

Supplemental material

Anti-Poliovirus activity of *Nerium oleander* aqueous extract

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Nerium oleander (NO), a member of the Apocynaceae family, is an ornamental plant. In this study, we evaluated the antiviral activity of hot and cold extract of NO against six different viruses such as herpes simplex virus type 1 (HSV-1), poliovirus type 1 (Sb-1), vesicular stomatitis virus (VSV), reovirus type-1 (Reo-1), human immunodeficiency virus type-1 (HIV-1), and yellow fever virus (YFV). Interestingly the results of plaque reduction assay demonstrated that both, hot extract and cold extract (breastin) of NO inhibited Sb-1 viral infection. In order to identify the mechanism, by which NO exerts its antiviral activity, the virucidal effect, the time of addition and the adsorption assay were carried out. Results demonstrated that NO exerts its effect after infection period, particularly during the first two hours post infection.

Keywords: Apocynaceae , *Nerium oleander*, Hot extract, Cold extract, Poliovirus, Antiviral activity

Experimental

Plant material

Nerium oleander was collected from Vally Shoab which is located on the road to Jordan valley and dead sea and is about 4 Km south of the city Salt, Jordan. The plant was taxonomically identified by Prof. D. Al-Eisawi based on anatomy and internal structure of organs. Voucher specimen (Ner-Tf) was deposited in the herbarium of the Biology Department, University of Jordan, Jordan.

Hot extract (NO1) preparation

Sterile freshly ground leaves (200 g) of NO were boiled in distilled water (100 ml) for 3h and subsequently cooled for more than 6 h. Then, the extract was filtered, and the filtrate was boiled again for 1 h and cooled for 4 h and filtered three times. The volume was adjusted to 350 ml to obtain a clear, light brown filtrate (pH 5.84). The filtrate was concentrated using a rotary evaporator (Ozel 1992).

Cold extract (NO2) preparation

Sterile, freshly ground leaves (200 g) of NO were soaked in distilled water (1000 ml) under sterile conditions for at least 8 h. The solution was filtered and the volume was adjusted to 350 ml

to obtain a clear, dark brown filtrate. The sterile filtrate was lyophilized under sterile condition (Fiebig and Rashan 2010, Rashan 2011).

Cells and viruses

Cell lines were purchased from American Type Culture Collection (ATCC). The absence of mycoplasma contamination was checked periodically by the Hoechst staining method. Cell lines supporting the multiplication of viruses were the following: human CD4⁺ T-cells containing an integrated HTLV-1 genome (MT-4); Baby Hamster Kidney (BHK-21) [ATCC CCL 10 (C-13) *Mesocricetus auratus*]; Monkey kidney (Vero-76) [ATCC CRL 1587 *Cercopithecus aethiops*]. Viruses were purchased from American Type Culture Collection (ATCC), with the exception of human immunodeficiency virus type-1 (HIV-1), and yellow fever virus (YFV). Viruses representative of positive-sense, single-stranded RNAs (ssRNA⁺) were: i) Retroviridae: the IIB laboratory strain of HIV-1, obtained from the supernatant of the persistently infected H9/IIB cells (NIH 1983); ii) Flaviviridae: yellow fever virus (YFV) [strain 17-D vaccine (Stamaril Pasteur J07B01)]; iii) Picornaviridae: enterovirus C [poliovirus type-1 (Sb-1), Sabin strain Chat (ATCC VR-1562)]. Viruses representative of negative-sense, single-stranded RNAs (ssRNA⁻) were: iv) Pneumoviridae: human respiratory syncytial virus (hRSV) strain A2 (ATCC VR-1540); v) Rhabdoviridae: vesicular stomatitis virus (VSV) [lab strain Indiana (ATCC VR 1540)]. The virus representative of double-stranded RNAs (dsRNA) was: vi) Reoviridae reovirus type-1 (Reo-1) [simian virus 12, strain 3651 (ATCC VR-214)]. The virus representative of DNA virus was: vii) Herpesviridae: human herpes 1 (HSV-1) [strain KOS (ATCC VR-1493)]. Viruses were maintained in our laboratory and propagated in appropriate cell lines. The viruses were stored in small aliquots at -80 °C until use.

