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Full Length Research Paper

Antiviral activity of leaves extracts of *Marrubium alysson* L.

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Petroleum ether, ethyl acetate, chloroform, butanol and methanol extracts of the leaves of *Marrubium alysson L.* were screened for *in vitro* antiviral activity against human cytomegalovirus (HCMV) strain AD-169 (ATCC Ref. VR 538) and coxsackie B virus type 3 (CoxB-3) using a cytopathic effect (CPE) reduction assay. Total phenolic content of the extracts were determined by the Folin-Ciocalteu colorimetric method. The most potent inhibition was observed with methanol extract, which inhibited HCMV and Cox B3 viruses replication at 100 µg/ml without showing cytotoxic effects. Good activities were also found with the ethyl acetate, butanol and chloroform extracts which exhibited antiviral effect against both HCMV and Cox B3 at 100 to 250 µg/ml.

Key words: *Marrubium alysson* L., antiviral activity, total phenolic content.

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 the 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 virally related diseases is of growing concern. For that 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 Berghe et al., 1986; Vlietinck et al., 1995; 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; Marchetti et al., 1996; Hayashi et al., 1997).

The genus Marrubium is represented by 97 species which are widely spread over the temperate and warm regions. Many Marrubium species are reported in the literature to be used in folk medicine (Anastasia et al., 2003, Anastasia et al., 2005). Most of the species are annual or rhizomatous perennial herbs with a distinct indumentums of often very complex hairs, and verticillasters subtended by floral leaves. *Marrubium alysson* is a common plant in Tunisia and is used in traditional medicine in the form of a decoction as a remedy for asthma, diabetes and as a diuretic. Recent research demonstrated the antibacterial activity of *M. alysson* extracts (Edziri et al., 2007). To the best of our knowledge the antiviral activity of *M. alysson* has not been studied yet.

The aim of this study was to investigate the antiviral

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property of methanol extracts of *M. alysson L.* growing in Tunisia.

MATERIALS AND METHODS

Plant materials

Fresh aerial parts were collected in 2010 in kerker (Tunisian centre). The plant was identified by Pr. Mohamed Chaieb botanist in the University of Science (Sfax, Tunisia). A voucher specimen was deposited in the herbarium of our laboratory.

Extraction

750 g of dried *M. alysson* were extracted with MeOH through maceration for five days. The resulting extract was evaporated at reduced pressure to obtain a green residue (279 g) which was successively fractionated with, petroleum ether, chloroform and ethyl acetate and these fractions were used for biological tests.

Determination of total phenolic content

Total phenolic content in each extract was determined using Folin-Ciocalteus reagent according to the method of Singleton et al. (1965). Forty microliters of extract (1 mg/ml) was mixed with 200 µl Folin-Ciocalteus reagent (Sigma-Aldrich, Germany) and 1160 µl of distilled water, followed by 600 µl 20% sodium carbonate (Na_2CO_3) 3 min later.

The mixture was shaken for 2 h at room temperature and absorbance was measured at 765 nm. All tests were performed in triplicate. Gallic acid (Sigma-Aldrich, Germany) was used as a standard. The concentration of total phenolic compounds (TPC) was determined as µg gallic acid equivalents (GAE)/mg extract using the following equation obtained from a standard gallic acid graph ($R^2 = 0.9877$):

Absorbance = 0.0012 × gallic acid (µg) - 0.0034

Antiviral activity

Cell line and growth condition

Human diploid embryonic lung fibroblasts (MRC-5) (Biomerieux-France) were grown in MEM-D (Seromed, Germany, Ref. T041-05) supplemented with 10% fetal calf serum (FCS Ref. S0115, Seromed, Germany), 100 units/ml penicillin, 100 mcg/ml streptomycin. FCS was reduced to 2% for the viral infection. The cells were incubated for 3 days at 37°C in a humidified atmosphere of 5% CO_2 incubator until forming a confluent monolayer cells.

