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Antiviral-guided fractionation and isolation of phenolic compounds from *Limonium densiflorum* hydroalcoholic extract

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ABSTRACT

A preliminary antiviral plate assay of the green solvent (hydro-ethanolic) shoot extract of *Limonium densiflorum* showed a potent activity against the herpes simplex virus type 1 (HSV-1). In order to isolate the active compounds, an *in vitro* bio-guided fractionation was undertaken by preparative chromatographic techniques. On the basis of nuclear magnetic resonance techniques, the structure of the isolated compounds was determined as gallic acid, epigallocatechin gallate, quercitrin, dihydrokaempferol, pinoresinol, *N-trans*-ferulolyl tyramine and (myricetin 3-*O*-α-rhamnopyranoside and myricetin 3-*O*-L-arabinofuranoside). Moreover, all isolated molecules were evaluated for their virucidality against HSV-1. Results showed that gallic acid and epigallocatechin gallate have strong activity, while pinoresinol and *N-trans*-ferulolyl tyramine have moderate activity. Whereas, the other molecules were inactive.

1. Introduction

Herpes simplex virus type 1 (HSV-1) is a highly successful human pathogen. It is responsible for a broad range of human infectious diseases persisting during the lifetime of the host, often in a latent form. HSV-1 infection can cause several clinical conditions such as gingivo-stomatitis or cutaneous sequella [1]. Prolonged therapy with the available anti-herpes drugs has resulted in some undesirable effects and has induced the emergence of drug-resistant virus strains [2]. Therefore, the development of new anti-herpetic agents and complementary therapy with currently available drugs are still needed.

Natural products, such as plant extracts, standardized extracts or pure compounds, provide numerous opportunities for new drug discoveries because of the unmatched availability of chemical diversity [3]. According to the World Health Organization (WHO), more than 80% of the world's population relies on traditional medicine for their primary healthcare needs [4]. In fact, plant derived drugs have served as the foundation of a large fraction of the current pharmacopoeia [5]. Between 1981 and 2006, approximately 44% of the anti-viral drugs approved were natural products [6]. For these reasons, phytochemicals are gaining in importance as potential sources for anti-viral agents. When one considers that a single plant may contain up to thousands of constituents, the possibilities of making new discoveries becomes evident. The crucial factor for the ultimate success of an investigation on bioactive plant constituents is thus the selection of plant materials

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and the appropriate extraction and purification process of the active compounds [7].

The analyte extraction from plants is an important step before analyzing the composition of the vegetal matrix. Various methods have already been used for this purpose and most of them are being assisted with a solvent extraction, such as soxhlet, under reflux and maceration. A large number of organic solvents are volatile, flammable, often toxic and responsible for environmental pollution while contributing to the greenhouse effect. Thus, safeties, as well as environmental and economical concerns are forcing industries to turn to greener solvents [8–10]. Several innovations towards green extraction have been developed: solvent-free technology [8,11], use of water as an alternative solvent [12] or use of ionic liquids that have low vapor pressure and consequently occur producing a lesser emission of the volatile organic compounds [13,14]. Ethanol is a worthy candidate for investigation as an alternative solvent since its cost is low and it may be produced from a large variety of biological materials using simple technology [15]. In addition, although flammable, this alcohol is recognized as non-toxic and has less handling risks than petroleum solvents [15]. It can also be obtained by fermentation and therefore labeled as "natural". This solvent is widely used as a solvent for substances intended for human contact or consumption. It is a readily miscible with water and able to dissolve many polar compounds [16].

In reality, to adapt to the green extraction six principles should be viewed for industries and scientists as a guideline to establish an innovative and be a driving-force to innovate not only in the process but also in all aspects of solid-liquid/liquid–liquid or other types of extraction [17].

The green extraction's principles are: (i) innovation by selection of varieties and use of renewable plant resources, (ii) reduction of energy consumption by using innovative technologies, (iii) the use of alternative solvents and principally those from agricultural resources (iv) the reduction of unit operations through technical innovation and favor safe, robust and controlled processes, (v) the production of co-products instead of waste to include the bio- and agro-refining industry and (vi) obtaining a denatured extract without contaminants [17].

Extraction is particularly affected by environmental and economic factors that require a massive reduction of energy consumption and wastes produced. There are four routes to minimize energy consumption: optimizing existing processes, recovering the energy liberated during the extraction process, assisting existing processes with intensification, and a full process innovation [17]. Besides, the "Bio-refinery" concept is becoming widely accepted as the world's natural resources are being used up and can be considered as a facility that combines the biomass conversion process with equipment to produce a wide range of bio-based products such as biofuels and biomaterials. Recently, producing useful chemicals from biomass instead of petroleum has been attracting much attention [18]. In addition, plants are made up of an enormous number of substances that may be refined: each constituent of the plant can be extracted and functionalized to produce green fuels, building materials, packaging, maintenance products, beauty creams, *etc.* [17].

In spite of its small area, Tunisia has a large plant biodiversity [19]. Its flora counts for more than 2150 species growing in various bioclimatic zones from humid to Saharan regions [20]. Among them, medicinal halophytes, living in extreme environments dealing with frequent changes in the salinity level, are used to treat various diseases, microbial and viral infections and aging processes particularly in the rural areas, where the folk medicine remains a major source to cure minor ailments [21]. The genus *Limonium* is also known in the traditional medicine and is widely distributed in different salt regions. Screening of *Limonium* plant extracts has led to the detection of effective *in vitro* viral replication inhibitors. For example, *Limonium sinense* and *Limonium densiflorum* showed a potent anti-herpes capacity [22,23]. *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 deficiencies. Previous work demonstrated that the chemical composition of the ethanolic shoot extract of *L. densiflorum* showed excellent radical's scavenging and antioxidant properties [24]. Furthermore, it represents a rich and growing source of natural target molecules, such as phenolic compounds [25]. Phenolic compounds, especially flavonoids, have gained much attention due to their antioxidant activities and free radical-scavenging abilities, which potentially have beneficial implications in human health [25].

