In vitro effect of *Dodonaea viscosa* extracts on the replication of coxackievirus B3 (Nancy) and rotavirus (SA-11)

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ABSTRACT

Objective: To determine the *in vitro* antiviral activity of different extracts from *Dodonaea viscosa* leaves against coxackievirus B3 (CVB3) and rotavirus SA-11 (RV SA-11) infections.
Methods: The cytotoxic effect of the extracts on GMK and MA 104 cells was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) reduction colorimetric assay. To evaluate the antiviral effect of the plant extracts, three different strategies were used.
Results: *D. viscosa* exhibited ttherapeutic index (TI) ranging from 0.3 to 25 with reduction in virus titre ranging from 0.25 to 5 $\log_{10}$ $TCID_{50}$/0.1 ml for CVB3 whereas TI ranging from 0.4 to 29.2 with reduction in virus titre ranging from 0.25 to 5.25 $\log_{10}$ $TCID_{50}$ for RV SA-11**.** Crude extract provided the potent inhibition of CVB3 and RV SA-11 replication by binding to a viral capsid of CVB3 and viral receptor of RV SA-11 preventing viruses entry into host cells for both viruses.
Conclusions: We conclude that the crude extract of *D. viscosa* leaves shows the potential antiviral effect to CVB3 and RV SA-11 infections.

KEY WORDS: Coxsackievirus
Rotavirus
Antiviral activity
Tetrazolium reduction assay

Introduction

Coxsackieviruses B, of family *Picornaviridae*, cause acute and chronic human diseases ranging from nonspecific febrile illnesses, exanthematic fever to meningitis, cardiomyopathy, pancreatitis, severe neonatal diseases, and neurological disorders [1, 2]. The disease manifestation depends on the infection of target organ and the ability of the virus to destroy particular tissues [3]. Currently, there are no effective antiviral drugs to prevent and/or treat diseases caused by coxsackievirus infections. So, it is a necessity to search for effective antiviral drugs against coxsackievirus infections. Numerous synthetic compounds exhibited *in vitro* antiviral activity by binding in a hydrophobic pocket beneath the canyon floor in the centre of the viral protein 1 (VP1), preventing viral attachment or uncoating. However none have been yet approved for clinical use. Rotavirus, family of *Reoviridae*, is a major pathogen which causes diarrhoea in infants and young children worldwide, mostly in developing countries [4, 5]. Each year, more than 450,000 children younger than five years old die due to rotavirus infections [6].

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Rehydration, intake of fluids and electrolytes are the only way to tread rotavirus (RV) dehydration [7]. Currently, there are two vaccines (RotaTeq and Rotarix) to prevent RV infection [8]. These vaccines are costly, and effective against certain strains of RV, not completely safe in immuno-compromised patients [9]. Rotavirus treatment remains yet unsolved. Herbs are interesting antiviral candidates as antiviral agents, based on their low cost and minimum side effects. *D. viscose* belongs to the family of *Sapindaceae* is a shrub [10]. Pharmacological studies have confirmed that *D. viscosa* possess antibacterial [11], antifungal [1], antidiabetic [13], anti-inflammatory [14], antiulcer [15], antioxidant [16], anti-diarrheal [17], anti-

hyperlipidemic and hepatoprotective [18], antinociceptive [19], anti-coxsackie virus B3 [20], anti-Influenza A virus [21], anti -Human immunodeficiency virus type-1 and 2 [22], and anti-Herpes simplex virus type-1 (HSV-1) [23] activities. Phytochemical studies shown that its leaves contain tannins, heterosides cardiotonic, essential oils, flavonoids, saponins, coumarins, mucilage, gum, organic acids, and diterpenoid acids [24-27]. In the *D. viscose* leaves can also be determined terpenoids, p-coumarin acid ester, sterols, and volatile oil [28-32]. The aim of our study was to determine the *in vitro* antiviral effect of a natural extracts from a traditional Egyptian medicinal plant on CVB3 and RV SA-11.

Materials and Methods

Plant collection

D. viscosa leaves were collected from Botanical Garden of the National Research Centre (NRC), Giza, Egypt during May and June 2011 and were kindly identified by Mrs. Tersea Labib, taxonomist at Orman botanical garden, Giza and Dr. Mona Marzok, Researcher at National Research Center (NRC).

