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Antiviral efficacy of *Limonium densiflorum* against HSV-1 and influenza viruses

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ABSTRACT

Viral infections remain a major threat to humans and animals and there is a crucial need for new antiviral agents especially with the development of resistant viruses. Several *Limonium* genus members (Plumbaginacea) have been widely used in traditional medicine for the treatment of infections. In this study, we investigated the antiviral activities of different fractions after successive extraction (hexane, dichloromethane, ethanol and methanol) of the halophyte *Limonium densiflorum* against H1N1 influenza and HSV-1 herpes viruses. In addition, TLC phytochemicals of the shoot extracts were analyzed. All extracts were tested for their cytotoxicity using a fluorometric resazurin assay. The antiviral activity of extracts was tested using four modes of action: virucidal test, pretreatment of cells with samples before infection, attachment assay and plaque reduction test. A good antiviral activity was found with ethanol and methanol extracts. They were most potent in HSV-1 inhibition than H1N1 influenza virus. The most potent inhibition was observed with ethanol extract, and it exhibited high levels of virucidal activity against HSV-1 (IC_{50} = 6 μg/mL). It inhibits the replication of the virus by 75% when added after penetration of the virus, and by 100% when added during the viral attachment. It protects MDCK cells against influenza virus by abolishing virus to entry into the host cell (IC_{50} = 55 μg/mL). After attachment of influenza virus, the ethanol extract displayed an appreciable inhibition of virus replication (IC_{50} = 193 μg/mL). Methanol extract showed a moderate antiviral capacity against both viruses. While dichloromethane has excellent antiherpes potential, results were inappropriate because it was toxic to Vero cells, hexane extract has no effect. TLC analysis of these extracts showed that flavonoids and saponins were the major classes of natural products found in the shoot extracts that may be responsible for these antiviral activities.

1. Introduction

Frequent human infections of viral origin could be exemplified as common HIV, AIDS, cold influenza, HSV etc. (Orhan et al., 2009). Herpes simplex virus (HSV) causes a variety of diseases in humans, with different degrees of severity, ranging from mild to severe, and in certain cases, it may even lead to life-threatening conditions. Herpes simplex virus infection is characterized by watery blisters on the skin or mucous membranes of the mouth, lips or genitals. It stands out because of the wide variety of clinical symptoms they provoke, including oral and genital vesicular lesions, eczema, encephalitis, pneumonia and hepatitis (Alvareza et al., 2009). After the primary infection, HSV-1 trends to persist in the neuron of the ganglia. Reactivation of latent HSV which is very common during the deficiency of immunity causes recurrent herpes infection (Loizzo et al., 2008). According to epidemiological surveys, the HSV infection rate has continuously increased in most countries (Chuanasa et al., 2008). Influenza is a serious global contagious disease that leads to hundreds of thousands of deaths every year (Zhao and Akber, 2012). There are currently three types of influenza A, B and C based on the antigenic and genetic differences of the inner proteins and genome structure. Influenza A is an important human pathogen that has an impact on the global health. After the pandemic period, the viruses still occur at high levels in winter as seasonal flu strains.

Viral vaccines and anti-viral drugs are the primary means for protection against and treatment of infections. For instance, nucleoside analogs, acyclovir (ACV) and other nucleoside derivatives, valaciclovir, and ganciclovir have been approved for treatment of HSV infections worldwide (Leung and Sacks, 2000; Brady and Bernstein, 2004). Oseltamivir phosphate, an orally administered drug, was used for the prophylaxis and treatment of human influenza A and B and it is the most promising drug for the treatment of influenza virus (Moscana, 2005). However, in the appearance of resistant viruses, virus is a current problem. The failure of treatment of HSV-1 is also due to the recurrence of latent viruses (Field and Biron, 1994). Besides, the high mutation rate of influenza virus also limits the effect of influenza vaccine. The vaccine

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cannot be effective in elderly people and children; it has been reported that the influenza virus has shown resistant to oseltamivir (Zhao and Akber, 2012). For these reasons, there is still a need in the future to search for new and more effective antiviral agents that can substitute or complement currently used antiviral medicines.

Ethopharmacology provides scientists with an alternative approach for the discovery of antiviral agents, namely the study of medicinal plants with a history of traditional use as a potential source of substances with significant pharmacological and biological activities (Cos et al., 2011). According to the literature, a great number of salt-tolerant medicinal plants (halophytes) are used to treat various diseases, viral infections and aging processes particularly in the rural areas, where the traditional folk medicine remains a major source to cure minor ailments (Ksouri et al., 2011). Halophyte plants are able to withstand hard climatic conditions which generate oxidative stresses by the production of antioxidant system. There are more than 2500 halophyte species known worldwide to possess some salinity tolerance, and among them, several could be suitable candidates to be used as cash crops. For instance, *Cakile maritima* had diuretic, antiscorbutic, digestive and purgative properties (Davy et al., 2006), *Limonium* spp. is known also in a folk medicine. *Limonium* Mill is a genus of 180 species, widely disturbed in different salt regions of the world for example, *Limonium brasiliense* is known as cardioprotective for anti-bacterial and anti-inflammatory properties (Murray et al., 2004) and *Limonium wrightii* is used for the treatment of fever or arthritis (Aniya et al., 2002). In addition, *Limonium sinense* and *Limonium tetragonum* show antiviral activity (Yuh-Chi et al., 2002), whereas, *Limonium axillare* and *Limonium californicum* show cytotoxic and antibacterial activities (Kandil et al., 2000; Sakagami et al., 2001).