Cell toxicity assay

The evaluation is based on the reduction of MTT, (3-(4.5-dimethylthiazol-2-yl) – 2,5 diphenyltetrazolium bromide) by the mitochondrial deshydrogenase of viable cells, to give a blue formazan product which can be measured spectrophotometrically. The MTT colorometric assay was performed in 96 -well plates (Litchfield and Wilcoxon, 1949). Cells were seeded in 96-wells plate at a concentration of 5 x 10^4 cells/ well and incubated for 24 h at 37°C in a 5% CO_2 enriched atmosphere.

After treatment with various concentration of each extract (100, 200, 400 and 800 µg/ml), the cells were incubated for an additional 48h at 37°C. After that, the medium was removed and cells in each well were incubated with 50 µl of MTT solution (5 mg/ml) for 4 h at 37°C. MTT solution was then discarded and 50 µl dimethyl sulfoxide (DMSO) were added to dissolve insoluble formazan crystal. Optical density was measured at 540 nm. Data were obtained from triplicate wells.

Titration of viruses

Human cytomegalovirus (HCMV) strain AD-169 (ATCC Ref. VR 538) and coxsakie virus type B3 were used for the antiviral activity. Serial 10- fold dilution (10^{-1} - 10^{-5}) were prepared in MEM containing 2% FCS. Virus dilutions 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 CPE in the virus -infected cells was observed microscopically, $TCID_{50}$ was determined by the method of Reed and Muench (1938).

Antiviral activity assay

We have adopted a cytopathic effect (CPE) reduction assay for screening the antiviral activities of the plant extracts. In brief to confluent cell monolayers in a 96-well plate, 100 TCID50 (50% tissue culture-infective dose) virus suspension and serial two-fold dilutions of crude extracts were added simultaneously. As positive control, cells were infected with the same concentration of virus but without the addition of extract, and as a negative or cell control, only MEM-D was added to the cells. The plates were incubated at 37°C in a humidified CO_2 atmosphere for 3 to 5 days. The concentration that reduced 50% of CPE in respect to the virus control was estimated from the plots of the data and was defined as the 50% inhibitory concentration (IC_{50}). The selective index (SI) was calculated from the ratio CC50/IC50 (Kujumgier et al., 1999).

RESULTS AND DISCUSSION

The TPC was expressed in equivalent of gallic acid (mg CE/g of extract). The total phenolic content of extracts of *M. alysson* L. is given in Figure 1. The total phenolic content varied from 123.98 to 26.6%. We can conclude that methanol and butanol extracts had the highest total phenolic contents than the other extracts.

The antiviral activity was estimated on the basis of cytopathic effect (CPE) of the virus-infected confluent monolayer of MRC 5 cells. The mean IC_{50}, CC_{50} and SI (CC_{50}/IC_{50}) values are shown in Table 1. We can conclude that all extracts were not toxic against MRC5 cells (IC50 > 500 µg/ml). The most active extract was the methanol extract, which inhibited HCMV and Cox B3 viruses replication at 100 µg/ml without showing cytotoxic effects and with selective index upper to 5. Good activities were also found with the ethyl acetate, butanol and chloroform extracts which exhibited antiviral effect against both HCMV and Cox B3 viruses at concentrations ranging from 100 to 250 µg/ml.

Viral infections are accompanied by profound changes in cell/tissue metabolism, which lead to severe generation of reactive oxygen species. The latter may cause aggravation in the pathogenesis of the infection. It has been found recently that the main cause of mortality from