Extracts preparation

Leaves of *D. viscosa* were air-dried at room temperature, dissolved in methanol. This methanol extract was dissolved in hot distilled water, making a suspension to which chloroform, ethyl acetate or n-butanol were added using a separating funnel till complete extraction was achieved. All the extracts, including the residue which remained in water, were concentrated to dryness in a rotary evaporator at 40^{o}C. Dried extracts were weighed to calculate the percentage yield (concentration). Stock solutions were prepared by dissolving 100 mg of the lyophilized extract in 0.5 ml distilled dimethyl sulphoxide (DMSO) and volume was made up to 10 ml with DMEM or EMEM. The stock solutions were sterilized by membrane filtration (Millipore 0.45 µm), then stored at 4^{o}C in a refrigerator until use.

Cell lines and viruses

Green monkey kidney cell line (GMK) and CVB3 were a kind gift from the National Reference Center of the Enterovirus Laboratory, Faculty of Medicine, Slovak Medical University, Prague under the government project (SAIA). Rhesus monkey kidney cell line (MA 104) with Simian rotavirus SA-11 stock were given by Department of Virology, National Institute for Cholera and Enteric Diseases (NICED), Kolkata, India under the government project INSA – JRD TATA. GMK and MA 104 Cells were cultivated in Eagle's minimum essential medium (MEM), and Dulbecco's Modified Eagle Medium (DMEM), respectively. The media were supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 units/ml penicillin, 100µg/ml streptomycin and 1% HEPES (4-2-hydroxyethyl-1-piperazineethanesulfonic acid) and incubated in humidified 5% CO_2 atmosphere. The medium used for the cytotoxicity and antiviral assays contained 2% of fetal bovine serum. Viral CVB3 stocks were prepared in GMK cells, RV SA-11 stocks in MA 104 cells. The stock viruses were stored in small aliquots at -80^{o}C until use.

Virus titration

RV SA-11 (with trypsin 10 mg/ml trypsin for 30 min at 37^{o}C) and CVB3 stocks were titrated using MA 104 and GMK cells, in 96-well microtiter plates as described previously [33]. The viral titers were calculated as $TCID_{50}$/0.1 ml (50% tissue culture infectious doses/0.1 ml) using standard Spearman Kärber formula [34].

Cytotoxicity assay

Various concentrations from the prepared stock solutions of the leaves extracts (7.8, 15.6, 31.25, 62.5, 125, 250, 500, and 1000 µg/ml) were prepared in DMEM or EMEM (containing 2% antibiotics and 2% FBS). The cytotoxic activity of the *D. viscosa* extracts was determined in MA 104 and GMK by using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method [35]**.** Briefly, MA 104 and GMK cell lines were grown in 96-well microtiter plates at concentration 5 x 10^3 and 5 x 10^4 cells/well, respectively for 24 hour in 5% CO_2 incubator at 37 °C. 24 h cell monolayers were treated with each extract dilutions (each dilution in triplicate) while cell control contained only medium. The cells were incubated for 48 h at 37 °C in a 5% CO_2 incubator and were examined microscopically daily to control the cell morphology. The culture medium was removed and

100 µL of MTT solution (5 mg/ml) was added to cells in each well for 4 h at 37°C. After removal of MTT, 50 µl DMSO was added to solubilize the formazan crystal and incubated for 30 min at 37°C. The optical densities (OD) were measured at 540 nm using an ELISA reader (MRX microplate reader, Dynex technologies, USA). The percentage of cytotoxicity is calculated as [(A - B / A) x 100], where A and B indicate the mean of three optical density of cell control and treated cells, respectively. The 50% cytotoxic concentration (CC_{50}) is defined as the extract concentration (µg/ml) that can reduce 50% of cell viability to compare with cell control.

Antiviral activity of *D. viscosa* extracts on RV SA-11 and CVB3 by MTT method:

MA 104 (5×10^3 cells/well) and GMK (5×10^4 cells/well) cells were grown in 96-well microtiter plates for 24 h in CO_2 incubator at 37 ^oC. The culture media were removed and three non-toxic concentrations of each extract were chosen to test. Three different ways were used to study antiviral activity:

Virucidal activities

50 µl of 10^6 $TCID_{50}$ virus suspensions (CVB3 or activated RV SA-11) was incubated with an equal volume of culture media (with or without the test compound) for 1 h at 37°C in humidified 5% CO_2 atmosphere. 100µl of the mixed solution was incubated with the 24 h cells monolayer for 1 h then the mixed solution was removed. The cell lines were washed with culture medium twice, 200 µl of EMEM containing 2% FBS for GMK and 200 µl of FBS free DMEM containing 2 µl of trypsin for MA 104 cells was added.