Cytotoxicity assays

Exponentially growing MT-4 cells were seeded at an initial density of 4×10^5 cells/ml in 96-well plates in RPMI-1640 medium, supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin G and 100 µg/ml streptomycin. BHK-21 cells were seeded in 96-well plates at an initial density of 1×10^6 cells/ml, in Minimum Essential Medium with Earle's salts (MEM-E), L-glutamine, 1 mM sodium pyruvate and 25 mg/l kanamycin, 10% foetal bovine serum (FBS) (BHK-21). Vero-76 cells were seeded in 96-well plates at an initial density of 4×10^5 cells/ml, in Dulbecco's Modified Eagle Medium (D-MEM) with L-glutamine and 25 mg/l kanamycin, supplemented with 10% FBS. Cell cultures were then incubated at 37 °C in a humidified, 5% CO₂ atmosphere, in the

absence or presence of serial dilutions of test extracts. Cell viability was determined after 48-96 h at 37 °C by MTT method for MT-4, Vero-76 and BHK-21 (Sanna et al. 2015).

Antiviral assays

Extracts activity against HIV-1 was based on inhibition of virus-induced cytopathogenicity in exponentially growing MT-4 cell acutely infected with a multiplicity of infection (m.o.i.) of 0.01. Extracts activity against YFV and Reo-1 was based on inhibition of virus-induced cytopathogenicity in BHK-21 cells acutely infected at an m.o.i. of 0.01. After a 3 or 4-days incubation at 37 °C, cell viability was determined by the MTT method, as described earlier (Carta et al. 2018). Extracts activity against Sb-1, VSV, HSV-1 and RSV was determined by plaque reduction assays as described earlier (Carta et al. 2018). Briefly, monolayer of Vero-76 cells was grown overnight on 24-well plate. The cells were then infected for 2 h with 250 µl of proper virus dilutions to give 50-100 PFU/well. Following removal of non-adsorbed virus, 500 µl of medium [D-MEM with L-glutamine and 4500 mg/l D-glucose, supplemented with 1% inactivated FBS] containing 0.75% methyl-cellulose, with serial dilutions of test extracts, were added. The overlaid medium was also added to non-treated wells as non infection controls. Cultures were incubated at 37°C for 2 (Sb-1 and VSV), 3 (HSV-1) or 5 days (RSV) and then fixed with PBS containing 50% ethanol and 0.8% crystal violet, washed and air-dried. Plaques in the control (no inhibitor) and experimental wells were then counted. Concentrations resulting in 50% inhibition (CC₅₀ or EC₅₀) were determined by linear regression analysis. For the purpose of calculating selectivity index (SI), CC₅₀ values were taken at day 2. Selective activities of the compounds were calculated as follows: Selectivity index (SI) = CC₅₀ in µg/ml/EC₅₀ in µg/ml.

Virucidal activity assay

A Sb-1 suspension containing 5×10^5 PFU/ml was incubated with or without different concentrations of extracts for 1 h at 4 or 37 °C. At the end of incubation, the residual infectivity was determined by plaque assay in Vero-76 cells after dilution below the inhibitory concentration.

Adsorption assay

Vero-76 cells grown in 24-well plate were pre-chilled at 4° C for 1 hour and then infected with Sb-1, at an m.o.i. of 5, in the presence or absence of NO extracts. Plates were incubated for 60 min at 4 °C. Medium containing non-adsorbed virus was then removed, cell monolayer was washed

twice with PBS and overlaid with fresh medium, incubated for 24 h and then examined by plaque counting (Visintini Jaime et al. 2013).

