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Full Length Research Paper

Antiviral activity of *Conyza canadensis* (L.) Cronquist extracts grown in Tunisia

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Ethyl acetate, chloroform, butanol and methanol extracts of the aerial parts of *Conyza Canadensis* L. Cronquist were investigated for their antiviral activity against human cytomegalovirus (HCMV) AD-169 and Cox-B3 viruses by modification of the widely used shell-vial assay. The results showed that butanol and methanol extracts had the most potent antiviral activity against HCMV and Cox-B3 viruses.

Key words: *Conyza canadensis* (L.) Cronquist, antiviral activity, human cytomegalovirus (HCMV), Coxsackie B virus type 3 (CoxB-3).

INTRODUCTION

Herbal medicine represents one of the most important fields of traditional medicine all over the world. There has been growing interest in the investigation of natural products from plants for the discovery of new antimicrobial agents. The appearance of viral resistant strains to antiviral agents is an emerging problem. Thus, the prevalence of viral related diseases is of great concern. For this reason, the development of new and better antiviral compounds is vital and desirable. It has been demonstrated that natural products are preferable to synthetic compounds as sources of new antiviral agents (Vanden et al., 1986; Vlietinck and Vanden, 1991; Abad et al., 1997). A number of compounds with inhibitory activity against the replication of several viruses have been extracted from a number of medicinal plants (De Rodriguez et al., 1990, 1996; Marchetti et al., 1996; Hayashi et al., 1997). *Conyza canadensis* (L.) Cronquist belongs to the family of Compositae and is native to North America (Weaver, 2001). This plant also grows spontaneously in central Tunisia. Whole plant has been used as a Tunisian folk medicine for the treatment of rheumatism. It also has antidiarrhoeal and antiha-emorrhoidal activities (Chevallier, 1996; Chiej, 1984; Sastri, 1952). In our previous work, we reported that *C. canadensis* extracts possess antibacterial and antioxidant activities (Edziri et al., 2008).

In searching for natural products as potential antiviral agents, we have examined the antiviral action of *C. canadensis* L. Cronquist extracts against human cytomegalovirus (HCMV) and Coxsackie B3 viruses.

MATERIALS AND METHODS

Plant material was collected in the flowering season from Monastir region (Tunisia). The plant was identified by Doctor Mohamed Chaieb, a botanist in the University of Science (Sfax, Tunisia). A voucher specimen was deposited in the herbarium of our laboratory.

Plant extraction

Powdered plant tissues (700 g) were extracted three times by maceration with methanol, the resultant extract was concentrated under reduced pressure. The methanol extract was extracted successively with equal volumes of four organic solvents of increasing polarity of petroleum ether, chloroform, ethyl acetate and butanol. The different extracts were concentrated to dryness and kept at 4°C.

Cell cultures

Human diploid embryonic lung fibroblasts (MRC-5) (Biomerieux-

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Table 1. Cytotoxic activity of *Conyza canadensis* (L.) cronquist extracts on MRC-5 cells

Extract	CC_{50}[a] (µg/ml)
Petroleum ether	250
Ethyl acetate	250
chloroform	250
Butanol	250
Methanol	250
Ganciclovir	0.8
Ribavirin	130

CC_{50}[a]: is the concentration of the 50% cytotoxic effect.

France) were grown in MEM-D (Seromed, Germany, Ref. T041-05) supplemented with 100 units/ml penicillin, 100 mcg/ml streptomycin and 10% fetal calf serum (FCS Ref. S0115, Seromed, Germany). FCS was reduced to 2% for the viral infection.

MTT assay

The MTT colorimetric assay was performed in 96-well plates (Polydoro et al., 2004). Briefly, each well of a 96-well plate (NUNC, Ref. 167008) contained a continuous monolayer of MRC-5 cells at a concentration of 10^5 cells/well. After that, the medium was removed and various concentrations of each extract were added. After 48 h, the extract was eliminated, the cells were washed twice with phosphate buffer saline and 15 ml of a 5 mg/ml solution of MTT (3-dimethylthiazol-yl)-2 and 5-diphenyltetrazolium bromide (Sigma M5655) was added. The plate was covered with self-adhesive tape and incubated at 37°C for 2 h. After incubation, the MTT solution was removed and 50 µl dimethyl sulfoxide (DMSO) was added to dissolve insoluble formazan crystal. The absorbance was read in a microplate reader (Reader 530, Organon Teknika) at 570 nm. CC_{50} parameter is defined as the concentration (µg/ml) of substrate that causes 50% death of cells.

Titration of viruses

HCMV strain AD-169 (ATCC Ref. VR 538) and Coxsackie B virus type 3 (CoxB-3) were used for the antiviral activity. Serial 10-fold virus dilutions (10^{-1} - 10^{-5}) were prepared in MEM-D containing 2% FCS, were inoculated into confluent cells in quadruplicate wells of 96-well plates and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 3 to 5 days. When a cytopathic effect (CPE) in the virus-infected cells was observed microscopically, virus titers were expressed as 50% tissue culture-infective dose (TCID50) and were determined by the method of Reed and Muench (1938). The mouse monoclonal antibodies directed against the immediate-early CMV 76 kD antigen (clone E-13, Ref. 11-003, ARGENA-Biosoft, France) was diluted 1:50 in PBS. The same source provided the goat-anti-mouse FITC-conjugated antibody (Ref. 50- 012).