In this study, we report the anti-herpetic activity-guided fractionation as well as the characterization of purified molecules from the hydro-ethanolic extract of *L. densiflorum*.

2. Materials and methods

2.1. Plant material

L. densiflorum (Guss.) Kuntze was collected in the Sebkha Sidi El Hani region in the center of Tunisia (saline area). The plant material was identified by Prof Abderrazek Smaoui (Botanist in the Laboratory of Extremophile Plants) and a voucher specimen (PLM30) was deposited at the Herbarium of the Laboratory. Plants were air-dried, ground and stored at 4 °C until extraction.

2.2. Bioassay-guided fractionation and isolation of biomolecules

2.2.1. Extraction

The powder of *L. densiflorum* (800 g) was extracted under reflux with portions of increasingly diluted aqueous ethanol (95, 90, 85 and 80%) for 1 h 30 min each time. The extracts were filtered and pooled, evaporated, lyophilized, and then evaluated for cytotoxicity and anti-viral activity.

2.2.2. Fractionation

The extract was fractionated on a silica gel column (9 cm diam. × 55 cm height) eluted with EtOAc/EtOH/H_2O (100/16.5/13.5 v/v/v) to obtain 69 fractions (NB1 to NB69).

According to their elution by TLC, sub-fractions have been pooled as follow: NB 1 to NB10 (F1); NB11 to NB17 (F2); NB18 to NB27 (F3); NB28 to NB33(F4); NB34 to NB38 (F5); NB 39 to NB 49 (F6); NB 50 to NB 63 (F7); NB 64 (F8); NB 65 + NB 66 (F9); NB 64 (F10); NB68 + NB69 (F11); NB70(F12).

Subfractions F9 (1 g), F10 (900 mg), F11 (800 mg), and F12 (12 g), which were the most active, were pooled and submitted to a second fractionation on a silica gel column (2 cm diam. × 95 cm height) eluted with EtOAc/MeOH/H_2O (100:10:4) then (100:10:7) to yield seven new sub-fractions (Fr-A to Fr-G). The sub-fraction F2 was separated with Low pressure liquid chromatography with ETOAC/MeOH (25/1) to recover six sub-fractions from F2-A to F2-F.

The sub-fractions Fr-A, Fr-B, Fr-C, F2-A and F2-B were the most active against HSV-1. They were fractionated by preparative HPLC (High Performance Liquid Chromatography) using an Agilent 1100 system. Methods developed on an analytical column (Zorbax Eclipse C18, 250 × 4.6 mm) were transferred to a preparative column (Inertsil prep-ODS, 250 × 10 mm, 5 μm particle size) using multiple wavelength detectors and an automatic fraction collector. Chromatograms were monitored at 280, 254, 210 and 300 nm.

2.3. Chemical analysis

The sub-fractions were analyzed by thin layer chromatography (TLC) performed on silica gel (60 F254, 0.25 mm pre-coated TLC plates Silicycle, Québec, Canada). Visualization was made by spraying the plates with NP-PEG (natural products reagent/polyethylene glycol) and observing them under UV light (365 nm).

Structural identification of the compounds was based on phytochemical tests and spectroscopic analyses (UV and NMR) by direct comparison of data with authentic samples or reference data. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance spectrometer at 400 MHz (^{1}H) and 100 MHz (^{13}C), equipped with a 5 mm QNP probe. Elucidations of chemical structures were based on the analysis of ^{1}H, ^{13}C, COSY, HMBC, HSQC and DEPT-135 experiments. Signals are reported as m (multiplet), s (singlet), d (doublet), t (triplet), dd (doublet of doublet), dt (doublet of triplet), and br s (broad singlet) and coupling constants *J* are reported in hertz (Hz). UV absorption spectra were recorded with an Agilent 8453 diode-array spectrophotometer. High resolution electrospray ionization mass spectra were obtained in a positive mode on an Applied Biosystems/MDS Sciex QSTAR XL Q-TOF MS system. HPLC-APCI MS (negative mode) spectra were obtained on an Agilent 1100 series system consisting of a degasser, a quatpump, an automatic injector, a temperature-controlled column compartment, a diode-array detector and a mass selective detector Agilent G1946 VL model equipped with an APCI source.

2.4. Evaluation of cytotoxicity

The cell viability was evaluated by the 7-hydroxy-3-*H*-phenoxazin-3-one 10-oxide (resazurin) method [26]. Briefly, Vero cell cultures were prepared in 96-well plates, at a density of 5×10^3 cells per well. After a 24 h incubation period, at 37 °C in a humidified 5% CO_2 atmosphere, the culture medium was removed and cells were treated with 200μL/well of samples at different concentrations prepared in the culture medium (1000, 500, 250, and 125 μg/mL for fractions). Untreated controls were performed by the addition of 200 μL of culture medium. Then, the cells were incubated for 96 h. The medium was removed and 100 μL of resazurin solution (1 mg/mL) was added. The plates were re-incubated for 2 h. After that, the resazurin solution was removed. The fluorescence was read 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. The results are expressed as the concentration inhibiting fifty percent of cell growth (IC_{50}).