In previous work *Limonium densiflorum* exhibited a potent antioxidant activity (Medini et al., 2011). Our research approach aimed to discover novel plant derived natural products as new leads, which could be developed for the treatment of viral infectious diseases. *L. densiflorum* is a rosette plant from coastal regions and salt flat. It can tolerate a wide range of environmental conditions and resist to abiotic stresses such as salt, high temperature, and water deficit stress.

In the course, we examined the inhibitory effect of different fractions of the halophyte *L. densiflorum* against herpes simplex type 1 (HSV-1) and influenza A viruses, and have an idea about mode of action of these extracts. Besides TLC phytochemicals was investigated.

2. Materials and methods

2.1. Cells for anti-herpes activity

The African Green Monkey Kidney cells (Vero cells ATCC: CCL81) were grown in monolayer culture with M199 medium supplemented with 10% fetal calf serum (Hyclone, Logan, USA), solution of vitamins (1×), sodium pyruvate (1×), non-essential amino acids (1×), penicillin (100 IU) and streptomycin (100 μg/mL). The monolayers were removed from their plastic surfaces and serially passaged whenever they became confluent.

2.2. Cells for anti-influenza activity

Madin-Darby Canine Kidney (MDCK cells: CCL34) were kept in minimum essential medium (MEM) supplemented with 5 mL penicillin/streptomycin, 10% BSA (1%), 0.025% trypsin solution and 0.025% glucose solution.

2.3. Viruses

Herpes simplex virus type 1 (HSV-1; 15577 strain) and influenza A viruses (H1N1 strain) were purchased from the American Type Culture Collection (ATCC, Rockville, MD). They were kindly provided by Laval University, Canada. All virus stocks were stored at −80 °C until use.

2.4. Plant material and preparation of crude plant extract

L. densiflorum specimen was harvested from salt flat (Sebkha) from Kairouan city (semi arid climate). The harvested plants were identified at the Biotechnology Center at the Technopark of Borj-Cedria, and a voucher specimen (PLM30) was deposited at the herbarium of the Laboratory of Extremophile Plants at the Biotechnology Center. Shoots were rinsed with distilled water, left at room temperature for 7 days in the dark, and then oven dried for 24 h at 50 °C. Extracts were obtained by successive extraction in Soxhlet using several solvents (hexane, dichloromethane, methanol and ethanol). Afterwards, the extracts were filtered, and solvent was evaporated under reduced pressure using rotary vacuum evaporator. At last, shoot extracts were stored and the residue was reconstituted in dimethyl sulphoxide (DMSO) before testing.

2.5. Cytotoxicity assay

For cytotoxicity assay, cells were seeded into 96-well plates at a density of $5 * 10^3$ cells per well and incubated for 24 h at 37 °C. The medium was removed and fresh DMEM containing the appropriate concentration of the *L. densiflorum* extracts was added onto confluent appropriate cells. All extracts were dissolved in DMSO and added to the medium at a final concentration of 1% DMSO, well containing 1% DMSO but without drug or extract was also included on each plate as control. After 4 days of incubation, the cytotoxicity was assessed using the resazurin reduction test as described by O'Brien et al. (2000). Measurements of fluorescence were carried out after 96 continuous hours of contact between extracts and cells on an automated 96-well Fluoroskan Ascent Fl™ plate reader (Labsystems) using an excitation wavelength of 530 nm and an emission wavelength of 590 nm. Results are expressed as the concentration inhibiting 50% of the cell growth (IC_{50}). The cell morphology was observed under the microscope.

2.6. Antiherpes activity

Cells and viruses were incubated with acyclovir or extracts at different stages during the viral infection cycle in order to determine the mode of antiviral action. All extracts were dissolved in DMSO and added to the medium at a final concentration of 1% solvent; wells containing 1% solvent but without drug were also included on each plate as controls.

2.6.1. Direct virucidal effect of extracts on HSV-1

Viral suspension was incubated with different concentrations of extracts at 37 °C for 1 h. (Camargo et al., 2008). The mixture was then used to infect Vero cells for 1 h. After that, it was removed; wells were washed with PBS (pH 7.4) and incubated with methylcellulose/medium 0.5% for 72 h. After 3 days, medium was aspirated and the infected cells were fixed with 10% formalin, stained with 1% crystal violet, and the number of plaques was counted (Rechter et al., 2006). The wells overlaid with medium without test sample were used as the controls. The percentage of inhibition of plaque formation was calculated as follows: [(mean number of plaques in control) − (mean number of plaques in sample)] × 100/mean number of plaques in control. Results are expressed as IC_{50} (μg/mL).

2.6.2. Effect of test samples before virus infection

Confluent Vero cells in 24-well tissue culture plates were washed with PBS. 100 μL of culture medium containing different concentrations of test extracts were added to each well, and the cells were incubated for 1 h at 37 °C and 5% CO_2. After removal of the extract, the cells were washed with PBS and then infected with 30 pcf/well of HSV-1. After 1 h incubation, the cells were washed with PBS twice to remove any unabsorbed virus, and further incubated in medium containing 0.5% methylcellulose for 72 h. Next, cells were fixed with formaldehyde

and stained with crystal violet. And subsequently lysis plaques were counted (Papazisis et al., 1997).

