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In vitro anti-influenza screening of several Euphorbiaceae species: Structure of a bioactive Cyanoglucoside from *Codiaeum variegatum*

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ABSTRACT

A bio-guided screening against influenza A virus (FLUAV) was carried out with seven Euphorbiaceae species. The results showed that chromatographic fractions from *Phyllantus niruri*, *Euphorbia pulcherrima* and *Codiaeum variegatum* had relevant anti-FLUAV activity, although only chromatographical subfractions from *C. variegatum* kept the activity. From this plant, the active compound against FLUAV was isolated. Its structure was assigned as 2-(3,4,5)-trihydroxy-6-hydroxymethyltetrahydropyran-2-yloxymethyl)acrylonitrile (**1**) on the basis of NMR, mass spectrometry and X-ray diffraction analysis. The compound displayed virucidal activity without impairment of haemagglutination properties of the used virus strain. This is the first report indicating antiviral activity of a cyanoglucoside.

1. Introduction

From approximately 200 viral respiratory pathogens, the most important ones are influenza viruses, causing around half a million deaths globally each year (Brooks et al., 2004). Influenza A virus (FLUAV) belongs to the *Orthomyxoviridae* family and is an enveloped single negative-stranded RNA virus. One of the most important viral proteins, the haemagglutinin (HA) protein, is responsible for virus attachment to the sialic acid receptor for the subsequent fusion of viral and cellular membranes (Palese and Shaw, 2006). Available antiviral drugs frequently lead to the development of viral resistance, characterized by their high mutational rate (Palese and Shaw, 2006). The search for natural antiviral compounds from plants is a promising approach in the development of new therapeutic agents (De Clercq, 2006; Wang et al., 2006). Natural compounds such as fucan (Udhikari et al., 2006), lignans (Asano et al., 1996), flavonoids (Du et al., 2003) and phytoestrogens (Martin et al., 2007) displayed activities against several types of viruses. Regarding Euphorbiaceae species, extracts of *Euphorbia paralias, E. serpens* and *Phyllantus* spp. showed antiviral activity (Abdelgaleil et al., 2001; Ruffa et al., 2004; Bagalkotkar et al., 2006).

In this paper, we report a bioassay guided screening for anti-FLUAV compounds and the isolation and identification of an active compound from *Codiaeum variegatum.*

2. Results and discussion

2.1. Structural elucidation

Through column chromatography of the active fractions I-2 and I-4 from *C. variegatum* a new compound was isolated. According to the high resolution mass spectrometry measurements, compound **1**, which we propose to name codiacyanoglucoside, has the elemental composition $C_{10}H_{15}NO_6$. Since no melting point could be determined due to molecular carbonization, exhaustive acetylation provided a peracetate derivative for further characterization, which indicated four of the six oxygen atoms to be hydroxyl groups. The ^{1}H NMR spectrum of **1** showed two signals at δ 6.25 (1H, *t*, J = 1.4 Hz) and δ 6.13 (1H, br s), corresponding to protons of an sp^2 terminal methylene group, which were long-range coupled to an allylic oxygen bearing methylene group that appears as two double triplets (J_d = 13.4 Hz, J_t = 1.4) at δ 4.49 and at δ 4.33. Other resonances, suggesting the presence of a sugar residue, were a doublet (J = 7.7 Hz) at δ 4.34, a double doublet (J = 12.1 and

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1.4 Hz) at δ 3.90, another double doublet (J = 9.2 and 7.7 Hz) at δ 3.25, a double double doublet (J = 12.1, 4.2 and 1.4 Hz) at δ 3.69, and complex multiplets for one hydrogen atom at δ 3.37 and for two hydrogen atoms at δ 3.30.

Six signals observed in the ^{13}C NMR spectrum of **1** at δ 104.3 (*s*, anomeric), δ 75.7 (*d*, C-2′), δ 78.8 (*d*, C-3′), δ 72.4 (*d*, C-4′), δ 79.0 (*d*, C-5′), and δ 63.6 (*t*, C-6′) were indicative of a glucopyranosyl moiety. The remaining four resonances were an oxygen bearing allylic methylene group at δ 69.9 and three at δ 134.1 (CH_2), δ 122.3 (C), and δ 119.1 (CN), corresponding to a conjugated $H_2C{=}C{-}CN$ residue, in agreement with the CN band at 2220 cm^{-1} in the IR spectrum, and a strong absorption at 201 nm (ε, 12,600) in the UV spectrum. The high frequency chemical shift of the sp^2 methylene carbon is due to its conjugation to the cyano group. A gHSQC plot gave evidence of the hydrogen atoms being directly attached to carbon atoms, while the gHMBC spectrum showed long-range coupling of the doublets owing to the terminal methylene protons at δ 6.25 and δ 6.13 with the methylene group at δ 69.9 and the quaternary carbons at δ 122.3 and at δ 119.5. In turn, the methylene protons at δ 4.49 and δ 4.33 showed connectivities to the signals at δ 134.1, δ 122.3, δ 119.2 and δ 104.3. The nitrile carbon atom is shielded at δ 119.1, due to conjugation with the methylene group. Homonuclear ^{1}H–^{1}H COSY and spin–spin decoupling experiments performed on **1** and its tetraacetate provided the assignments given in the Experimental. For the latter molecule, gHSQC and gHMBC measurements were also performed. The structure of **1** was independently confirmed by single crystal X-ray diffraction analysis (Fig. 1).