Treatment before virus infection (Pre-treatment)

50 µl of the extract was incubated at various concentrations with GMK or MA 104 cells in 96-well microtiter plate for 24 h at 37°C in 5% CO_2 atmosphere. After removal of the tested extracts and washing the cells, 50 µl of 10^6 $TCID_{50}$ virus suspension (CVB3 or activated RV SA-11) was added to each well for 1 h incubation period. The unabsorbed virus was removed, the cell lines were washed twice then incubated with EMEM containing 2% FBS for GMK or FBS free DMEM containing 2 µl of trypsin for MA 104.

Treatment after virus infection (Post-treatment)

10^6 $TCID_{50}$ of CVB3 or RV SA-11 were incubated with cell monolayers for 1 h at 37°C in 5% CO_2 atmosphere. After removal of unabsorbed viruses, the cells were washed and incubated with three various concentrations of each extract for 24 h in CO_2 incubator. After removing the extracts and washing the cell lines, EMEM with 2% FBS was added for GMK whereas FBS free DMEM containing 2 µl of trypsin was added for MA 104 cells.

Positive virus controls (the virus suspension without extract) and negative cell controls (culture medium without extracts) were included in all assays. All plates were incubated at 37^{o}C in CO_2 incubator for 72 h; the inhibition of virus-induced cytopathic effect (CPE) was checked daily and measured by the MTT as described. The percentage protection was calculated as [(A-B)/(C-B) x 100], where A, B and C indicate the spectrophotometric absorbance readings (as OD_{540}) of the tested extract with virus infected cells, virus and cell controls,. 50% inhibitory concentration (IC_{50}) was defined as the compound concentration that protects 50% of treated infected cells to compare with cell control. Therapeutic index (TI) of the extract for the antiviral activity was determined by calculating the ratio CC_{50} over IC_{50}.

Antiviral activity of *D. viscosa* extracts on RV SA-11 and CVB3 by measurement of cytopathic effect

For $TCID_{50}$ determination, 300 µg/ml of methanol, chloroform, ethyl acetate, and aqueous extracts as well as 10 µg/ml of butanol extract were used to be evaluated against CVB3 infection. Whereas, 500 µg/ml of each extract were used to be evaluated for their antiviral effect against RV. 10-fold dilution of CVB3 and activated RV SA-11 were prepared in EMEM and DMEM, respectively. 100 µl of viral dilutions $10^{-4} – 10^{-9}$ were treated with 100 µl of each extract in three different ways as described in the antiviral MTT assays. Positive virus control (virus dilutions without extracts) were included. Virus dilution either with or without extracts was added into four parallel wells. All plates were incubated at 37^{o}C in CO_2 incubator for 72 h, then the cytopathic effect was observed under light microscope and virus titration was calculated and expressed as 50% tissue culture infection dose ($TCID_{50}$) by using Spearman Kärber method [34]. The reduction in virus titer

was calculated as differences between the values of treated and untreated virus.

Results

Cytotoxicity of *D. viscosa* extracts in GMK and MA-104 cells

Five extract from *D. viscosa* leaves were investigated for their cytotoxic effects on GMK and MA 104 cells using MTT colorimetric assay. CC_{50} value for each extract after 48 h incubation with GMK and MA 104 cells was calculated. The cytotoxicity of ethyl acetate, chloroform and aqueous extract exhibited less toxicity on GMK cells with CC_{50} more than 1mg/ml compare to methanol and butanol extracts which had higher CC_{50} of 851.2 and 747.4 µg/ml, respectively. On the other hand, all extracts except for the chloroform showed low cytotoxicity on MA 104 cells (Table 1).

Table 1. The cytotoxicity effect of *D. viscosa* leaf extracts on GMK and MA 104 cell lines.