Antisera

The mouse monoclonal antibodies directed against the immediate-early CMV 76 kD antigen and Cox-B3 (clone E-13, Ref. 11-003, ARGENA-Biosoft, France) was diluted 1:50 in PBS. The same source provided the goat-anti-mouse FITC-conjugated antibody (Ref. 50-012). This antibody was diluted 1:100 in PBS-supplemented with 0.002% Evan's blue.

Antiviral activity

The test used in this paper is a modification of the generally used 'shell-vial' culture method for the early detection of the CMV infection (Alpert et al., 1985). The diagnostic method was sensitive (Pepin et al., 1991) and quantitative (Mazeron et al., 1991). Extracts of the plant were diluted in MEM-D, then the virus was added, and centrifuged for 1 h at 1000 g. After centrifugation, the wells were incubated for 48 h at 37°C, and the viral antigens were revealed by immunofluorescence.

The culture medium was aspirated and the cells were infected with 600 µl of the virus dilution followed by centrifugation at 1000 g for 1 h. After centrifugation, the mixture was incubated for 48 h at 37°C, and then the viral antigens were revealed by immunofluorescence.

The immunofluorescence detection of the viral antigens was common for the earlier mentioned procedures and was as follows: at the end of incubation, the content of the wells was aspirated and the cells were fixed with cold methanol at 20°C for 10 min. The methanol was aspirated and 250 µl of anti immediate-early antibody was added and incubated for 45 min at 37°C in a humidified chamber. At the end of this time, the wells were washed three times for 5 min with PBS. Then the FITC labelled anti-mouse antibody was incubated for 40 min and finally washed under the same conditions three times as previously done. After that, the cells were observed under an immunofluorescence microscope. Because the extracts were dissolved in dimethylsulphoxide (DMSO) which positively affects the infectivity of the viruses (Li and Fong, 1990), DMSO was also included in the wells without any extract. The antiviral activity was determined by counting the number of cells infected by the virus (presenting fluorescent nuclei) in comparison with the number of infected cells in the reference well (no presence of any antiviral agent). The reference well contained cells infected by the virus without any antiviral agent. The earlier described experiments were done three to four times.

RESULTS AND DISCUSSION

The results of the MTT assay are presented in Table 1 and show that all extracts of *C. Canadensis* were not cytotoxic against MRC5 cells (CC_{50} is above 250 µg/ml). The results of the antiviral activity are presented in Table 2. As shown in this table, ethyl acetate, chloroform, butanol and methanol extracts had anti-HCMV and anti-Cox-B3 activity but the most active extract seemed to be methanol extract (Table 2). The active concentration of the methanol extract was 100 µg/ml with 99% activity against CMV virus and 100% activity against Cox-B3 virus.

To our knowledge, this study represents the first demonstration of an inhibitory activity of *C. canadensis* extracts against the CMV and Cox-B3 viruses. Many studies have shown the antiviral action of the plant or its constituents: hypericin and pseudohypericin on different viruses. Human CMV is a frequent cause of infection in immunocompromised people. The available anti-CMV drugs are not very effective in some patients and/or safe. Drug resistance is also a growing problem. Coxsackie B-

Table 2. Anti-HCMV and Anti-Cox-B3 activity of *Conyza canadensis* (L.) cronquist extracts

Extract	Concentration (µg/ml)	Antiviral activity (%)	
		HCMV	CoxB-3
Petroleum ether	200	nd	nd
	100	nd	nd
Ethyl acetate	200	80.12	75
	100	78.65	60
chloroform	200	87.54	78
	100	75.98	75
Butanol	200	95.75	99
	100	90.10	97
Methanol	200	100.0	100
	100	99.10	100
Ganciclovir	0.8	50	-
Ribavirin	130	-	50

3 viruses are single-strand positive-sense RNA viruses that are resistant to a wide variety of chemical treatments.

Since HCMV is a very slow growing virus, we attempted the modified 'shell-vial' diagnostic method to reduce the culture time to 48 h, in this way limiting most of the inconvenient artefacts induced by a long culture step. This method is quantitative as the number of positive nuclei is directly proportional to the number of viruses growing.

The most active extract seemed to be methanol with active concentration of 100 µg/ml which also had no cytotoxic effect against MRC-5 cells. The ED50 of ganciclovir against a laboratory strain of CMV, AD-169 was 0.8 mg/ml (Tocci et al., 1984). Thus, for the current investigation, it is obvious that the methanol extract of *C. canadensis* had a potent anti-HCMV action on the laboratory strain AD-169 and anti-Cox B3 virus. Screening of chemical groups in the methanolic extract of *C. canadensis* (L.) Cronquist revealed the presence of flavonoids, terpenoids and tannins. The observed antiviral activity may be due to the higher amount of phenolic compounds, particularly flavonoids and tannins which are known to possess good antiviral activities (Fukuchi et al., 1989; Namba et al., 1998). However, it is difficult at the moment to indicate the exact mode of this action, which requires further examination. It is concluded that *C. canadensis* could contain one or more products and almost non-cytotoxic compounds, showing a strong antiviral activity. Further studies are in progress with the aim of understanding the exact mechanism of action of the plant and of isolating and identifying the active principle(s).

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