2.6.3. Effect of test samples during the infection

The assay was performed as described above, with the exception that the test extract was added together with the virus. After 1 h incubation, the solution containing unabsorbed virus was removed, and the cell monolayer was washed with PBS and further incubated in DMEM/methylcellulose for 72 h. The virus-induced cellular lysis was expressed as inhibition percent (%) (Camargo et al., 2008).

2.6.4. Plaque reduction assay

Infection assays were performed with HSV-1 on Vero cells in 24-well plates. Following viruses were adsorbed at 37 °C for 60 min, inoculi were removed and 0.5% methylcellulose overlays, optionally supplemented with drug or extracts, were added to the wells. After incubation for 3 days at 37 °C, the infected cells were fixed with 10% formalin, stained with 1% crystal violet, and the number of plaques was counted (Rechter et al., 2006). The wells overlaid with medium without test sample were used as the control. The percentage of inhibition of plaque formation was calculated as follows: [(mean number of plaques in control) − (mean number of plaques in sample)] × 100/mean number of plaques in control. Acyclovir was used as a positive control drug in the anti-HSV1 study.

2.7. Anti-influenza activity

2.7.1. Virucidal effect

Influenza A viruses were incubated in medium containing the extract for 1 h at room temperature prior to infection of MDCK cells. After 1 h of adsorption of the mixture (virus-extract) at 37 °C, the inoculum was removed and cells were overlaid with medium MEM/BSA for 4 days. The anti-influenza virus capacity was assayed by quantifying of the fluorescent production in response to the 'chemical reduction' of the medium resulting from cell growth or by mitochondrial enzymes in host cells (resazurin test). After 4 days, the fluorescence of the medium was recorded for the excitation wavelength 530 nm and emission wavelength 590 nm. The inhibition ratio was obtained using the equation: Inhibition activity (%) = $(F_{extract} - F_{virus}) / (F_{control} - F_{virus}) * 100$ where F_{Virus} is the fluorescence of the influenza virus control (virus, buffer and medium), $F_{control}$ is the fluorescence of the drug control (buffer and drug), and $F_{extract}$ is the fluorescence of the tested samples (virus, sample solution, medium). Subsequently, the 50% inhibitory concentration (IC_{50}) was determined by extrapolation of the results from various doses tested using a linear equation. At least three independent measurements were collected to determine the mean and SD values.

2.7.2. Effect of test samples before virus infection

Cells monolayer was pretreated with the *L. densiflorum* extract or oseltamivir, for 1 h at 37 °C. The extract was aspirated and cells were washed immediately before the influenza A inoculum was added. After 1 h of adsorption, inoculum was removed and cells were overlaid with medium MEM/BSA for 4 days. After 96 h of incubation at 37 °C, the fluorescence of the medium was read (ex 530 nm, em 590 nm). Results were expressed as 50% inhibitory concentration (IC_{50}) (μg/mL).

2.7.3. Effect of test samples during the infection

For analyzing the antiviral inhibition during the adsorption period, influenza was mixed with the drug or extract and added to the cells immediately. After 1 h of adsorption at 37 °C, the inoculum was removed and cells were overlaid with medium MEM/BSA for 4 days. The anti-influenza capacity was detected using resazurin test.

2.7.4. Direct plaque assay

MDCK cell monolayer grown in MEM/BSA in 96 well culture plates was infected with influenza virus. After 1 h adsorption at 37 °C, viruses were removed and medium containing substances at various concentrations/or oseltamivir were overlaid to the plates. The anti-influenza virus capacity was assayed by quantifying of the fluorescent (ex 530 nm, em 590 nm). Results were expressed as 50% inhibitory concentration (IC_{50}) (μg/mL).

2.8. Qualitative phytochemical analysis

The identification of major chemical groups of the plant extracts was carried out by thin layer chromatography (TLC) on silica gel (60 F2SJ glass plates, 250 μm layer thickness, Silicycle, Québec. Canada). An aliquot of *L. densiflorum* extract in methanol solution (1 mg/mL, 10 μL) was directly deposited (as bands) onto the TLC plates. TLC plates were developed in a presaturated solvent chamber with ethyl-acetate, acetic acid/formic acid/water (100:11:11:26) as developing reagents until the solvent front reached 1 cm from the top of plates. The developed TLC plates were then removed from the chamber, and allowed to air-dry for 30 min, followed by spraying with 2-aminoethyldiphenylborinate/polyethylene glycol (NPPEG) to reveal the flavonoids and phenolic acids. For the detection of terpenoids, we use chloroform/methanol/water (26:14:3) as mobile phase. After that, TLC plates were sprayed with 5% H_2SO_4 and heated at 110 °C for 5 min. For the detection of steroidic saponins, plates were migrated in a mixture of solvents (chloroform/methanol) (25:1) and sprayed with 5% H_2SO_4 followed by 20% vanillin. All these plates were visualized by inspection of the plates under visible light. Each TLC plate was also monitored under UV light at 254 and 366 nm. The retention factor of these compounds was determined.

2.9. Statistical analysis

Means were statistically compared using the STATISTICA program. Analysis of variance (ANOVA) and Duncan's multiple range tests were carried out to test any significant differences between parameters at $P < 0.05$.