From a biogenetic point of view, codiacyanoglucoside (**1**) might be derived from valine, in analogy to the biosynthetic pathway (Forslund et al., 2004) of other cyanoglucosides found in nature. Nevertheless, this is the first report showing a cyanoglucoside with strong anti-FLUAV activity.

2.2. Anti-FLUAV activity

The chemical fractions (crude latex, or soluble and insoluble leaf fractions) from *Hura crepitans, Codiaeum variegatum, Caryodendron orinocense, Phyllanthus niruri, Euphorbia pulcherrima, Euphorbia cotinifolia* and *Euphorbia tirucalli* plants were tested using the conventional colorimetric MTT [3-(4,5-dimethylthiazol–2-yl)-2,5-diphenyltetrazolium bromide] assay to evaluate the anti-FLUAV activity. A bio-guided fractionation strategy to screen the extracts and chromatographic fractions was used. A selective index (SI) $\geqslant$ 2 or inhibitory concentration 50% (IC_{50}) $\leqslant$ 100 μg/mL (Cos et al., 2006) was established as stringent endpoint selection criterion for relevant antiviral activity. According to the above selection parameters, three crude extracts exhibited antiviral activity: the soluble fraction in the hexane-dichloromethane-methanol mixture of *P. niruri* and the insoluble fractions in the same mixture solvents of *E. pulcherrima* and *C. variegatum* (Table 1). The antiviral activities of *P. niruri* and *E. pulcherrima* were probably lost after fractionation since a putative synergist effect in the antiviral activity could disappear when individual constituents are evaluated.

After purification of the active fractions by Sephadex LH-20 column chromatography, four subfractions from *P. niruri* and *E. pulcherrima* were each obtained, but none had relevant antiviral activity against FLUAV, thereby precluding the identification and characterization of any active compound. However, two subfractions (I-2 and I-4) obtained from *C. variegatum* exhibited relevant antiviral activity, both with similar IC_{50} and SI values. Morphological changes in treated non-infected MDCK (Madin–Darby Canine Kidney) cells were not detected and cytotoxic activity was not found since CC_{50} was above the higher concentration tested (Table 1).

MTT test only measures cell viability, but it does not directly determine virus production (Cheng et al., 2004). Therefore, to determine if subfractions I-2 and I-4 from *C. variegatum* were able to reduce viral replication, the haemagglutination assay was carried out. The results showed a significant dose-dependent decrease of the viral progeny, as measured by hemagglutinating activity, in the supernatant from treated cells, as compared to those from infected but non-treated cells, thus indicating that these subfractions reduced viral production. This virucidal assay measures the ability of **1** to reduce viral infectivity, although the virus retains its ability to induce haemagglutination. The haemagglutination decrease reflects a decrease in production of viral particles in treated cells, while the assays in the next section measure the haemagglutinating capacity of the virus after pretreatment with **1**, but do not measure viral progeny.

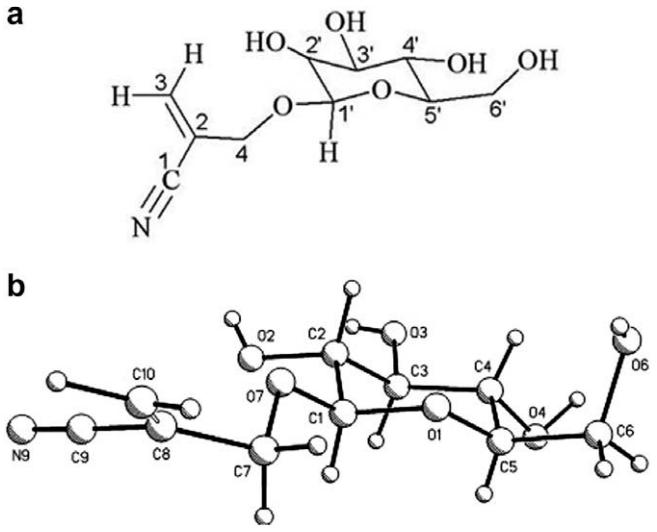

Fig. 1. (a) Structure and (b) X-ray PLUTO plot of codiacyanoglycoside (1). X-ray and systematic molecular numbering differ.