Extract	CC_{50} (µg/ml)[a]	
	GMK cells	MA 104 cells
Methanol	851.2	6231
Chloroform	>1000	553
Ethyl acetate	>1000	9045
Butanol	747.4	1060
Aqueous	>1000	5084

a: Concentration of extract that is cytotoxic to 50% of cells

Antiviral activity of *D. viscosa* extracts against CVB3

All tested extracts showed some degree of protection against virus in three different ways of infection. Methanol, chloroform, and ethyl acetate extract of *D. viscosa* leaves showed higher antiviral activity when were pre-incubated with virus prior to cell infection with TI= 25, 8.5, 8 and 5, 1.75 $\log_{10}$ reduction in virus titers, respectively. Whereas, butanol and aqueous extracts were more effective against CVB3 infection during post-infection treatment with TI= 6 and 0.75 $\log_{10}$ virus titer reduction and TI= 20.6 and 4.75 $\log_{10}$ reduction in virus titers, respectively. Moderate antiviral effect of these extracts were showed when methanol and ethyl acetate extracts were added to cells after virus infection with TI= 7 reducing the virus titer by 1 $\log_{10}$ $TCID_{50}$/0.1 ml and TI=1.2 reducing the virus titer by 0.25 $\log_{10}$ $TCID_{50}$/0.1ml, respectively. The chloroform extract showed its moderate inhibitory effect when pre-incubated with cells prior virus infection with TI= 7.7 and 1.5 $\log_{10}$ $TCID_{50}$/0.1 ml reduction in virus titers. Butanol and aqueous extract exhibited their moderate antiviral activity against CVB3 when they pre-incubated with virus prior to infection with TI= 2.4 resulting in 0.25 $\log_{10}$ $TCID_{50}$/0.1 ml reduction in virus titers and TI= 4.3 resulting in 0.75 $\log_{10}$ $TCID_{50}$/0.1 ml reduction in virus titers, respectively. The weak inhibitory effect of these extracts were shown when methanol, ethyl acetate, butanol, and aqueous extracts were pre-incubated with cells. Moreover, no reduction in virus titer was observed for neither ethyl acetate nor butanol extract during the cell pre-treatment. The chloroform extract showed a weak inhibitory effect when added to cells after CVB3 infection with TI=6 and 0.75 $\log_{10}$ titer reduction. Our *in vitro* results indicated that cells treatment with most extracts before CVB3 infection was the least effective in terms of studied antiviral activity (Table 2).

Antiviral activity of *D. viscosa* extracts against RV SA-11

Three different ways of antivirus effects of *D. viscosa* leaf extracts on RV SA-11 were examined. Methanol, ethyl acetate, and aqueous extracts showed the greatest inhibitory effect when pre-incubated with cells prior to virus infection with TI=29.2, 23, and 12 reducing the virus titers by 5.25, 4.75, and 2.5 $\log_{10}$ $TCID_{50}$/0.1 ml, respectively. The maximum inhibitory effects of chloroform and butanol extract were demonstrated in the treatment after viral infection. Theses extracts showed moderate antiviral activity when methanol and aqueous extracts were added to cells after infection with TI= 23 and 5 $\log_{10}$ $TCID_{50}$/0.1 ml reduction in virus titers, TI=3.7 and 0.25 $\log_{10}$ $TCID_{50}$/0.1 ml reduction in virus titers, respectively.

Table 2. The antiviral activity of *D. viscose* extracts on CVB3 determined by MTT and Karber methods.

Extract	Virucidal			Treatment before infection			Treatment after infection		
	IC_{50} (µg/ml)[a]	TI[b]	R	IC_{50} (µg/ml)[a]	TI[b]	R	IC_{50} (µg/ml)[a]	TI[b]	R
Methanol	34	25	5	361	2.3	0.25	120	7	1
Chloroform	5970	8.5	1.75	6530	7.7	1.5	8531	6	0.75
Ethyl acetate	140	8	1.75	3071	0.4	0	925	1.2	0.25
Butanol	307	2.4	0.25	2151	0.3	0	125	6	0.75
Aqueous	1038	4.3	0.75	1504	2.9	0.25	215	20.6	4.75

a: Concentration of extracts that inhibit viral infectivity (Cytopathic Effect) by 50%; b: Therapeutic index = CC_{50}/IC_{50} = The mean values of triplicate experiments. R: Reduction in virus titre calculated as the difference between treated and untreated virus and expressed in $\log_{10} TCID_{50}$/0.1 ml.

Table 3. The antiviral activity of *D. viscose* extracts on RV SA-11 determined by MTT and Karber methods.