3. Results

3.1. Cytotoxicity effect of different extracts

Evaluation of cytotoxicity is an important part of the assessment of a potential antiviral agent since the beneficial extract should be selective for virus-specific processes with little or no effects on the metabolisms of host cells. All extracts were dissolved in DMSO and added to the medium at a final concentration of 1%. DMSO by itself did not exhibit any toxic effect on Vero and MDCK cells when applied to 1%. The effect of the extract on the growth of Vero and MDCK cells was examined by the microscopic analyses and determination of IC_{50} value of each extract using resazurin fluorometric test. Microscopically, it was observed that all extracts do not alter the morphology or cell viability except for dichloromethane and methanol extracts, when their concentration increased; morphological changes (loss of monolayer) were visible. Dichloromethane extract decreased the viability of Vero cells. Methanol extract has no effect on Vero cells but it reduces that of MDCK cells. This result was confirmed by the determination of the cytotoxic IC_{50} value of each extract (Table 1). The IC_{50} values were 13.5 and 60 μg/mL for dichloromethane and methanol extracts respectively. The remainder extracts were not toxic for the appropriate cells.

3.2. Anti-herpes activity

Herpes virus replication is characterized by a cascade of coordinately regulated events. The inhibitory effect of *L. densiflorum* extract was

Table 1
Cytotoxicity assay and virucidal potential of *Limonium densiflorum* extracts expressed as IC_{50} values (μg/mL).

	Methanol extract	Ethanol extract	Dichloromethane extract	Hexane extract
Vero cells	>500	>500	13.5 ± 4	>500
MDCK cells	60 ± 15	>500	>500	>500
Herpes virus	85 ± 8	6 ± 0.5	60 ± 3	_
Influenza A	200 ± 15	189 ± 10	_	_

Data are presented as the mean values of three independent experiments including their standard deviations.
_: absence of virucidal activity.

determined following the addition at different times during viral infection. To identify the step at which replication might be inhibited, cells were infected with HSV-1 after its incubation with plant extract, pretreatment of the virus with the extract prior to infection and addition of the extract during or after adsorption period. In all experiments untreated virus infected cells were used as control. The number of plaque formation and percent reduction was calculated relative to the amount of virus produced in the absence of the compound. All experiments were performed parallel with acyclovir to test the suitability of the assay. Acyclovir achieved the highest antiviral effect when applied during the replication period. Plaques were reduced by 90.2% at a concentration of 0.75 μg/mL. When this drug was added to the cells prior to infection, or during the infection or incubated with the herpes virus, no antiviral effect could be observed.

3.2.1. Virucidal potential of Limonium extracts against HSV-1

The potential virucidal effect of *L. densiflorum* extracts against HSV-1 was determined by pretreatment of the virus with various concentrations of extract ranging from 3.25 to 100 μg/mL, subsequent to an infection of Vero cells was done. Results displayed that ethanol, methanol and dichloromethane extracts drastically diminished viral infectivity. Hexane extract has no effect. Table 1 showed a dose-dependent activity of the tested extracts. Ethanol extract exhibited levels of virucidal activity greater than methanol and dichloromethane. The titers of HSV-1 are reduced by 50% at a concentration of 6.5 μg/mL for ethanol extract and at a concentration of 85 μg/mL for methanol one. Dichloromethane extract was inappropriate because this extract exhibited cytotoxicity towards Vero cells at dose from 20 μg/mL. Solvent at a final concentration of 1% had no effect on virus titers.

In order to analyze a possible time-dependent virucidal effect, HSV-1 was incubated with *L. densiflorum* extracts for different amounts of time, ranging from 10 min to 24 h. After 10, 30, 60 min and 2, 18, and 24 h, an aliquot was removed and assayed for the remaining infectivity on confluent monolayer of Vero cells. For the two extracts (ethanol and methanol), a clearly time-independent activity ($p < 0.05$) was demonstrated (Fig. 1A) and this activity was assumed to be irreversible. Moreover, to test the effect of temperature on the potential virucidal extracts, the mixture of virus-extract was treated at two different temperatures 37 °C and 26 °C. Results showed that no significant differences ($p < 0.05$) in virion inactivation titers were found when the virus suspensions were treated with the plant extracts at these two temperatures for the period of 10 min, 60 min and 24 h (Fig. 1B and C). In general, all active extracts significantly reduced HSV-1 infectivity at different conditions (time and temperature).

3.2.2. Effect of test samples before virus infection

The preventive effect of *L. densiflorum* extracts on Vero cells was determined by pre-incubation of cells with plant extract for 1 h, and after that they were infected with HSV-1. The number of lysis plaques formed by HSV-1 in all extracts (hexane, dichloromethane, ethanol and methanol) pretreated Vero cells was not significantly different from the number formed in untreated Vero cells (Fig. 2). This suggested that plant extracts may not protect cells from infection which clearly