2.5. Virucidal effects of extracts or compounds on HSV-1

A viral suspension was incubated with fractions (6 μg/mL) at 37 °C for 1 h [27]. The mixture was then used to infect Vero cells for 1 h. After that, it was removed; the wells were washed with PBS (pH 7.4) and incubated with methylcellulose/medium 0.5% for 72 h. After 3 days, the medium was aspirated and the infected cells were fixed with 10% formaldehyde, stained with 1% crystal violet, and the number of plaques was counted [28]. The wells overlaid with medium without the test sample were used as the controls. The percentage of inhibition of lysis plaque formation (Inhibition percent IP) was calculated as follows: [(mean number of plaques in control) − (mean number of plaques in sample)] × 100/mean number of plaques in control.

3. Results

3.1. Bio-guided fractionation

The crude hydro-ethanolic extract of *L. densiflorum* shoot that showed a strong inhibitory activity against HSV-1 was non-toxic towards Vero cells (Table 1) and was rich in

Table 1
Cytotoxic and anti-HSV-1 activities of *L. densiflorum* hydro-ethanolic extract (HE) and its fractions.

	Yeald (%)	IC_{50} (μg/mL)	IP (%)
HE		>500	50
F1	0.1	>500	26
F2	0.05	>500	73
F3	0.09	>500	33
F4	0.1	>500	40
F5	0.15	>500	26
F6	0.2	>500	35
F7	0.016	>500	42
F8	0.09	>500	47
F9	0.16	>500	72
F10	0.15	>500	82
F11	0.14	>500	80
F12	0.03	>500	71
Acyclovir		>500	0

IC_{50}: Concentration inhibiting 50% of cell growth (μg/mL).
IP: Virucidal activity expressed as the percent inhibition of lysis plaques in comparison with infected untreated cells.

flavonoids (yellow bands in TLC) and phenolic acids (blue bands) (Fig. 1). Fractionation of the extract over silica gel gave 12 fractions, which were all non-cytotoxic while showing some anti-viral activity (Table 1). In particular, the sub-fractions F2, F9, F10, F11 and F12 were the most (actives) active with plaque inhibition activities (IP) of 73, 73, 83, 81, and 71% respectively. In addition, F9 to F12 contained polar flavonoids whereas F2 contained less polar flavonoids (Fig. 2). Subsequently, F9, F10, F11 and F12 were regrouped in a single fraction (Fr) and fractionated, resulting in seven new sub-fractions: Fr-A to FrG. These fractions contained different classes of flavonoids with various polarities (yellow to orange and green bands, Fig. 3). The most anti-viral activity was observed with Fr-A (IP = 100%), Fr-B (IP = 75%) and Fr-C (IP = 70%) (Table 2). As for the sub-fraction F2, six new fractions (F2-A to F2-F) were obtained after chromatography. They present different classes of molecules such as flavonoids (yellow bands), and phenolic acids (blue bands) (Fig. 4). Among them, F2-A (75%) and F2-B (80%) were the most actives against HSV-1 (Table 2).

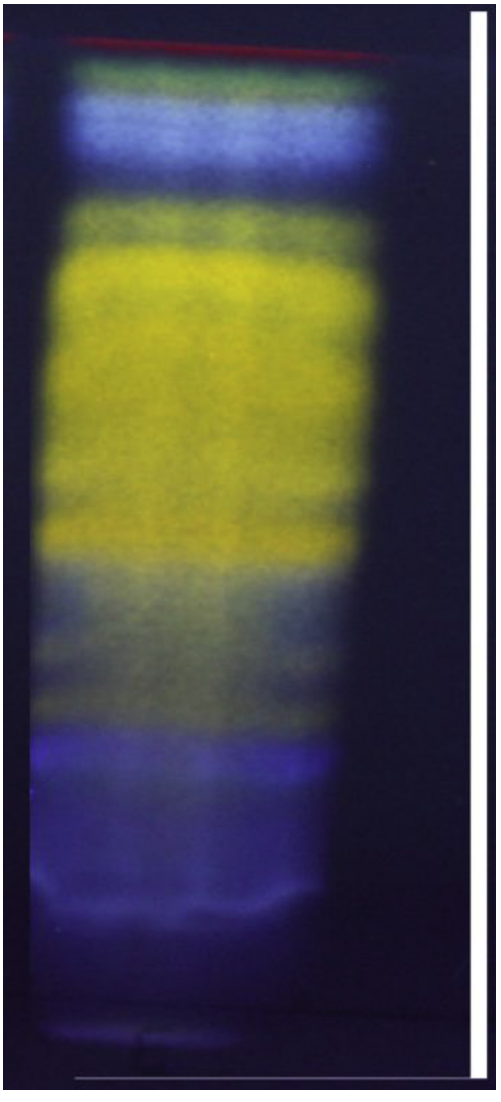

Fig. 1. CCM of hydro-ethanolic extract of *L. densiflorum*.