Extract	Virucidal			Treatment before infection			Treatment after infection		
	IC_{50} (µg/ml)[a]	TI[b]	R	IC_{50} (µg/ml)[a]	TI[b]	R	IC_{50} (µg/ml)[a]	TI[b]	R
Methanol	5263	1.2	0.25	213	29.2	5.25	270	23	5
Chloroform	62.5	9	1.75	437.5	1.2	0.25	43	13	3
Ethyl acetate	1750.5	5	0.75	390	23	4.75	1885	4.8	0.5
Butanol	470.7	2.2	0.25	ND	ND	0	443.5	2.4	0.25
Aqueous	12209	0.4	0	414.5	12	2.5	1370	3.7	0.25

a: Concentration of extracts that inhibit viral infectivity (Cytopathic Effect) by 50%; b: Therapeutic index = CC_{50}/IC_{50} = The mean values of triplicate experiments. R: Reduction in virus titer calculated as the difference between treated and untreated virus and expressed in $\log_{10} TCID_{50}$/0.1 ml.

Whereas the moderate effect of chloroform, ethyl acetate, and butanol extracts exhibited when they were pre-incubated with virus prior to infection with TI=9 and 1.75 $\log_{10}$ $TCID_{50}$/0.1 ml reduction in virus titers, TI=5 and 0.75 $\log_{10}$ $TCID_{50}$/0.1 ml reduction in virus titers, TI=2.2 and 0.25 $\log_{10}$ $TCID_{50}$/0.1 ml reduction in virus titers, respectively. Methanol, butanol and aqueous extracts were the least efficient when pre-incubated with virus prior to virus infection; even in aqueous extracts, no reduction in viral titre was seen. The chloroform extract showed the lowest inhibitory effect when pre-incubated with cells before virus infection with TI= 1.2 and 0.25 $\log_{10}$ titer reduction in virus titres whereas the ethyl acetate extract when treatment after infection (Table 3).

Discussion

CV and RV infections, represent a big worldwide problem resulting in morbidity and deaths, particularly in developing countries [36- 38]. Currently, there are no vaccines or antiviral agents approved for prevention and/or treatment of CVB3-induced acute or chronic infections [39]. Similarly, no effective drugs are available to control diarrhea caused by RV [40]. Cheap, safe, and effective natural compounds from food and herbal medicine are ideal candidates to be used as preventive and therapeutic agents against CVB3 and RV infections in developing countries. In our *in vitro* study, five extracts (methanol, chloroform, ethyl acetate, butanol, and aqueous) of *D. viscosa* leaves were tested to determine the antiviral activities against CVB3 and RV infection (in three different ways). We

explored the *D. viscosa* inhibitory effectof *D. viscosa* on CVB3 and RV replication. Viral replication cycle includes various steps such as attachment, penetration, replication of viral proteins and genetic materials, assembly and viral escaping from infected cells. These steps can be used as targets of anti-coxackievirus B3 and anti-rotavirus SA-11 agents [7]. Supposing that the effect of *D. viscose* extracts antiviral activity could involve three steps: 1) binding to the virus capsid, 2) blocking of receptors on the cell surface (and so influence the viral attachment and prevent virus entry into cells), 3) inhibit some steps of the viral life cycle after viral entry into host cells. For this purpose, extracts were added to the cell system after incubation with virus (virucidal activity), before (pre-treatment), and after (post-treatment) virus infection.

Our results demonstrated that the methanol crude extract of *D. viscosa* leaves showed the strongest effect against both viruses when compared with other tested extracts. Methanol extract was most effective against CVB3 mainly via the virucidal activity (inhibition of attachment). Whereas the cell pre-treatment (before infection) was more effective against RV-SA-11 by blocking of cell receptors. These results may be attributed to the presence of many compounds in the crude extract rather than the purified extract. This can be interpreted as synergistic which produces greater antiviral activity than the purified extract [41]. Our results correspond with Getie *et al* [42] who report the anti-CVB3 activity of this plant. However, the antiviral activity of *D. viscosa* against rotavirus has not been previously tested. The anti-RV effect recorded by us may be due to the properties of saponins, to which the extracts belong. Tam and Roner [43] reported that saponins reduced rotaviral infection *in vivo* by blocking the viral receptors. Generally, our results indicated that the crude chloroform and ethyl acetate have a higher inhibitory effect on enveloped RV than the non-enveloped CVB3. In contrast, the butanol and aqueous extracts have higher antiviral activity on non-enveloped than enveloped viruses. We conclude that the extract of *D. viscosa* leaves shows antiviral activity and can be effective in the treatment of coxackieviruses and rotaviruses infections. More detailed *in vivo* studies are required to assess the antiviral activity of these extracts.

Conflict of Interest

We declare that we have no conflict of interest.

Acknowledgements

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