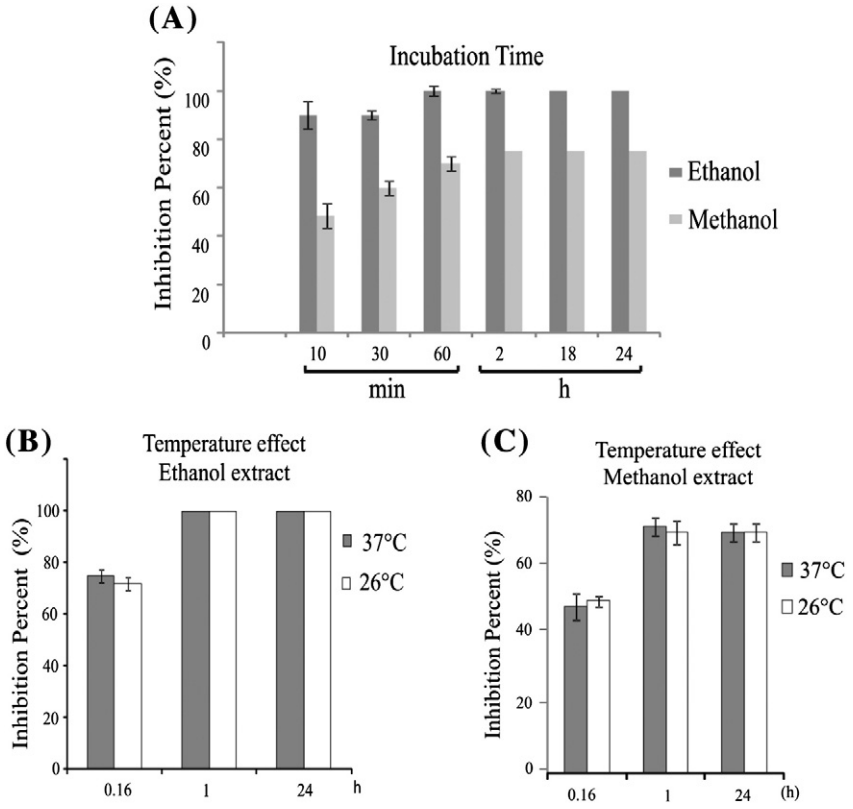

Fig. 1. Influence of exposure time (A) and temperature (B and C) on HSV virucidal effect using 100 μg/mL *Limonium densiflorum* extract. Data are represented as the mean of three independent experiments including their standard deviation values.

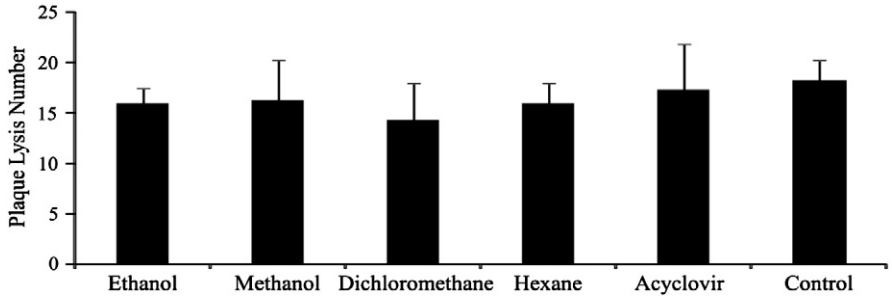

Fig. 2. Effect of *Limoium densiflorum* extracts on HSV-1 propagation in Vero cells when added 1 h before cell infection. No significant difference between extracts and control ($P < 0.05$). Data are represented as the mean of three independent experiments including their standard deviation values.

indicates that the activity of extract is caused by direct interaction with the virus.

3.2.3. Effect of test samples during infection

Viruses were allowed to attach to cells in the presence of different extracts of *L. densiflorum* for 1 h. The assay was terminated by removing the mixture (virus-extract) with PBS (pH 7.4). Results showed that the shoot extracts suppressed HSV-1 infection when added during the virus inoculation except for hexane extract; the activity was dose dependant. At 100 μg/mL of extract, the inhibitory rate was higher than 70% for dichloromethane extract and 80% for methanol extract and it reached 100% for ethanol extract (Fig. 3A).

3.2.4. Effect of test samples after infection

Limonium extracts was assayed for anti-HSV-1 during replication phase by the plaque reduction assay. Following virus infected cells for 60 min, inoculi were removed and 0.5% methylcellulose overlays, supplemented with drug or extract, were added to the wells. After incubation for 3 days at 37 °C, lysis plaques were counted and Inhibition Percent (IP) was calculated. Results displayed that ethanol extract has a good effect against replication virus. At 100 μg/mL, its inhibition percent (IP) against HSV-1 was 75%. Methanol extract showed a moderate activity; at the same concentrations it inhibits 42% of virus replication. After 72 h of dichloromethane extract incubation, it was toxic for Vero cells (Fig. 3B).

3.3. Anti-influenza activity

3.3.1. Virucidal potential of Limonium extracts

A low effect on viral growth was observed when virus was pre-incubated with extracts before cell infection. IC_{50} values were 200 and 189 μg/mL respectively for methanol and ethanol extracts (Table 1). In all steps, dichloromethane and hexane extracts did not reduce influenza A virus production.

3.3.2. Effect of test samples before virus infection

Interestingly, pretreatment of cells with ethanol and methanol extracts has a good inhibition of virus production (IC_{50} = 55.5 and 100 μg/mL respectively) (Fig. 4A).

3.3.3. Effect of test samples during infection

When plant extracts were added during the adsorption period, virus titers were reduced by 50% at a concentration of 100 μg/mL for ethanol extract, and 155 μg/mL for methanol one (Fig. 4B).