All active fractions were analyzed with LC-MS and injected to preparative HPLC to obtain the isolated compounds. Compounds **1** and **2** were obtained from sub-fraction Fr-A and compounds **3** and **4** were isolated from sub-fraction Fr-B. Compound **5** was recuperated from sub-fraction Fr-C. Compound **6** was purified from F2-A and compound **7** from F2-B. Isolated compounds **1**–**7** were characterized and identified based on their NMR spectra compared to literature. Indeed, compound **1** was identified as gallic acid [29]. Compounds **2**–**5** belong to the flavonoid group and were identified, respectively, as epigallocatechine gallate (**2**), mixture of myricetin 3-*O*-L-arabinofuranoside with myricetin 3-*O*-*R*-L-rhamnopyranoside (**3**), quercetin 3-*O*-L-rhamnoside (**4**) and dihydrokaempferol (**5**) [30–34]. Compounds **6** and **7**, which were lignans, were identified as pinoresinol and *trans*-*N*-feruloyl tyramine [35,36].

3.1.1. Compound **1**: gallic acid

^{1}H NMR 6.91 (2H, s, H-2, 6), ^{13}C NMR: 121.0 (C-1), 109.0 (C-2 and C-6), 145.9 (C-3 and C-5), 138.3 (C-4), and 168.0 (C-7).

3.1.2. Compound **2**: epigallocatechin

^{1}H NMR, 3.00–3.16 (m, 2H, CH_2), 4.93 (m, CH), 5.29 (m, 1H, CH), 5.81 (d, 1H, CH arom, J = 4 Hz), 5.91 (d, 1H, CH arom, J = 4 Hz), 6.40 (s, 2H, CH arom), and 6.80 (s, 2H, CH arom); ^{13}C NMR spectral data : 165.69 (1C, C=O), 157.3 (1C, C–OH arom), 156.32 (1C, C–O arom), 155.04 (1C, C–OH arom), 146.50 (1C, C–OH arom), 145.83 (1C, C–OH arom), 139.07 (1C, C–OH arom), 133.10 (1C, C–OH arom), 128.90 (1C, C arom), 119.36 ((1C, C arom), 109.00 (2C, CH arom), 105.3 (2C, CH arom), 97.78 (1C, C arom), 95.77 (1C, CH arom), 94.39 (1C, CH arom), 77.32 (1C, HC–O), 69. 31 (1C, HC-O), and 29.27 (1C, CH_2).

3.1.3. Compound **3** (mixture): myricetin 3-O-R-L-rhamnopyranoside

^{1}H NMR, δ 0.95 (3H, d, 6.4 Hz, H-6″), 3.3 (1H, t, 9.2 Hz, H-4″), 3.55 (1H, dq, 9.2, 6.4 Hz, H-5″), 3.78 (1H, dd, 9.2, 3.2 Hz, H-3″), 4.21 (1H, dd, 3.2, 1.8 Hz, H-2″), 5.31 (1H, d, 1.8 Hz, H-1″), 6.19 (1H, d, 2.3 Hz, H-6), 6.35 (1H, d, 2.3 Hz, H-8), 6.94 (2H, s, H-2′, 6′); ^{13}C NMR, δ: 17,70 (C-6″), 71,90 (C-2″), 72.06 (C-5″), 72.12 (C-3″), 73.36 (C-4″), 94.7 (C-8), 99.81 (C-6), 103.64 (C-1″), 105.58 (C-10), 109.59 (C-2′,6′), 121.93 (C-1′), 136.33 (C-3), 137.9 (C-4′), 146.86 (C-3′,5′), 158.51 (C-9), 159.43 (C-2), 163.07 (C-5), 165.0 (C-7), 159.43 (C-2), 179.68 (C-4); FAB-MS (positive), m/z 465 $[M+H]^+$

3.1.4. Compound **3** (mixture): myricetin 3-O-L-arabinofuranoside (betmidin)

^{1}H NMR (CD_3OD); 3.54 (2H, m, CH2 AB sys, J = 7, J = 12.4), 3.93 (1H, m, CH), 3.94 (1H, td, CH, J = 7 Hz), 4.36 (1H, m, CH), 5.47 (1H, d, CH), 6.18 (1H, d, CH, J = 2 Hz), 6.36 (1H, d, CH, J = 2 Hz), 7.14 (2H, s, CH). ^{13}C NMR, δ 179.9 (s, C-4), 166.1 (s, C-7), 163.0 (s, C-5), 159.3 (s, C-2), 158.5 (s, C-9), 146.8 (s, C-3′, 5′), 137.9 (s, C-4′), 134.8 (s, C-3), 122.0 (s, C-1′), 109.4 (d, C-2′, 6′), 109.4 (d, C-1″), 105.5 (s, C-10), 99.9 (d, C-6), 94.8 (d, C-8), 88.0 (d, C-4″), 83.2 (d, C-2″), 78.7 (d, C-3″), and 62.5 (t, C-5″); ESI/MS (neg.) m/z 449 ([M–H]), ESI/MS (pos.) m/z 473 ($[M+Na]^+$), 451 ($[M+H]^+$).

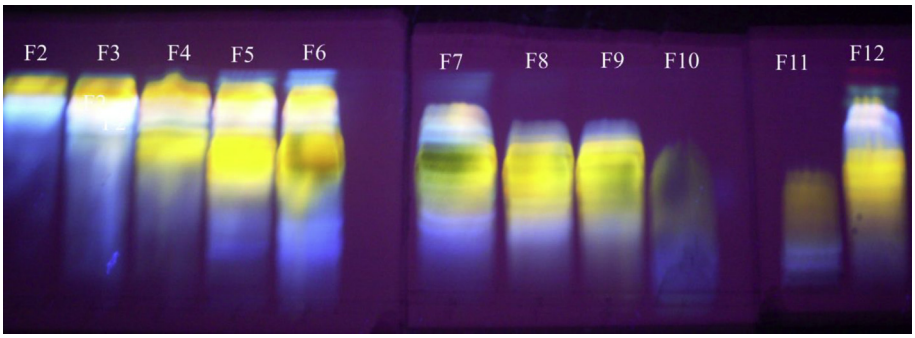

Fig. 2. CCM of fractions obtained from the hydro-ethanolic extract of *L. densiflorum* hydro-ethanolic extract.