Table 1
Anti-FLUAV activity of fractions and subfractions obtained from *Phyllanthus niruri*, *Codiaeum variegatum* and *Euphorbia pulcherrima* in MDCK cells

Species	Code	CC_{50} (μg/mL)	IC_{50} (μg/mL)	SI
Phyllanthus	S[a]	202 ± 23	40 ± 4	5.1
niruri (leaves)	S-1	>250	NA[c]	NC[d]
	S-2	171 ± 1	NA	NC
	S-3	153 ± 12	NA	NC
	S-4	>250	NA	NC
Codiaeum	I[b]	**>250**	**91 ± 14**	**>2.7**
variegatum (latex)	I-2	**>250**	**60 ± 14**	**>4.1**
	I-4	**>250**	**59 ± 22**	**>4.2**
	Cyanoglucoside 1	**>250**	**17 ± 5**	**>14.9**
Euphorbia	I	**>250**	**89 ± 6**	**>2.8**
pulcherrima (leaves)	I-1	116 ± 5	NA	NC
	I-2	106 ± 3	NA	NC
	I-3	155 ± 4	NA	NC
	I-4	>250	NA	NC

The values represent the mean ± SD of three independent experiments.
[a] S: portion of the crude soluble fraction in the hexane–dichloromethane–methanol (2:1:1, v/v) mixture.
[b] I: insoluble portion of the above triple mixture.
[c] NA: No activity; the percentage of protection was <20% at a non-toxic concentration.
[d] NC: Not calculated. Solvent control (DMSO) showed no activity.

2.3. Antiviral activity of cyanoglucoside **1** from C. variegatum latex

A cyanoglucoside was isolated from the active fractions subfractions I-2 and I-4 of *C. variegatum*. Through the MTT assay, we found that this compound affected viral replication in a dose-dependent manner and the antiviral activity was more potent than that exhibited by the initial fraction. At 250 μg/mL, the protection was 100%. Similar to subfractions I-2 and I-4, morphological changes were not observed in treated non-infected MDCK cells. The IC_{50} and SI values were higher than the ones exhibited by the active subfraction, indicating that this compound is responsible for the anti-FLUAV activity (Table 1).

To explore the antiviral mechanism, a virucidal assay was performed. Two-fold serial dilutions of the compound were incubated at room temperature with the virus and then this mixture was used to infect MDCK cells. The results indicated that the infectivity of FLUAV was lost before the virus was in contact with the cells (Fig. 2). To test whether pre-incubation of the virus with codiacyanoglucoside **1** would result in impairment of the FLUAV haemagglutination capacity, two-fold serial concentrations of the compound were incubated with or without 10 HAU (haemagglutination units) of the virus. The results indicated that at 250 μg/mL cyanoglucoside **1** reduced the influenza haemagglutination activity only by one HAU, but at lower concentrations no reduction was observed. The non-inhibitory effect on viral adsorption onto human red blood cells, suggests that this compound exhibits virucidal activity without impairing the haemagglutination ability of FLUAV.

Cyanogenesis is especially common in Euphorbiaceae and is found in many genera including *Bridelia*, *Euphorbia* and *Phyllanthus*, as well as in the economically important species, *Hevea brasiliensis* and *Manihot esculenta* (Miller et al., 2006). The poisonous effect of cyanogenic glycoside compounds is caused by enzymatic cyanide liberation, which inhibits the respiratory chain. Despite the fact that compound **1** has a nitrile group, it is a non-cyanogenic glycoside, because a hydrolytic process is unable to produce free cyanide due to the presence of the conjugated methylene group. Thus, a potential toxic effect is excluded.