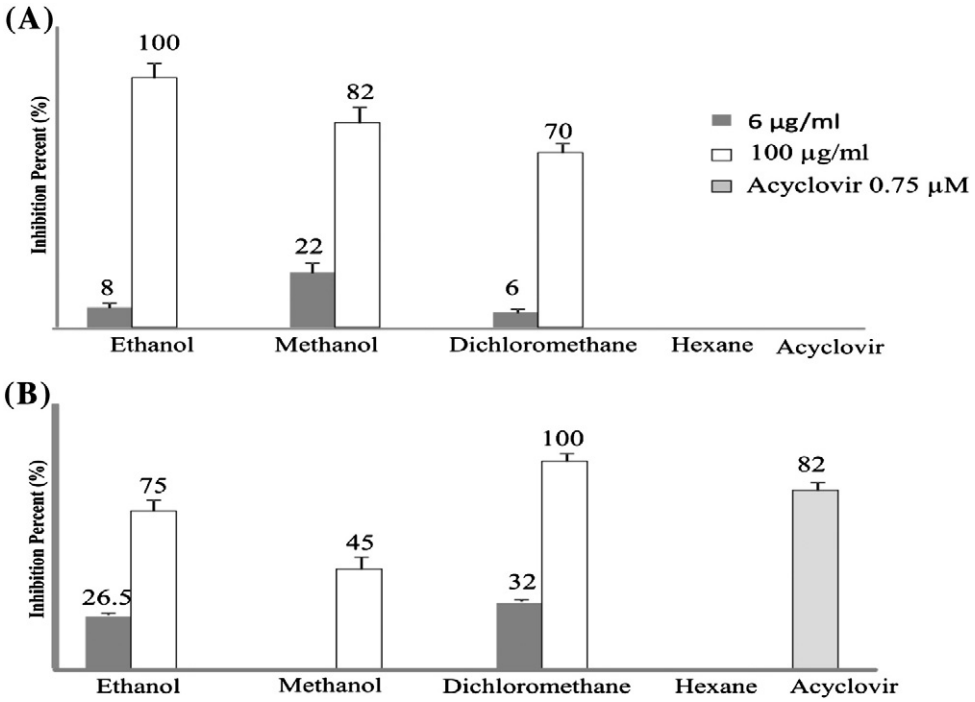

Fig. 3. Inhibitory effect of different concentrations of *L. densiflorum* on HSV-1 replication when added during viral infection (A) and after HSV-1 penetration (B). Data are represented as the mean of three independent experiments including their standard deviation values.

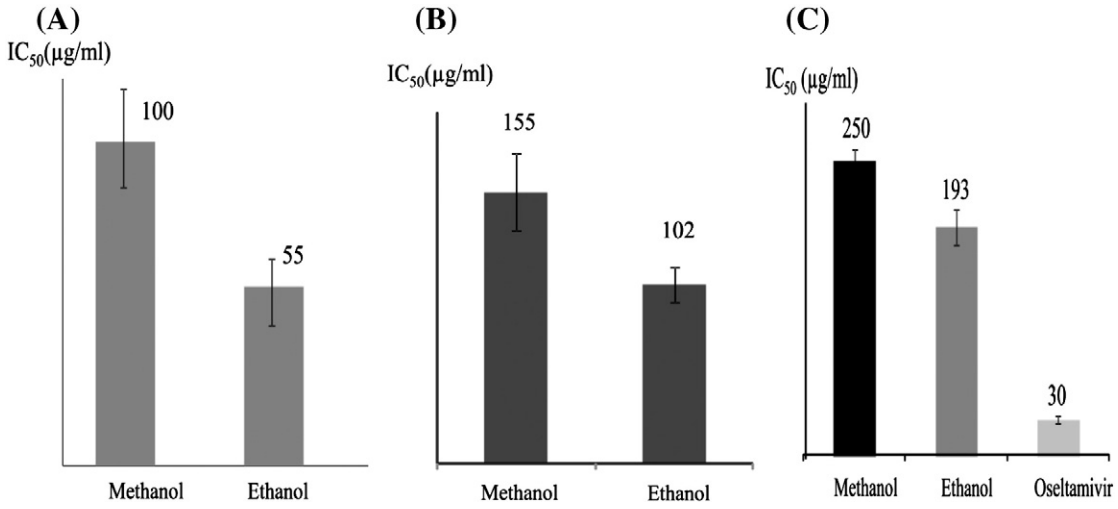

Fig. 4. Efficacy of shoot extracts on H1N1 influenza virus, when applied before viral attachment (A), or during infection (B) or after penetration of virus (C). Results are expressed as IC_{50} (µg/mL).

3.3.4. Inhibitory activity on influenza virus replication after cells infection

MDCK cells were incubated with influenza virus and subsequently with the plant extract at various concentrations. Maximum reduction was observed with ethanol extract (IC_{50} = 193 µg/mL). Methanol extract was also effective against H1N1 influenza virus however, a higher concentration was needed to achieve the same degree of reduction of virus titers (IC_{50} = 250 µg/mL). Both extracts have a good anti-influenza A activity by comparing to oseltamivir (IC_{50} = 30 µg/mL). None of the hexane and dichloromethane extracts have an effect on influenza replication (Fig. 4C).