*3.1.5. Compound **4**: quercetin 3-O-L-rhamnoside (Quercitrin)*

^{1}H NMR (300 MHz, δ: 6.24 (1H, d, 2.3 Hz, H-6), 6.41 (1H, d, 2.3 Hz, H-8), 7.28 (1H, d, 2.4 Hz, H-2′), 6.86 (1H, d, 7.8 Hz, H-5′), 7.25 (1H, dd, 2.4,8 Hz, H-6′), 5.26 (1H, d, 1.6 Hz, H-1″), 3.98 (1H, m, H-2″), 3.10–3.53 (3H, m, H-3″, 4″, 5″), 0.82 (3H, d, 6.4 Hz, CH_3); ^{13}C NMR (100 MHz,) δ: 158.1 (C-2), 134.4 (C-3), 180.2 (C-4), 160.8 (C-5), 98.5 (C-6), 164.4 (C-7), 93.4 (C-8), 157.2 (C-9), 104.5 (C-10), 120.1 (C-1′), 115.8 (C-2′), 144.9 (C-3′), 148.1 (C-4′), 115.3 (C-5′), 120.9 (C-6′), 101.6 (C-1″), 70.7 (C-2″), 70.4 (C-3″), 71.2 (C-4″), 70.1 (C-5″), and 17.2 (C-6″).

*3.1.6. Compound **5**: dihydrokaempferol*

^{1}H NMR, 5.35 (d, 1H, CH, J = 8 Hz), 5.95 (d, 1H, CH, J = 8 Hz), 5.81 (d, 1H, CH arom, J = 4 Hz), 6.84 (d, 1H, CH arom, J = 4 Hz), 6.74 (d, 2H, CH arom, J = 8 Hz), and 7.27 (d, 2H, CH arom, J = 8 Hz). ^{13}C NMR spectral data : 71.76 (1C, C–OH), 83.18 ((1C, C–O), 95.38 (1C, CH arom), 95.39 (1C, C arom), 100.69 (1C, CH arom), 115.22 (2C, CH arom), 127.89 (1C, C arom), 129.90 (2C, C arom), 158.05 (1C, C–OH arom), 162.69 (1C, C arom), 163.63 (1C, C–OH arom), 167.40 (1C, C–OH arom), and 198.03 (1C, C=O).

*3.1.7. Compound **6**: pinoresinol*

^{1}H NMR spectral data: d 3.14 (m, 2H, CH), 3.85 (s, 6H, 2. CH_3O), 3.91 (s, 6H, OCH_3), 3.82 and 4.23 (m, 4H AB syst), 4.71 (d, 2H), 6.76 (d 2H, J = 8.1 Hz), 6.81 (dd, 2H, J = 1.8 Hz, J = 1.8 Hz), and 6.95 (d, 2H, J = 1.8 Hz).

^{13}C NMR spectral data: δ 55.36 (2C, CH); 56.39 (2C, CH_3O (72), 72.6 (2C, CH2), 87,51 (2C, CH), 110,95 (2C, CH arom), 116,06 (2C, CH arom), 120.05 (2C, CH arom), 133,78 (2C, C arom), 147,3 (2C, C–O arom), and 149 (2C, C–0H arom). EIMS (direct probe, *m/z* rel. int.): 358 (70), 327 (11), 235 (15), 205 (30), 163 (38), 151 (100), and 137 (72).

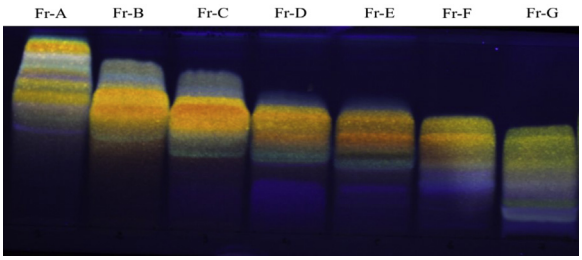

Fig. 3. CCM of different sub-fractions obtained from the Fr fraction.

*3.1.8. Compound **7**:* N-trans-*feruloyl tyramine*

^{1}H NMR 2.65 (m, 2H, CH_2), 3.33 (m, 2H, CH_2), 3.78 (s, 3H, OCH_3), 6.41 (d, 1H, CH), 6.68 (d, 2H, CH, J = 8.1 Hz), 6.78 (d, 1H, CH, J = 8.1), 6.97 (dd, 1H, CH, J = 8.3 Hz), 7.0 (d, 2H, CH, J = 8.3HZ), 7.1 (d, 1H, CH, J = 1.3 Hz), and 7.30 (d, 1H, CH, J = 15.7 Hz).^{13}C NMR spectral data: 34,45 (1C, CH2); 40.71 (C, CH2), 55.56 (1C, CH3), 110.76 (1C, CH arom), 115.16 (2C, CH arom), 115,68 (1C, CH), 119.03 ((1C, CH), 121.057 (1C, CH), 126.44 (1C), 129.52 (2C, CH), 129.56 (C), 138.94 (1C, CH arom), 147.86 (1C), 148.26 (1C), 155.67 (1C), and 165.37 (1C, C=O).