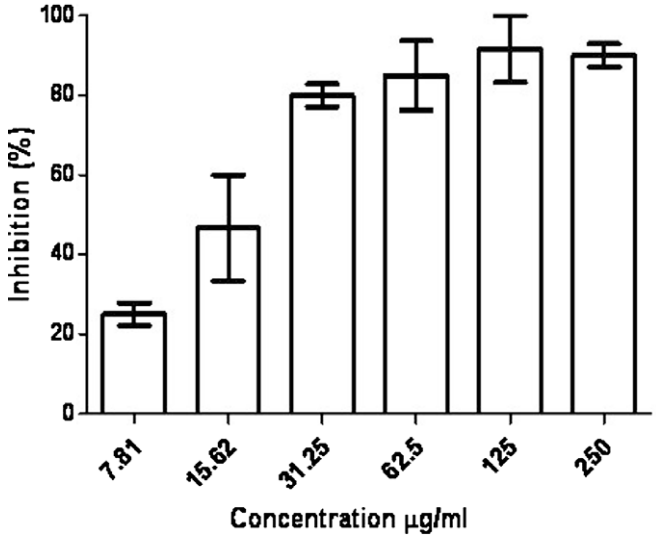

Fig. 2. Virucidal activity of **1** against FLUAV. This assay shows viral infectivity reduction on MDCK cells. Cells were infected with FLUAV (1000 PFU/mL) and incubated with or without two-fold serial dilutions of **1**. Each mixture was then diluted 1:500 and 200 μL were added to the cells and incubated for 1 h at 37 °C. Cells were then overlaid for 72 h with a low-viscosity overlay medium, the virus was inactivated with 10% formaldehyde and the cell monolayer was stained. Plaques were counted to determine the virucidal activity of 1. Each bar represents the mean ± SD of three independent experiments.

The functional analysis indicates that the antiviral activity of the cyanoglucoside is independent of the HA protein, since this compound exhibited virucidal activity without impairing the haemagglutination induced by FLUAV. There are no reports indicating antiviral activity of this or similar compounds that might suggest a mechanism of action of cyanoglucoside **1**. Considering that different reports indicated that a single compound might interfere with more than one step in the viral replication cycle (Cheng et al., 2004), it is necessary to explore whether this compound might impair subsequent steps during viral replication and interfere with other influenza strains.

3. Conclusion

A new cyanoglucoside was isolated from *Codiaeum variegatum* and the structure was determined through 2D NMR, HRMS and X-ray diffraction. For the first time this type of secondary metabolite is reported as a virucidal compound. In addition, the mechanism of action did not involve a biochemical interaction with the virus haemagglutinin. It is important to underline that according to the structural characteristics, this metabolite should not be associated to toxic effects considering that chemical or enzymatic hydrolysis of this compound does not result in the production of the toxic ion, cyanide, due to the presence of an exocyclic methylene in the molecule. Other metabolites isolated from *Euphorbiaceae* family, such as putranjavaina A (Cheng et al., 2004), hippomanin A (Yang et al., 2007) and niruriside (Qian-Cutrone et al., 1996), have shown virucidal activity and/or capacity to inhibit viral replication at post-entry steps; however, their chemical structures differ from the compound reported in this study.

4. Experimental

4.1. General

The melting point was determined on a Fisher–Johns apparatus and is uncorrected. UV spectra were recorded on a Perkin Elmer Lambda 12 spectrophotometer. The IR spectrum of **1** was obtained on a Perkin Elmer FTIR-1000, while that of tetraacetate **2** was obtained on a Buck Scientific model 500 spectrophotometer. Optical rotations were measured on a Perkin Elmer model 341 polarimeter. ^{1}H NMR spectra were recorded at 300 MHz and ^{13}C NMR at 75 MHz on Bruker AMX-300 and Varian Mercury 300 spectrometers. Chemical shifts are reported in δ units (ppm) and coupling constants (J) in Hz. High resolution mass spectra were determined by electrospray ionization/atmospheric pressure chemical ionization on an Agilent LCTOF spectrometer at the UCR mass spectrometry facility, University of California, Riverside, CA.

4.2. Plant material

Plants and latex were collected during 2005 and 2006 from several farms of Universidad de Antioquia, located in San Pedro, Barbosa, Porce and Caucasia, Colombia. Voucher specimens are in deposit at the Herbario Universidad de Antioquia (HUA): *Hura crepitans* 142565, *Codiaeum variegatum* 142566, *Caryodendron orinocense* A94316, *Phyllanthu niruri* 6743, *Euphorbia pulcherrima* 6715, *Euphorbia cotinifolia* 2707 and *Euphorbia tirucalli* 6797.