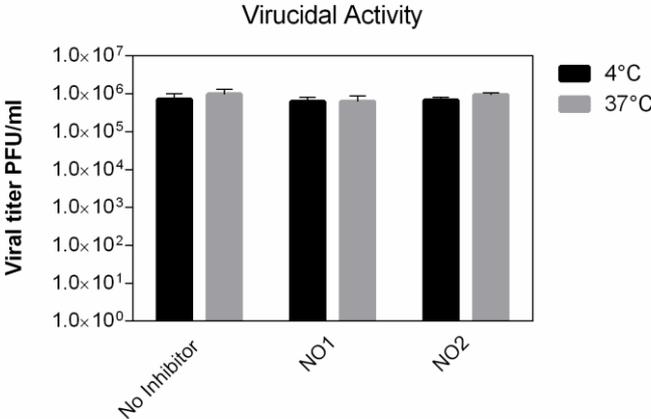
Effect of Time Addition of NO extracts on the Sb-1 Replication Cycle

The confluent monolayers of Vero-76 cells in 24-well tissue culture plates were infected for 1h at room temperature with Sb-1 dilutions to give a final m.o.i. of 5. After adsorption, the monolayers were washed three times with PBS and incubated with D-MEM medium with L-glutamine, supplemented with 1% inactivated FBS, 1mM sodium pyruvate and 0.025 g/l kanamycin. Monolayers were then treated with NO extracts ($5 \times EC_{50}$ $\mu\text{g/ml}$) or reference for 1 h during infection period (at -1 to 0) and at specific time point, 0 to 2, 2 to 4, 4 to 6 and 6 to 8 h post infection. After each incubation period, the monolayers were washed two times with PBS and incubated with fresh medium. Then, the monolayers were frozen at $-80\text{ }^{\circ}\text{C}$ at 12 h p.i. and the extracellular virus production was determined by plaque assay.

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Fig. 2. Virucidal activity of NO extracts on Sb-1



Data presented as mean ± SD of triplicates.

Table 2. Cytotoxicity and antiviral activity of hot extract (**NO1**) and cold extract (breastin) (**NO2**) of *Nerium oleander* and reference compounds against representatives of ssRNA⁺ (HIV-1, YFV, Sb-1), ssRNA⁻ (RSV), viruses.

Inhibitors	<u>MT-4</u>	<u>HIV-1</u>	<u>BHK-21</u>	<u>YFV</u>	<u>Reo</u>	<u>Vero-76</u>	<u>RSV</u>	<u>VSV</u>	<u>HSV-1</u>
	^a CC ₅₀	^b EC ₅₀	^c CC ₅₀	^d EC ₅₀	^d EC ₅₀	^e CC ₅₀	^f EC ₅₀	^g EC ₅₀	^g EC ₅₀
NO1-hot	5	>5	>100	>100	>100	4	>4	>4	>4
NO2-cold (breastin)	1.5	>1.5	>100	>100	>100	2.5	>2.5	>2.5	>2.5
Efavirenz*	37	0.001							
2'-C-Me-Guo*			>100	10	0.7				
6-Aza-Uridine*						14	2.2		
Acycloguanosine*						>100			3

^a Extract concentration (µg/ml) required to reduce by 50% the proliferation of mock-infected MT-4 cells, as determined by the MTT method at day 4 following treatment.

^b Extract concentration required to achieve 50% protection of MT-4 cells from HIV-1-induced cytopathogenicity, as determined by the MTT method at day 4 p.i.

^c Extract concentration (µg/ml) required to reduce by 50% the viability of mock-infected BHK cells, as determined by the MTT method at day 3 following treatment.

^d Extract concentration (µg/ml) required to achieve 50% protection of BHK cells from YFV^(d) and Reo^(d)-induced cytopathogenicity, as determined by the MTT method at day 3 p.i.

^e Extract concentration (µg/ml) required to reduce by 50% the viability of mock-infected Vero-76 cells, as determined by the MTT method at day 5 following treatment

^f Extract concentration (µg/ml) required to reduce by 50% the plaque number of RSV in Vero-76 cells at day 5 p.i.

^g Extract concentration (µg/ml) required to reduce by 50% the plaque number of VSV and HSV-1 in Vero-76 cells at day 2 and 3 p.i. respectively

* Reference Compounds CC₅₀ and EC₅₀ in µM