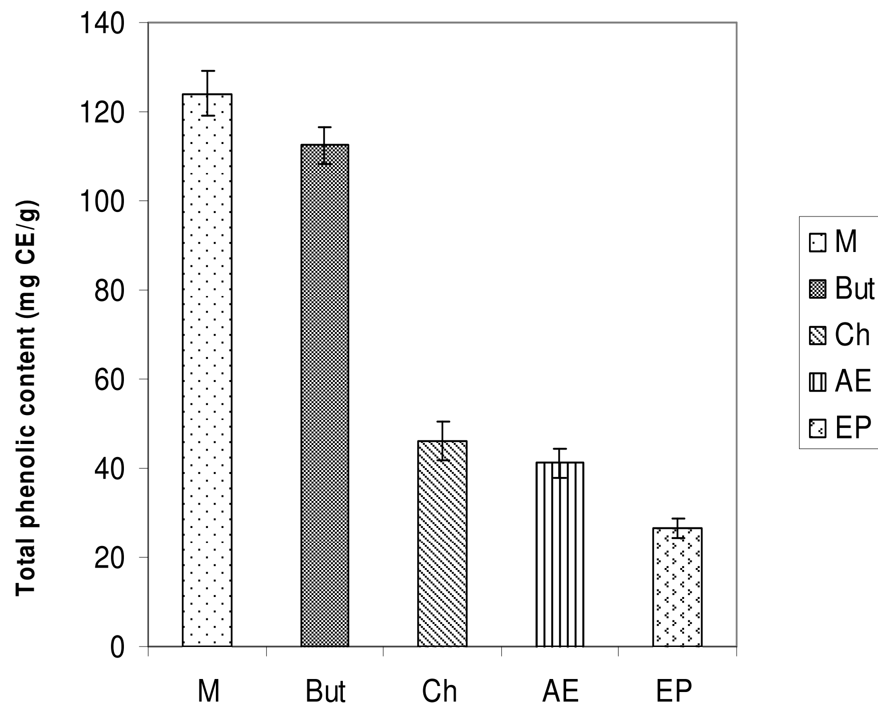

Figure 1. Total phenolic content of *M. alysson* extracts. mg CE/g: milligram catechin equivalent per gram extract; M: methanol extract; But: butanol extract; Ch: chloroform extract; AE: ethyl acetate extract; EP: petroleum ether extract.

Table 1. Antiviral activity of *M. alysson* extracts.

	Anti -CMV			Anti-CoxB3	
Extracts	[a]IC_{50} (µg/ml)[a]	[b]CC_{50} (µg/ml)[b]	SI[c]	IC_{50} (µg/ml)	SI[c]
Petroleum ether	350	>500	>1.41	300	>2.5
Ethyl acetate	250	>500	>2.5	250	>3,3
Chloroform	200	>500	>2.5	150	>3,33
Butanol	200	>500	>2.5	100	>5
Methanol	100	>500	>2.5	100	>5
Ribavirine[d]	nd	>250	nd	130	>1.92
Ganciclovir[d]	0.8	>200	>250	nd	nd

[a] IC50 is the concentration of the sample required to inhibit 50% virus-induced CPE. The blank parts in the table denote no inhibitory effect on virus-induced CPE in MNCC. [b] CC50 is the concentration of the 50% cytotoxic effect. [c] SI (selective index) is the ratio CC50/IC50. [d] Ribavirin and Ganciclovir, which are clinically used anti-CoxB-3 and anti-HCMV drugs, respectively, were used as positive controls in the antiviral activity. nd: not determined.

influenza virus-induced pneumonia is the cytotoxicity, which is determined by the considerably increased levels of O_2 before the viral replication in the bronchial epithelial cells (Akaike et al., 1996). Therefore the use of antioxidants could be of great value in preventing the inception or the progression of the disease to play an important role in the mechanism of antiviral activity to reduce the action of reactive oxygen species (ROS) in tissue damage.

Methanol, butanol and ethyl acetate extracts showed significant anti-Coxsaki B3 activity. This observed activity may be due to the higher amount of phenolic compounds particularly flavonoids and tannins known to possess good antiviral activities (Fukuchi et al., 1989; Namba et al., 1998).

The substances responsible for the antiviral activity must be isolated and chemically characterized. Further analysis, including additional purification of the extracts, along with further antiviral testing, are currently being conducted.

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