3.2. Cytotoxic and anti-herpes activities of the isolated compounds

The isolated compounds were, separately, evaluated regarding their *in vitro* ability to inhibit HSV-1 infection (Table 3) and their effect on Vero cells. Results showed that at 50 μg/mL all molecules were no toxic for Vero cells. Compound **1** and **2** showed a strong antiviral activity with an IP of 100%. Pinoresinol (compound **6**) and *N-trans*-feruloyl tyramine (compound **7**) have a moderate capacity

Table 2
Cytotoxicity and anti-HSV-1 activity of *L. densiflorum* Fr and F2 sub-fractions.

Sub-fractions	Masse (mg)	IC_{50} (μg/mL)	IP (%)
Fr fractions			
Fr-A	45	354	100
Fr-B	100	220	75
Fr-C	50	>500	70
Fr-D	32	>500	59
Fr-E	53	>500	60
Fr-F	140	>500	25
Fr-G	270	>500	18
F2 sub-fractions			
F2-A	138	>500	75
F2-B	218	>500	80
F2-C	133	>500	25
F2-D	100	>500	32
F2-E	41	>500	12
F2-F	33	>500	10
Acyclovir		>500	0
HE		>500	48

IC_{50}: Concentration inhibiting 50% of cell growth (μg/mL).
IP: Virucidal activity expressed as the percent inhibition of lysis plaques in comparison with infected untreated cells.

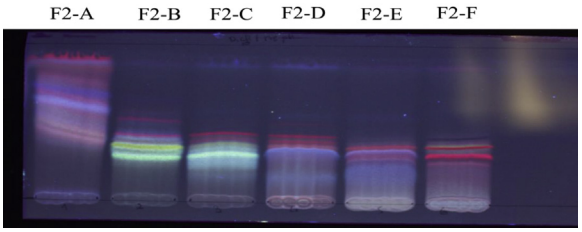

Fig. 4. CCM of sub-fractions recuperated from the F2 fraction.

with an IP of 26%. However, quercetrin (compound **4**), the mixture of myricetin 3-*O*-α-rhamnopyranoside and myricetin 3-*O*-L-arabinofuranoside (compound **3**) and dihydrocaempferol (compound **5** had no effect on HSV-1 infection.

4. Discussion

In a study that defines "green" solvents, it was shown that simple alcohols (ethanol, methanol) or alkanes (heptane, hexane) are more environmentally preferable solvents compared to dioxane, acetonitrile, acids, formaldehyde and tetrahydrofuran [37]. Furthermore, when comparing solvent mixtures it was shown that ethanol–water or methanol-water mixtures are environmentally favorable compared to pure alcohol or propanol–water mixtures. The use of solvent mixtures was found to enhance the extraction yields by improving the solubility and increasing interaction of the targeted analyte with the extraction solvent [38,39]. In a dual mixture, one solvent could improve the solubility of the analyte, while the other would enhance the analyte desorption. In this context, water is usually important to aid breaking matrix and matrix–analyte (hydrogen) bonding. For instance, the water/ethanol mixture was possibly the most suitable solvent system for the extraction of sage polyphenols due to the different polarities of the bioactive constituents, and the acceptability of this solvent system for human consumption. Moreover, it improves the recoveries of catechins from tea leaves and grape seeds and also phenolic compounds from grapes [40,41].

In our previous screening for biological activity of plants from Tunisian salt flat, hydro-ethanolic extract of *L. densiflorum* exhibited an interesting anti-viral activity against HSV-1. For this reason, a bio-guided fractionation was carried out in order to identify the compounds responsible for this activity. Preliminary TLC analysis of the crude extract revealed the presence of phenolic compounds (flavonoids) in the plant which appeared as yellow spots, using the (NPPEG) reagent. The intensity of these yellow spots seemed to be correlated with the *in vitro* antiviral activity. Samples derived from the extract by submission to a succession of silica chromatographic columns were screened and the most promising anti-herpes activity was detected for six sub-fractions. Finally, seven compounds were isolated after an additional preparative HPLC step and were characterized by NMR. All compounds were identified as phenolic compounds. They are aromatic compounds with one or more hydroxyl groups attached to the aromatic ring. They belong to three groups: phenolic acids (gallic acid (**1**)), flavonoids (epigallocatechine gallate (**2**), quercitrin (**3**), mixture of myricetin 3-*O*-α-rhamnopyranoside + myricetin 3-*O*-L-arabinofuranoside (**4**) and dihydrokaempferol (**5**)) and the group of lignans (pinoresinol (**6**) and *N-trans*-ferulolyl tyramine (**7**)).

Table 3
Cytotoxic and anti-HSV-1 activities of phenolic compounds isolated from *L. densiflorum*.

Compound	IC_{50} (μg/mL)	IP (%)
Compound **1**	>100	100
Compound **2**	>100	100
Compound **3**	>100	NA
Compound **4**	>100	NA
Compound **5**	>100	NA
Compound **6**	>100	26
Compound **7**	>100	26

NA: Not active.
IC_{50}: Concentration inhibiting 50% of cell growth (μg/mL).
IP: Virucidal activity expressed as the percent inhibition of lysis plaques in comparison with infected untreated cells.