4.3. Extraction and isolation of compounds

Milled dry leaves from *H. crepitans, P. niruri, E. pulcherrima* and *C. orinocense* (500 g each) were extracted by percolation with MeOH (6 L) at room temperature during three days. After filtration and solvent evaporation in vacuo, residues containing 105.0, 102.3,

100.9 and 101.6 g, respectively, were obtained. A sample of 15 g of each residue was individually dissolved in H_2O and extracted with 400 mL of hexane-CH_2Cl_2–MeOH (2:1:1, v/v), with each yielding a soluble (*S*) and an insoluble (I) fraction. *H. crepitans* gave 5.9 g of the soluble and 9.1 g of the insoluble fraction, *P. niruri* gave 8.1 g of the soluble and 7.2 g of the insoluble fraction, *E. pulcherrima* gave 6.5 g of the soluble and 7.5 g of the insoluble fraction, while *C. orinocense* gave 7.6 g of the soluble and 6.4 g of the insoluble fraction. All eight fractions were individually tested for cytotoxic and antiviral activities, but only the soluble fraction of *P niruri* and the insoluble of *E. pulcherrima* were virucidal. After separation of active fractions by Sephadex LH-20 CC eluting with 500 mL of hexane–CH_2Cl_2–MeOH and 200 mL of MeOH, four subfractions from each *P. niruri* and *E. pulcherrima* were obtained, which showed no relevant antiviral activity against FLUAV.

On the other hand, fresh latex (20 mL each) from *E. cotinifolia* and *E. tirucalli* were individually extracted with EtOAc (100 mL) in a funnel at room temperature. The residues, obtained after solvent evaporation in vacuo, were partitioned by silica–gel CC with C_6H_{14}:EtOAc of increasing polarity. Ten fractions (10 mL each) were collected and combined according to their thin-layer chromatography profiles with hexane:EtOAc (3:1, v/v) or $CHCl_3$:MeOH (9:1, v/v) to provide five fractions that were tested for antiviral activity. All were inactive against FLUAV.

In addition, fresh latex (50 mL) from *C. variegatum* was extracted as above with hexane–CH_2Cl_2–MeOH (2:1:1, v/v, 500 mL), and then with MeOH (200 mL). The former was then re-extracted with hexane, CH_2Cl_2, EtOAc and finally with MeOH:H_2O (3:1, v/v) (50 mL each), which after evaporation yielded 7, 25, 20 and 120 mg, respectively. They were evaluated against FLUAV as described below. The aqueous MeOH fraction (120 mg) was further separated using a Sephadex LH-20 eluted with MeOH to provide four fractions. Active fractions 2 and 4 (31 and 43 mg) were combined and purified through a RP-18 cartridge eluted with H_2O, H_2O:MeOH (1:1, v/v) and MeOH to yield compound **1** (20 mg) after vacuum evaporation. The pure sample was obtained by crystallization from MeOH.

Acrylonitrile, codiacyanoglucoside (**1**): $[\alpha]_{589}$-26, $[\alpha]_{578}$-27, $[\alpha]_{546}$-33, $[\alpha]_{436}$-66 (*c*, 0.56 MeOH); $UV_{\lambda max}$ (ε) 201 nm (12,600); $IR_{\nu max}$ (KBr) 3540 and 3330 (hydroxyls), and 2220 (cyano) cm^{-1}; ^1H NMR (CD_3OD) δ 6.25 (1H, *t*, *J* = 1.4, H3*E*), 6.13 (1H, br s, H3*Z*), 4.49 (1H, *dt*, *J* = 13.4, 1.4, OCH_2–C=), 4.34 (1H, *d*, *J* = 7.7, H1′), 4.33 (1H, *dt*, *J* = 13.4, 1.4, OCH_2–C=), 3.90 (1H, *dd*, *J* = 12.1, 1.4, H6'), 3.69 (1H, *ddd*, *J* = 12.1, 4.2, 1.4, H6′), 3.37 (1H, *m*, H3′), 3.30 (2H, *m*, H4′ and H5′), 3.25 (1H, *dd*, *J* = 9.2, 7.7, H2′); ^{13}C NMR (CD_3OD) δ 134.1 (CH_2), 122.3 (C), 119.1 (CN), 104.3 (C1'), 79.0 (C5′), 78.8 (C3′), 75.7 (C2′), 72.4 (C4′), 69.9 (CH_2) and 63.6 (C6′); HRMS $[M + Na]^+$ *m*/*z* 268.0793 (calcd for $C_{10}H_{15}NO_6Na$: 268.0797).

