.7nm characteristic of a benzoid chromosphere. The positive and negative ion modes in the HPLC-MS revealed *m/z* of 453$[M+H]^+$ and 451 $[M-H]^-$ respectively (fig. 4).

DISCUSSION

6-O-galloyl salidroside was obtained as an off-white solid. The HPLC-MS spectrum exhibited a molecular peak at *m/z* 452 $[M+H]^+$ indicating a molecular formula of $C_{21}H_{24}O_{11}$ (Debbab *et al*., 2009) containing ten degree of unsaturation. The ^{1}H NMR spectrum showed signals typical of a galloyl moiety (7.09, 2H,d, *J*=4.3 Hz), one *para*-substituted aromatic ring (*δ* 6.98, 6.65, each 2H, d, *J*=8.5 Hz) representing two pairs of chemically equivalent protons for H-2"/6" and H-3"/5" respectively and two methylene which are coupled with each other (*δ* 2.80, 3.93, each 2H,dd, *J*=8.5 Hz). The occurrence of a sugar moiety, as well as its glycosidic nature, was demonstrated by the appearance of an anomeric proton signal at *δ* 4.32 (d, *J*=7.8Hz) (Clematis *et al.*, 2011) together with the ^{1}H-^{1}H COSY correlations. In the ^{1}H-^{1}H COSY spectrum, the anomeric proton (δ_H 4.32 d, H-1') was found to correlate with H-2' (δ_H 3.21, t) which further coupled to H-3' (δ_H 3.40, m). The latter proton showed correlation to H-4' (δ_H 3.65, s) which in turn correlates to H-5' (3.40m), thus revealing a typical coupling pattern of a glucosyl residue.

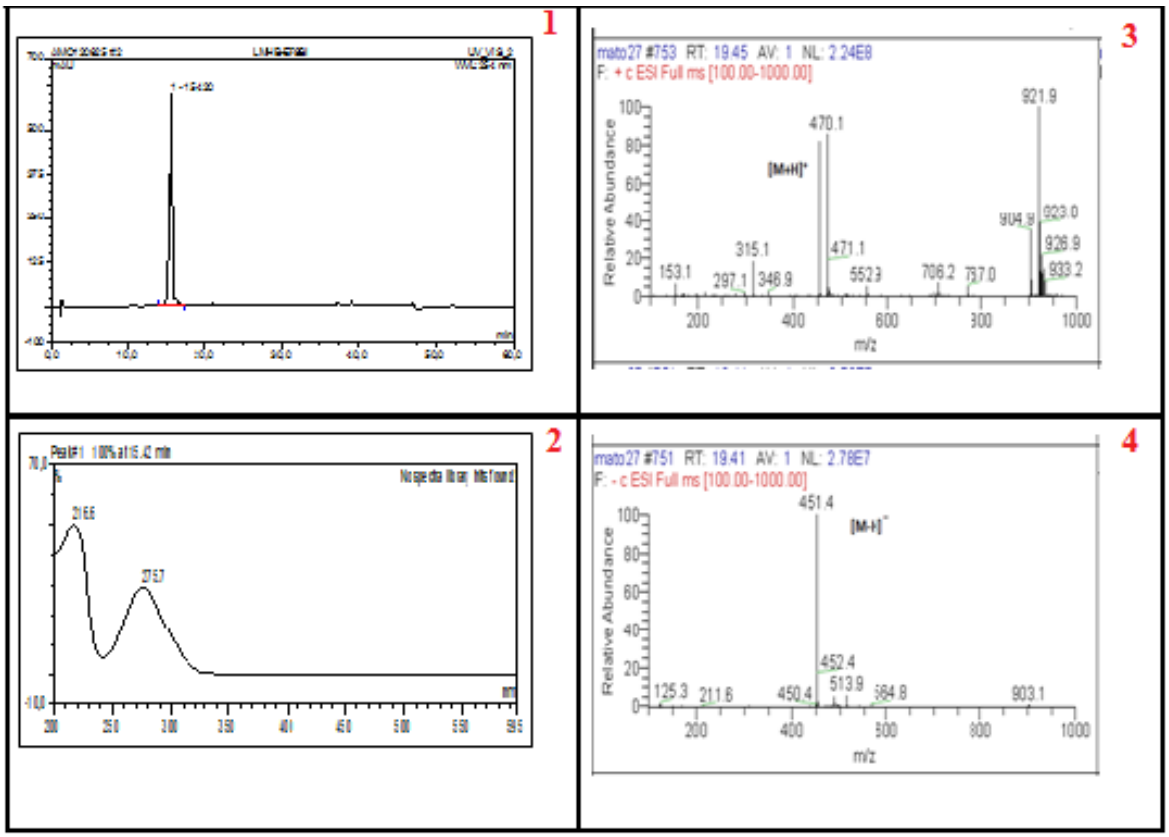


Fig. 4: HPLC Analysis of the isolated compound 1. HPLC Chromatogram 2= UV Spectrum 3. Positive ion and Negative ion (4) Modes HPLC-MS spectra