During the last decade, the potential benefits of flavonoids and phenolic acids in the control of different human diseases were increasingly investigated [25]. Phenolic compounds may assist regulatory metabolic processes against diseases by contributing, along with antioxidant vitamins and enzymes, to the total antioxidant defense system of the human body. Polyphenols possess ideal structural chemistry for free radical-scavenging activities, and have been shown to be more effective than vitamins E and C on a molar basis [42]. In addition, they possess diverse biological activities, such as antiulcerant, anti-inflammatory, anti-diabetic, anti-viral, cytotoxic and anti-tumor activities [24]. Among the isolated polyphenols isolated from *L. densiflorum*, gallic acid showed a powerful anti-herpes potential (IP = 100% at 50 μM). In accordance with the results of Kratez et al. [43], gallic acid caused complete inactivation of the HSV-1 virus in the absence of cells at low concentrations. Moreover, it is able to inhibit the virus adsorption and penetration to the cells (IC_{50} value of 23.9 μM) [43].

Epigallocatechin gallate (EGCG) is an ester of epigallocatechin and gallic acid and inactivates HSV-1 viruses at 50 μM by a direct effect on the virion. In fact, an incubation of EGCG with Vero cells for 48 h prior to infection with HSV-1 does not reduce HSV production [44]. Interestingly, a wide array of viruses of both veterinary and clinical importance was inhibited by vegetal alkyl gallates, including members from *Poxiviredea*, *Asfarviridea* and *Othomixviridea* [45].

In general, phenolic compounds are reported to have high affinity for proteins, forming unstable complexes. Therefore, the anti-viral effects of flavonoids and phenolic acids in the early stage of the HSV cycle could possibly be attributed to interactions with viral envelope glycoproteins [46], or inhibition of viral polymerase with consequent interference of viral genome synthesis [47]. However, in this study, the mixture of myricetin 3-*O*-α-rhamnopyranoside + myricetin 3-*O*-L-arabinofuranoside, quercitrin and dihydrokaempferol had no virucidal effect

on HSV-1. It is possible that the simultaneous presence of both myricetin molecules prevented the activity. The existence of a glycosyl group at C3 might also block the active site for anti-viral activity. In the case of dihydrokaempferol, by comparison with its structural analog kaempferol which exhibits powerful anti-herpetic activity ($IC_{50} = 0.4$ μg/mL) [48], the absence of potential anti-herpes for this molecule could be explained by the presence of a double link between C2 and C3 in its structure. In fact, according to Limem et al. [49] the number of the hydroxyl groups, compounds glycosylation and the alkene bond between C2 and C3 of phenolic compounds can all influence their biological activities.

Lignans constitute a large class of secondary metabolites produced by oxidative dimerization of two phenylpropanoid units. They possess anticancer and anti-viral properties and specifically inhibit certain enzymes and mediators involved in inflammation and immunity processes [50]. Results showed that pinoresinol, one of the structurally simplest lignans and *N-trans*-ferulolyl tyramine displayed a moderate virucidal activity (IP = 26% at 50 μg/mL) by acting directly on the glyproteins structure of the virus.

5. Conclusion

The *Limonium* genus has been reported to contain phenolic acids, flavonoids, lignans and phenolic glycosides. In accordance with these accounts, this is the first report of a green extraction and phenolic compound isolation from the halophyte *L. densiflorum*. The anti-viral activity, measured, suggests that this medicinal halophyte may be a promising species for the pharmaceutical industry.