3.4. TLC characterization of L. densiflorum

The phytochemical analysis of different extracts of *L. densiflorum* by TLC showed that ethanol and methanol extracts present high flavonoid concentrations (detected by the yellow and orange spots) and few phenolic acids (detected by blue spots), when plates were observed under UV-light at 254 nm (Fig. 5A). This may explain the good antiviral capacities of these extracts of *L. densiflorum*. Dichloromethane extract is rich with saponins (detected by the red spots) (Fig. 5B), however hexane contains few quantities of phenolic acids (Fig. 5B and C).

4. Discussion

Viral infections are the cause of many human and animal diseases that have a significant economic impact. On the basis of the fact that 80% or more of the adult human population may be sero-positive for herpes simplex virus (HSV), this virus must be judged as a highly successful pathogen (Cecilio et al., 2012). Furthermore, influenza continues to emerge and cause an important health problem associated with increase general of epidemics and occasionally pandemics, hospital admissions and excess death (Mooney and Tompkins, 2013). Many natural products are tested as antiviral agents because the control of viral infection remains a challenge for researchers (Gravina et al., 2011). Plants have a long evolutionary history with respect to developing resistance against viruses and are increasingly drawing attention as potential sources for the development of antiviral drugs. In the present investigation we reported for the first time the antiviral potential of the halophyte *L. densiflorum*. *Limonum* extracts have been widely used in traditional medicine (Ksouri et al., 2011). Experiments to assess the toxicity of different plant extracts indicate that hexane and ethanol extracts have no toxic behavior in cell cultures. However, dichloromethane and methanol extracts show a moderate toxicity respectively towards Vero and MDCK cells.

The antiviral activity of *L. densiflorum* extracts was dose related, strain specific and depended on the time of virus inoculums. Interestingly, extracts were quite more effective against HSV-1 than influenza. The timing of addition experiments, aiming at investigating the possible mode of action of *Limonium* extracts indicates that the pronounced inhibitory effect can be accounted for the protection cultures and the inhibition of early stages of viral replication or in adsorption and penetration of the virus. Pretreatment of the Vero cells with the *L. densiflorum* extracts had no effect on the inhibition of production of

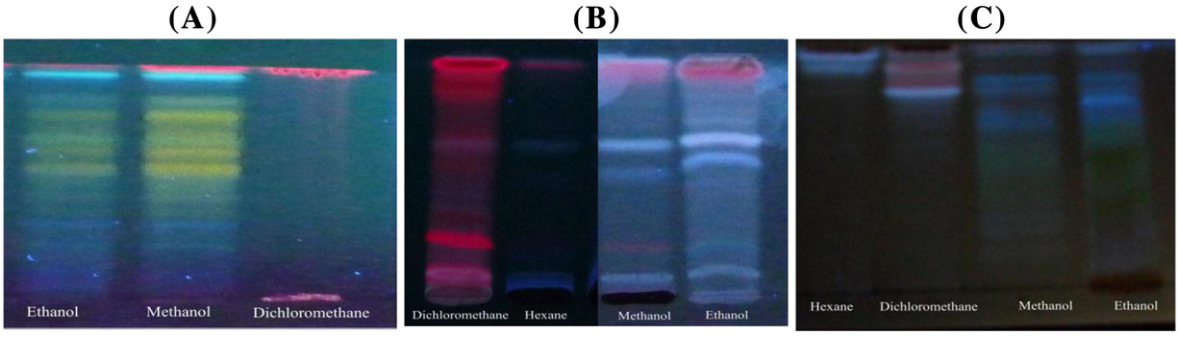

Fig. 5. TLC of *Limonium densiflorum* extracts. (A) Mobile phase: ethyl acetate/formic acid/acetic acid/water (100:11:11:26), revelation with NP/PEG, (B) mobile phase: chloroform/methanol/water (26:14:3), revelation with 5% H_2SO_4, (C), mobile phase: chloroform/methanol (25:1), revelation with 5% H_2SO_4 followed with 20%vanillin.

infectious virus and plaque formation was not affected. However, pretreatment of MCDK cells with the same extracts resulted in a good reduction of plaques by methanol and ethanol extracts suggesting that *L. densiflorum* extracts have a good preventive effect on MDCK against influenza. Probably, this extract protects the host cell infection by interfering with the action on the surface glycoproteins receptors and blocks the attachment of the virus to cells (Nizet and Esko, 2009). When plant extracts were added during the adsorption period, the amount of plaques for HSV-1 was reduced by 70%, 80% and 100% respectively for dichloromethane, ethanol and methanol extracts. A similar anti-adsorption effect was demonstrated for the methanol and ethanol extracts inhibiting the attachment of influenza to MDCK cells (IC_{50} values were respectively 155 and 102 μg/mL). This activity act can be explained by the inhibition of cellular signaling pathway (PKC/MAPK pathway) leading to retention of viral ribonucleoprotein in nucleus, thereby the reduction of virus replication (Ludwig, 2007). In fact, cell fate decisions in response to extracellular agents, including pathogenic invaders, are commonly mediated by intracellular signaling cascades that transducer signals into stimulus specific actions. In most cases the signal transduction within the cell is governed by kinases organized in different kinase cascades (MAPK) (Ludwig, 2007).