Table 1: ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Data of the isolated Compound

Position	δ_C	δ_H (J in Hz)	COSY
1	122.3		
2	110.9	7.09,d (4.3)	
3	147.3		
4	140.7		
5	147.3		
6	110.9	7.09,d (4.3)	
7	168.6		
1'	105.2	4.32,d (7.8)	2'
2'	75.8	3.21,t (8.4)	3'
3'	78.8	3.40,m	4'
4'	73.1	3.65,s	5'
5'	76.2	3.40,m	6'
6'	65.5	4.50,m	5'
		4.44,dd (5.8,11.8)	
1"	131.0		
2"	131.4	6.98,d (8.4)	3",5"
3"	117.1	6.65,m	2",6"
4"	157.5		
5"	117.1	6.65,m	2",6"
6"	131.4	6.98,d (8.4)	3",5"
7"	37.2	2.80,dd (8.5, 17.4)	8"
8"	72.6	3.93,dd (8.5,17.4)	7"

The ^{13}C NMR spectrum showed 21 carbon signals, which were divided into 1 carbonyl carbon, 12 sp^2-hybridized carbons,7 oxygenated sp^3-hybridized carbons (two methylene, and five methine carbons),1 sp^3 -hybridized carbon, as aided by the DEPT-135 experiment. The twelve sp^2-hybridized carbons include three chemically equivalent methines (δ_C110.9, CH-2/6; 147.3,CH-3/5; 131.4, CH-2"/6") and one equivalent oxygenated quaternary carbon (δ_C 117.1, C-3'/5'), as well as two further quaternary carbons at δ_C 122.3 (C-1) and δ_C 131.0 (C-1") and two oxygenated carbons at δ_C 140.7 (C-4) and δ_C 157.5 (C-4"). Therefore, the compound was elucidated as β-D-Glucopyranaside-2-(4-hydroxyphenyl) ethyl-6-(3, 4, 5-trihydroxy) benzoate (6-O-galloylsalidroside) (Kim *et al*., 2008).

The isolated salidroside was shown to exhibit strong anti-RSV activity with IC_{50} value of 10.3±1.5μg/mL. The outcome of RSV infectivity study with various concentrations of the isolated compound in HEp-2 cells (Human Larynx carcinoma cell line) culture showed that the compound inhibits RSV infectivity in a near concentration-dependent manner. Our present findings have shown the potential benefit of salidroside as a possible candidate to be developed into useful RSV therapy. Therefore, we proceeded to screening it for safety profile using a suitable mammalian cell line of

human airway epithelial origin. This is instructive given that RSV infection and replication in human host is largely restricted to the airway epithelial tissue, and in severe cases could affect other lower respiratory tract tissues.

A lot of pharmacological properties of salidroside (*p*-hydroxyphenylethyl-β-D-glucoside) isolated from *Rhodiola rosea* have been reported. The protective effects of salidroside isolated from *Rhodiola rosea* L against endogenous hydrogen peroxide (H_2O_2)-induced cytotoxicity in human endothelial cells (EVC-304) have been investigated (Zhao *et al*., 2013). Salidroside was found to attenuate H_2O_2 induced cytotoxicity in EVC-304 cells in a dose dependent pattern. The *in vivo* and *in vitro* antiviral effects of salidroside isolated from *Rhodiola rosea* L against coxsackievirus B3 (CVB3) has been reported (Wang *et al*., 2009). The isolated salidroside exhibited obvious antiviral effects in both *in vitro* and *in vivo* experiments and was found to modulate the *m*RNA expression of interferon-gamma (IFN-γ), interleukin-10 (IL-10), tumor necrosis factor-alpha (TNF-α), and interleukin-2 (IL-2), thus possesses antiviral activities against CVB3.

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