Single crystals of **1** were obtained by slow evaporation from MeOH. The X-ray data were collected on a Bruker–Nonius CAD4 diffractometer equipped with Cu Kα monochromated radiation (λ = 1.54184 Å), at 298 K in the ω-2θ scan mode. Unit cell refinements were done using the Express v2.0 software. $C_{10}H_{15}O_6N$, triclinic, P1 (No. 1), *a* = 6.4963(4) Å, *b* = 6.676(3) Å, *c* = 14.2350(5) Å, α = 102.91(1) deg, β = 92.764(4) deg and γ = 91.47(1) deg, *V* = 600.6(3) Å^3, *Z* = 2. The structure was solved by direct methods using the Sir2004 program and refined using SHELXL-97 to provide R_1 = 3.9% and wR_2 = 10.5%. For the structural refinement, the non-hydrogen atoms were treated anisotropically and the hydrogen atoms, included in the structure factor calculation, were refined isotropically. Atomic coordinates, bond lengths, bond angles, anisotropic thermal parameters, hydrogen coordinates, calculated and observed structure factors and torsion angles are in deposit at the Cambridge Crystallographic Data Center. The CCDC deposition number is 693504.

Tetraacetylcodiacyanoglucoside (**2**): A solution of **1** (8 mg) in pyridine (0.2 mL) was treated with Ac_2O (0.2 mL) and stored at room temperature for 4 h. The reaction mixture was poured over ice-water and extracted with $CHCl_3$. The organic layer was washed with water, diluted hydrochloric acid (10%), H_2O, aqueous $NaHCO_3$, and H_2O again, and finally dried (anhydr. Na_2SO_4), filtered, and evaporated. The residue was crystallized from $CHCl_3$–hexane to provide **2** (12.3 mg, 91%) as white plates, m.p. 112–114 °C; $[\alpha]_{589}$-26, $[\alpha]_{578}$-27, $[\alpha]_{546}$-32, $[\alpha]_{436}$-54 (c, 1.23 $CHCl_3$); $UV_{\lambda max}$ (ε) 201 nm (9700); $IR_{\nu max}$ ($CDCl_3$) 2263 (cyano), 1755 (carbonyls) and 1230 (C–O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 6.06 (1H, *t*, *J* = 1.1, H3*Z*), 6.00 (1H, *t*, *J* = 1.5, H3*E*), 5.25 (1H, *t*, *J* = 9.4, H3′), 5.10 (1H, *t*, *J* = 9.6, H4′), 5.05 (1H, *dd*, *J* = 9.7, 7.9, H2′), 4.57 (1H, *d*, *J* = 7.9, H1′), 4.42 (1H, *dt*, *J* = 13.4, 1.3, OCH_2–C=), 4.24 (1H, *dd*, *J* = 12.4, 4.5, H6′), 4.19 (1H, *dt*, *J* = 13.4, 1.3, OCH_2–C=), 4.17 (1H, *dd*, *J* = 12.4, 2.4, H6'), 3.71(1H, *ddd*, *J* = 9.6, 4.5, 2.4, H5'), 2.10 (3H, *s*, OAc), 2.08 (3H, *s*, OAc), 2.03 (3H, *s*, OAc), 2.01 (3H, *s*, OAc); ^{13}C NMR ($CDCl_3$) δ 170.6 (C=O), 170.2 (C=O), 169.4 (C=O), 169.3 (C=O), 132.4 (=CH_2), 119.2 (CN), 116.6 (C=), 99.7 (C1′), 72.4 (C3′), 72.0 (C5′), 70.7 (C2′), 68.2 (OCH_2), 68.1 (C4′), 61.6 (C6′), 20.7 (CH_3), 20.6 (CH_3), 20.5 ($2CH_3$); HRMS $[M + Na]^+$ *m*/*z* 436.1218 (calc for $C_{18}H_{23}NO_{10}Na$: 436.1220).

4.4. Preparation of extracts and bioassay

The extracts, chromatographic fractions, or pure compounds were diluted with DMSO (Sigma, St. Louis, USA) to obtain a stock concentration of 40 mg/mL which was stored in aliquots at −70 °C until used. A dilution of 1:80 (v:v) from the stock solution was prepared in Eagles' minimal essential media (EMEM) (Sigma), supplemented with 2% of heat-inactivated fetal bovine serum (FBS) (Sigma), to obtain a final concentration of 500 μg/mL. Two-fold serial dilutions were prepared to obtain test preparations at the following concentrations: 500, 250, 125, 62.5, 31.25 and 15.625 μg/mL. Dimethyl sulfoxide (DMSO) was used as a control in all cases.