After the adsorption period, the efficiency of *L. densiflorum* extract on viral replication was also demonstrated in the plaque reduction assay, since the good inhibition of HSV-1 was observed 96 h after treatment with both ethanol and methanol extracts, resulting in an inhibition percent of viral titer of 75 and 42%, the modest inhibition of influenza yield was expressed as IC_{50} (μg/mL); when treating with the same extracts IC_{50} values were respectively 250 and 193 μg/mL. Therefore, extracts of *L. densiflorum* extract showed appreciable antiviral activities on both HSV-1 and influenza A, 96 h after viral infection compared with the synthetic drugs ACV (100% of activity) and oseltamivir (IC_{50} = 30 μg/mL). When extracts were pre-incubated with virus before cell infection, HSV-1 showed sensibility to ethanol extract (IC_{50} = 6 μg/mL). The powerful virucidal activity against herpes virus (HSV-1) may be caused by the disintegration of the entire HSV particles, the solubilization of the virus envelope, and degradation or masking of some of the essential envelope protein which are necessary for adsorption or entry of the virus into host cells (Alvareza et al., 2009).

Accordingly, the ethanol extract tested was strongly active against herpes viruses which made us consider that polar compounds could be responsible for the antiviral activity. Consistent with our present data numerous researchers have found the ethanol extract from various plants to process antiviral activities against HSV-1 (Loizzo et al., 2008). In another hand, Yuh-Chi et al. (2002) has isolated a new flavonoid from *L. sinense* shoot which suppressed HSV-1 multiplication in Vero cells without apparent cytotoxicity. In addition, Lin et al. (2000) has isolated two flavonoids from the root of the same plant that inhibits the replication of herpes virus. This suggested that the genus *Limonium* could be a source of potent antiviral drugs.

Crude plant extracts contain diversity of constituents that may exert their antiviral effect either singularly or in concert with each other. The fact that some degree of inhibition was observed with these extracts may suggest that the extracts contain an active component(s) in low concentrations that may be responsible for the observed activity. These substances may, if present at higher concentrations, be capable to improve the inactivation of the virus as well as preventing its replication in the host system. Previous work displayed that *L. densiflorum* extract has very high polyphenol, flavonoid and tannin contents (Medini et al., 2011); so the antiviral of the halophyte *Limonium* may relate to the presence of polyphenolic compounds. Many ploypheno ls are known for their anti-herpes and anti-influenza activities (Van den Berghe et al., 1986). It is known that polyphenols bind with proteins to form unstable complex, therefore enveloped viruses and among them herpes and influenza A viruses may be the most vulnerable to the action of polyphenols because this class of natural substance can easily interact with the glycoproteins of the viral envelope (Haslam, 1996). We have showed that ethanol extract rich in polyphenols has a strong antiherpes virucidal potential. This kind of molecules exerts their anti-influenza activity by inhibiting intracellular metabolic pathways rather than viral proteins. Inactivation of host-cell functions that are essential for the virus replication offers two important advantages: not only is it more difficult for the virus to adapt to, but it can also be expected to affect viral replication (Fioravanti et al., 2012).

Usually, viral infections are accompanied with profound changes in cell/tissue metabolism, which leads to generation of reactive oxygen species. The latter may enhance the pathogenesis of the infection. For example, it was found that the main cause of mortality from influenza virus-induced pneumonia is cytotoxicity, which is determined by a considerably increased level of O_2 before viral replication in the bronchial epithelial cells occurs (Akaike et al., 1996). Several studies made a correlation evaluation of the antioxidant and the antiviral activities of plant extracts and natural substances, for example, Edziri et al. (2012) proved that extracts from a leaf of *Marrubium deserti* showed potential antioxidant capacity and it has good anti-cytomegalo and anti-coxsakie viruses.

Halophytic vegetation, includes all classes, and grows in different saline biotopes. In plants, unfavorable environmental conditions such as salt constraint increase production and accumulate the reactive oxygen species (ROS), leading to cellular damage. To overcome harmful environment, halophytes were equipped with a powerful antioxidant system including enzymatic and non enzymatic molecules mainly phenolics (Ksouri et al., 2011). This hypothesis was confirmed by the measurement of the antioxidant potential of different Tunisian halophytes via many tests. Results demonstrated the powerful antioxidant capacity of *L. densiflorum* (Medini et al., 2011). In the light of this result, we can conclude that *L. densiflorum* not only has an inhibitory effect against herpes and influenza viruses, but also can be of great value in preventing the inception or the progression of the disease.

The present study corroborates ethno-pharmacological data as a valuable source in the selection of plants with antiviral activity and to some extent validates their traditional uses.

5. Conclusion

This study has shown that *L. densiflorum* extracts can inhibit both (H1N1) influenza and HSV-1 viruses by inhibiting the early stage of HSV multiplication, along with a good protection of cells against influenza virus. These results lead to further investigation about the characterization of active compounds and their specific mechanism against these viruses. In addition, *L. densiflorum* was rich in phenolic compounds so it can be interesting in preventing the inception or the progression of the disease. Given the urgent need for new and abundantly available anti-influenza and anti-herpes drugs, *L. densiflorum* ethanol extract appear to be a promising option as a replacement or supplemental strategy to currently available antiviral therapies.

Conflict of interest

The authors declare that they have no conflict of interest.

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