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References

[1] D. Christopher, A. Conrady Douglas, J. Drevets Daniel, J. Carr, J. Neuroimmunol 220 (2010) 1–9.
[2] B. Roizman, D.M. Knipe, R.J. Whitley, Herpes Simplex Viruses. Fields Virology, 5th ed., 2007, pp. 2501–2601.
[3] B. Bhuwan, K. Mishra Vinod, J. Tiwari, Eur. J. Med. Chem. 46 (2011) 4769–4807.
[4] A. Hassan, S. Rahman, F. Deeba, S. Mahmud, J. Med. Plants. Res. 3 (1) (2009) 020–023.
[5] D.G.I. Kingston, J. Nat. Prod. 74 (2011) 496–511.
[6] D.J. Newman, G.M. Cragg, J. Nat. Prod. 70 (3) (2007) 461–477.
[7] K. Hostettmann, A. Marston, J.L. Wolfender, Phytochemistry of Plants Used in Traditional Medicine, Clarendon Press, Oxford, 1995, pp. 17–45.
[8] F. Chemat, Paris: Dunod. (2011) pp. 169–197.
[9] R.K. Henderson, C.J. Gonzalez, D.J.C. Constable, S.R. Alston, G.G.A. Inglis, G. Fisher, J. Sherwood, S.P. Binks, A.D. Curzons, Green. Chem. 13 (2011) 854.
[10] L. Moity, M. Durand, A. Benazzouz, C. Pierlot, V. Molinier, J.M. Aubry, Green. Chem. 14 (2012) 1132.
[11] T. Michel, E. Destandau, C. Elfakir, Food Chem. 126 (2011) 1380–1386.
[12] J. Kronholm, K. Hartonen, M.L. Riekkola, Trends Anal. Chem. 26 (2007) 396–412.
[13] J. Leveque, G. Cravotto, Chimia 8 (2006) 313–320.
[14] P. Scammells, J. Scott, R. Singer, J. Chem. 58 (2005) 155–169.
[15] S. Ferreira-Dias, D.G. Valente, J.M.F. Abreu, Grasas y Aceites 54 (2003) 378–383.
[16] F.M. Kerton, R. Marriott, Sales and Customer Care, Royal Society of Chemistry, Thomas Graham House, Science Park, Milton Road, Cambridge, CB4 0WF, UK, 2013.
[17] F. Chemat, M. Abert Vian, G. Cravotto, Int. J. Mol. Sci. 13 (2012) 8615–8627.
[18] M. Akizuki, T. Fujii, R. Hayashi, Y. Oshima, J. Biosci. Bioeng. 117 (1) (2014) 10–18.
[19] H.N. Le Houerou, Options Mediterr. Serie B: Etudes Reche. 10 (1995) 396.
[20] G. Pottier-Alapetite, Flore de la Tunisie. Angiospermes-Dicotyledones: Apetalesdialypetales- gamopetales, 1981, p. 1190.
[21] R. Ksouri, W. Megdiche-Ksouri, I. Jallali, A. Debez, C. Magné, I. Hiroko, C. Abdelly, Crit. Rev. Biotech. (2011) 1–38.
[22] K. Yuh-Chi, L. Lie-Chwen, T. Wei-Jern, C. Cheng-Jen, K. Szu-Hao, H. Yen-Hui, Antimicrob. Agents Chemother. 46 (9) (2002) 2854–2864.
[23] M. Faten, L. Jean, P. André, A. Chedly, K. Riadh, S Afr. J. Bot. 92 (2014) 65–72.
[24] M. Faten, K. Riadh, F. Hanen, M. Wided, T. Najla, A. Chedly, J. Med. Plants Res. 5 (31) (2011) 6719–6730.
[25] M. Saxena, D.J. Saxena, D.A. Pradhan, J. Pharm. Sci. Rev. Res. 16 (2) (2012) 130–134.
[26] J.I. O'Brien, T. Wilson, F. Orton, K. Pognan, Eur. J. Biochem. 267 (2000) 5421–5426.
[27] F. Camargo, D.A.G. Cortezb, T. Ueda-Nakamurac, C.V. Nakamurac, B.P. Dias Filho, Phytomed 15 (2008) 202–208.
[28] S. Rechter, T. Konig, S. Auerochs, S. Thulke, H. Walter, H. Dornenburg, C. Walter, M. Marschall, Antivir. Res. 72 (2006) 197–206.
[29] O.A. Eldahshan, Curr. Res. J. Biol. Sci. 3 (1) (2011) 52–55.
[30] I. Peres, S. Rocha, M.C. Pereira, M. Coelho, M. Rangel, G. Ivanova, Polymer 82 (2010) 861–866.
[31] S. Chung, Y.C. Kim, Y. Takaya, K. Terashima, M. Niwa, J. Agri. Food Chem. 52 (2004) 4664–4668.
[32] C.J. Shen, C.K. Chen, S.S. Lee, J. Chin. Chem. Soc. 56 (2009) 1002–1009.
[33] O.D.P. Philipe, G.D. Marie, Q. Gilbert, M. Anne Marie, Phytochemistry 47 (1997) 1171–1173.
[34] J. Kang, Z. Li, T. Wu, S. Jensen, G.S. Schauss, A.G.X. Wu, Food Chem. 122 (2010) 610–617.
[35] M. Gan, Y. Zhang, S. Lin, M. Liu, W. Song, J. Zi, C.Y. Yong, X. Fan, J. Shi, J. Hu, J. Sun, N. Chen, J. Nat. Prod. 71 (2008) 647–654.
[36] C.H. Juliana, M.X.L. Lucia, Molecules 15 (2010) 9462–9472.
[37] A. Mustafa, Ch. Turner, Anal. Chim. Acta 703 (2011) 8–18.
[38] P. Arapitsas, P.J.R. Sjöberg, C. Turner, Food Chem. 109 (1) (2008) 219–226.
[39] M. Mukhopadhyay, P. Panja, Int. J. Food Eng. 4 (8) (2008).
[40] M. Palma, Z. Pineiro, C.G. Barroso, J. Chrom A 968 (1–2) (2002) 1–6.
[41] Z. Pineiro, M. Palma, C.G. Barroso, J.Chrom A 1026 (1–2) (2004) 19–23.
[42] C. Rice-Evans, N. Miller, G. Paganga, Trend. Plants. Sci. 2 (4) (1997) 152–154.
[43] J.M. Kratz, C. Regina, A.F. Diese, J. Kolling, P.C. Leal, C.S. César Cláudio, Y. Rosendo Augusto, R.J. Nunes, E. Trybala, T. Bergström, I. Frugulhetti, M.B.C. Regina, C.M. Oliveira Simões, Mem. Instit. Oswal. Cruz 103 (5) (2008).
[44] C.E. Isaacs, W. Xu, G. Merz, S. Hillier, L. Rohan, G.Y. Wen, Antimicrob. Agents Chemother. (2011) 5646–5653.
[45] C. Hurtado, M.J. Bustos, P. Sabina, M.L. Nogal, A.G. Granja, M.E. Gonzalez, P.P. Gonzalez, Y. Revilla, A.L. Carrascosa, Antiv. Ther. 13 (2008) 909–917.
[46] E. Haslam, J.Nat. Prod. 59 (2) (1996) 205–215.
[47] P. Schnitzler, A. Schuhmacher, A. Astani, J. Reichling, Phytomedicine 15 (9) (2008) 734–740.
[48] M. Amoros, C.M. Simoes, L. Girre, F. Sauvager, M. Cormier, J. Nat. Prod. 55 (1992) 1732–1740.
[49] I. Limem, E. Guedon, A. Hehn, F. Bourgaud, L. Chekir Ghedira, J.M. Engasser, M. Ghoul, Process. Biochem. 43 (2008) 463–479.
[50] J.L. Ríos, R.M. Giner, J. M Prieto, Stud. Nat. Prod. Chem. 26 (2002) 183–292.