4.4.1. Cell cultures and viruses

MDCK (Madin–Darby Canine Kidney) cells and FLUAV (A/PR/8/34.H1N1) were purchased from the American Type Culture Collection (ATCC, Rockville, USA). The cell line was routinely grown and maintained in EMEM supplemented with 10% FBS, 1% penicillin/streptomycin (Sigma), 1% essential amino acids (Sigma), 1% vitamins (Sigma), 1% L-glutamine (Sigma), 0.5% $NaHCO_3$ (Sigma), and incubated at 37 °C in 5% CO_2. FLUAV was propagated in 11-day chick embryos and was adapted to MDCK cells. The virus was stored in small aliquots at −70 °C until used. Virus titration was performed by the limit dilution method, using 96-well microplates (Nunclon, NY, USA) and plaque formation using 24-well plate (Nunclon). The viral titer was estimated from cytopathogenicity of cells induced by viral infection and expressed as 50% tissue culture infectious doses/mL ($TCID_{50}$/mL) and PFU/mL.

4.4.2. Colorimetric MTT assay for cell toxicity and antiviral activity

The cytotoxicity and antiviral activity of the test fraction/compound were determined using quantitative colorimetric MTT (Smee et al., 2002). Briefly, MDCK cells were seeded in 96-well plates and grown to subconfluence. Serial two-fold dilutions of the test fraction were added. At each concentration, three wells were infected with ten $TCID_{50}$ of virus per well, while three wells were left uninfected for toxicity determination. Plates were incubated at 37 °C for 72 h, the culture medium was then removed and MTT (2 mg/mL, Sigma) was added. Each plate was incubated for further 2 h and DMSO was added. The optical densities (OD) were read on a microplate spectrophotometer, Dynex Revelation MRX Version 3.2 (Dynex Laboratories, S.R.O., Czech Republic) at 490 nm.

Results are expressed as: (i) Protection% = [(**A** − **B**)/(**C** − **B**)] × 100, where **A** corresponds to OD of cells treated and infected cells; **B** is OD of untreated and infected cells; and **C** is OD of untreated and uninfected cells; (ii) Cytotoxic% = [(**A** − **B**)/**B**] × 100, where **A** is the OD of untreated cells, and **B** is the OD of treated cells. The 50% cytotoxic (CC_{50}) and the 50% inhibitory concentrations (IC_{50}) of the test fractions were calculated by lineal regression analysis of the dose response curves generated from these data. Thus, the selective index (SI) for each compound was also determined from the CC_{50}/IC_{50} ratio.

4.4.3. FLUAV virucidal assay

The virucidal assay was performed according to Cheng et al., 2004, with minor modifications. Briefly, MDCK cells were seeded in 24 well culture plates with 150×10^3 cells/well in 500 μL of EMEM supplemented with 10% FBS and incubated at 37 °C, 5% of CO_2 for 24 h to reach at least 95% confluence. The virus (1000 PFU/mL) was incubated with or without two-fold serial dilution of fractions for 1 h at room temperature (~22 °C). This mixture was then diluted 1:500 and 200 μL were added to the wells and incubated for 1 hour at 37 °C. Later, the cells were overlaid for 72 h with a low-viscosity overlay medium containing 1.2% of microcrystalline cellulose AVICEL™ (FMC Biopolymer, Philadelphia, USA) (Matrosovich et al., 2006). Finally, the virus was inactivated with 10% formaldehyde in PBS and the cell monolayer was stained with 1% crystal violet. The plaques were counted by visual examination and the degree of plaque inhibition was calculated relative to the control in the absence of fractions. The virucidal activity of the extracts was determined by the following formula: Inhibition% = [1 − (plaque number)test/(plaque number)control] × 100.

4.4.4. Haemagglutination inhibition assay

Two-fold serial dilutions of the cyanoglucoside in PBS were mixed with an equal volume of FLUAV viral solution (10 hemagglutinating units (HAU)/50 μL). After incubation for 60 min at room temperature, 50 μL of 0.8% human erythrocytes type O RH positive, were added to an equal volume of the viral mixture and incubated for additional 60 min. A 1:80 dilution of DMSO was used as the control. Untreated erythrocytes precipitated to the bottom of the plate, while upon preincubation with the virus, the blood cells exhibit an even and diffuse distribution, indicating that the viral particles retained their erythrocyte-agglutinating properties.

4.5. Statistical analyses

Linear regression analyses were performed using the GraphPad Prism version 5.00 for Windows, GraphPad Software (San Diego, CA, USA). R^2 values greater than 0.80 were considered as statistically significant for extrapolation. Each experiment was repeated at least three times and all data are presented as mean ± SD (standard deviation) values.

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References

Abdelgaleil, S., Kassem, S.I., Doe, M., Baba, M., Nakatani, M., 2001. Diterpenoids from *Euphorbia paralias*. Phytochemistry 58, 1135–1139.

Asano, J., Chiba, K., Tada, M., Yosmil, T., 1996. Antiviral activity of lignans and their glycosides from *Justicia procumbens*. Phytochemistry 42, 713–717.

Bagalkotkar, G., Sagineedu, S.R., Saad, M.S., Stanslas, J., 2006. Phytochemicals from *Phyllanthus niruri* Linn. And their pharmacological properties: a review. J. Pharm. Pharmacol. 58, 1559–1570.

Brooks, M.J., Sasadeusz, J.J., Tannock, G.A., 2004. Antiviral chemotherapeutic agents against respiratory viruses: where are we now and what's in the pipeline? Curr. Opin. Pulm. Med. 10, 197–203.

Cheng, H.Y., Lin, T.C., Yang, C.M., Wang, K.C., Lin, L.T., Lin, C.C., 2004. Putranjivain A from *Euphorbia jolkini* inhibits both virus entry and late stage replication of herpes simplex virus type 2 in vitro. J. Antimicrob. Chemother. 53, 577–583.

Cos, P., Vlietinck, A.J., Berghe, D.V., Maes, L., 2006. Anti-infective potential of natural products: how to develop a stronger in vitro 'proof-of-concept'. J. Ethnopharmacol. 106, 290–302.

De Clercq, E., 2006. Antiviral agents active against influenza A viruses. Nature Rev. Drug. Discov. 5, 1015–1025.

Du, J., He, D.-Z., Jiang, R.-W., Ye, W.-C., Xum, H.-X., Hay But, P.-H., 2003. Antiviral flavonoids from the root bark of *Morus alba* L. Phytochemistry 62, 1235–1238.

Forslund, K., Morant, M., Jorgensen, B., Olsen, C.E., Asamizu, E., Sato, S., Tabata, S., Bak, S., 2004. Biosynthesis of the nitrile glucosides rhodiocyanoside A and D and the cyanogenic glucosides lotaustralin and linamarin in *Lotus japonicus*. Plant Physiol. 135, 71–84.

Martin, J.H.J., Crotty, S., Warren, P., Nelson, P.N., 2007. Does an apple a day keep the doctor away because a phytoestrogen a day keeps the virus at bay? A review of the anti-viral properties of phytoestrogens. Phytochemistry 68, 266–274.

Matrosovich, M., Matrosovich, T., Garten, W., Klenk, H.D., 2006. New low-viscosity overlay medium for viral plaque assays. Virol J. 63, 1–7.

Miller, R.E., Jensen, R., Woodrow, I.E., 2006. Frequency of cyanogenesis in tropical rainforests of far north Queensland. Aust. Ann. Bot. 97, 1017–1044.

Palese, P., Shaw, M., 2006. Orthomixoviridae. The viruses and their replication. In: Knipe, D.M., Howley, P.M. (Eds.), Fields Virology, fifth ed. LippIncott Williams and Wilkins, Philadelphia, pp. 1648–1689 (Chapter 47).

Qian-Cutrone, J., Huang, S., Trimble, J., Li, H., Lin, P., Alam, M., Klohr, S., Kadow, K., 1996. Niruriside, a new HIV REV/RRE binding inhibitor from *Phyllanthus niruri*. J. Nat. Prod. 59, 196–199.

Ruffa, M.J., Wagner, M.L., Suriano, M., Vicente, C., Nadinic, J., Pampuro, S., Salomon, H., Campos, R.H., Cavallaro, L., 2004. Inhibitory effect of medicinal herbs against RNA and DNA viruses. Antivir. Chem. Chemother. 15, 153–159.

Smee, D.F., Morrison, A.C., Barnard, D.L., Sidwell, R.W., 2002. Comparison of colorimetric, fluorometric, and visual methods for determining anti-influenza (H1N1 and H3N2) virus activities and toxicities of compounds. J. Virol. Methods 106, 71–79.

Udhikari, U., Mateua, C., Chattopadhyay, K., Pujol, C., Damonte, E., Ray, B., 2006. Structure and antiviral activity of sulfated fucans from *Stoechospermum marginatum*. Phytochemistry 67, 247–248.

Wang, X., Jia, W., Zhao, A., Wang, X., 2006. Anti-influenza agents from plants and traditional Chinese medicine. Phytother. Res. 20, 335–341.

Yang, C.M., Cheng, H.Y., Lin, T.C., Chiang, L.C., Lin, C.C., 2007. Hippomanin A from acetone extract of *Phyllanthus urinaria* inhibited HSV-2 but not HSV-1 infection in vitro. Phytother Res. 21, 1182–1186.