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Full Length Research Paper

In vitro antiviral activities of Jrani caprifig latex and its related terpenes

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The aim of this study was to search for new antiviral agents from Tunisian Jrani caprifig latex (*Ficus carica* L., Moraceae). Terpenes and coumarins were identified by gas chromatography–mass spectrometry (GC-MS) analysis in hexane and hexane ethyl-acetate (1:1, v/v) extracts, and used in experiments to test their influence on a series of viruses, namely *Herpes simplex* (HSV-1), *Adenovirus* (ADV) and *Echovirus* type 11 (ECV-11). To evaluate the capacity of the extracts inhibition of viruses replication cycles by preventing their adsorption and their penetrations in the cells (by interaction with the cellular receivers designed for these viruses) or by inhibition of their intracellular replication or by virus inhibition after a direct contact under microscopic observation of cytopathic effect (CPE). Extracts inhibited virus multiplication in tested techniques at the concentrations of 19.5 and 39 µg/ml, respectively. All extracts had no cytotoxic effect on Vero cells at all tested concentrations. In conclusion, some compounds of Jrani latex which possess antiviral activities may be due to the high level of Triterpens. Their mode of action against the tested viruses was found to be at all stages of multiplication, suggesting the potential use of this compound for treatment of the infection caused by these viruses.

Key words: *Ficus carica*, caprifig, latex, HSV-1, HSV-2, ECV-11, ADV, coumarins, terpens.

INTRODUCTION

The use of herbs and medicinal plant as the first medicines is a universal phenomenon. Every culture on the earth, through written or oral tradition, has relied on the vast variety of natural chemistries found in plants for their therapeutic properties. All drugs from the plant are substances with a particular therapeutic action extracted from plants (Serrentino, 1991). The usage of herbal plants as traditional health remedies is the most popular for 80% of the world population in Asia, Latin America and Africa and is reported to have minimal side effect (Doughari, 2006). In this study Jrani caprifig variety which belongs to the genus *Ficus carica* L. (Moraceae) has been selected. Unlike common figs, the caprifig is a fig tree which produces both male and female flowers and is used to fertilize the female trees of the species. The name caprifig is derived from caprificus (or “goat fig” in Italian) (Storey et al., 1977) produces three crops of syconia. These are known by their Italian terms, profichi,

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Abbreviations: CPE, Cytopathic effect; **PBS,** phosphate buffered saline; **MEM,** minimum essential medium; **FBS,** fetal bovine serum; **HSV-1,** herpes simplex virus type 1; **CPE,** cytopathic effect.

mammoni and mamme. The fig is a deciduous tree, which probably originated in Western Asia, and spread to the Mediterranean is commonly known as fig tree (Tous and Ferguson, 1996). Its products are widely used both as a food and in traditional medicine in the Mediterranean; the roots are used in treatment of leucoderma and ringworms and its fruits which are sweet, have antipyretic, purgative, aphrodisiac properties and have shown to be useful in inflammations and paralysis (Kirthikar and Basu, 1996). *F. carica* is claimed to be useful in liver and spleen disorders, to cure piles and in treatment of gout. The leaf decoction affected lipid catabolism in hyperglyceridemic rats (Perez et al., 1999). Several phytochemical investigations of *F. carica* leaves have been published, but with no biological data. Athnasios et al. (1962) have isolated prosalen, β-sitosterol and Bergapten. Others have isolated triterpenoids (Abu-Mustafa et al., 1964). *F. carica* latex released on picking the fruits is used to treat skin tumors and warts (Ghazanfar, 1994). The first scientific investigation of the activity of fig latex was done by Ullman et al. (1945, 1952). The dialysate of the latex contained the active ingredient. Although, isolation of the active components was not pursued further, some pharmacological work was reported by Ullman (1952) and Ullman et al. (1952). Fig latex has also been tested for its anthelmitic activity (De-Amorin et al., 1999).

In this modern age it is very important to provide scientific proof to justify the various medicinal uses of herbs. Herbal drugs are prescribed widely even when their biologically active components are unknown because of their effectiveness, fewer side effects and relatively low cost (Valiathan, 1998). However, we are not aware of a satisfactory remedy for serious viral diseases and search for effective and safe drugs.

In this paper we describe the identification of a potent antiviral terpenes and coumarins from Tunisian caprifig latex, Jrani variety.

MATERIALS AND METHODS

Latex collection

The Jrani caprifig latex was collected from unripe inedible fig fruit growing in Mesjed Aissa agricultural field located in the central cost of Tunisia. The latex was held in ice during the period of collection. The identification of this variety was established by Pr. Massoud MARS, professor of arboriculture at the high school of horticulture of Chott Meriam Sousse, Tunisia, Department of Agriculture, and Arboriculture, the code collection of the tree is JR1. This fig fruit was cat open from its top then slightly squeezed to collect few drops of latex directly into polyethylene centrifuge tubes, frozen immediately in Dry-Ice and maintained in frozen state at –30°C until the analyses were performed.

Extracts preparation

The gum, approximately 30% by weight in *F. carica* latex was removed from the aqueous solution by centrifugation in a refrigerated centrifuge (Bakemann Avanti TM30) at 15 000 rpm for 60 min at 0°C. The clear, straw-colored aqueous solution that was designated as a soluble material was frozen and stored at –30°C until required for analysis.

100 g of defrosted *F. carica* latex were macerated, (on cold) in 300 ml of methanol, over night, and repeated three times (Sarang et al., 2005), then evaporated under reduced pressure to afford (15 g) of yellow brown solid extract product 1 (P1).

The resulted residue was subjected to silica gel flash column chromatography eluted with hexane, hexane-ethyl acetate (1:1 v/v), and finally with ethyl acetate to obtain product 2 (P2), 3 (P3), and 4 (P4) of 4.10 g, 2.10 g and 0.60 g, respectively.

Furthermore, methanol was added to elute the polar compounds, which concentrated under reduced pressure, and then was re-dissolved in H_2O, and re-extracted with chloroform to afford product 5 (P5) of 3 g. All solvent were purchased from Merck, Germany.

GC-MS analysis

The analysis of Jrani caprifig latex hexane and ethyl-acetate (1/1, v/v) extracts was performed on a GC-MS HP model 1909S-433 inert MSD (Agilent Technologies, J and W Scientific Products, Palo Alto, CA, USA), equipped with an Agilent Technologies capillary DB-5MS column (30 m in length; 0.25 mm i.d.; 0.25 mm film thickness), and coupled to a mass selective detector (MSD1909S-433, ionization voltage 70 eV; all Agilent, Santa Clara, CA). The carrier gas was He and was used at 1 ml/min flow rate. The oven temperature program was as follows: 2 min at 150°C ramped from 150 to 240°C at 10°C/min and 1 min at 240°C then ramped from 240 to 280°C at 5°C/min and 15 min at 280°C. The chromatograph was equipped with a split/splitless injector used in the split mode. The split ratio was 1:100. Bis-(trimethylsilyl)-acetamide (BSTFA) (100 ml) was added to 100 ml of extract. The control of the GC/MS system and the data peak processing were carried out by means of MSDCHEM software. Identification of components was assigned by matching their mass spectra with Wiley and NIST library data, standards of the main components.

Cell and virus culture

The cell line used was *Cercopithecus aethiops* African green monkey kidney cells (Vero cell line ATCC CCL-81). The cellular lines maintained in the laboratory of transmissible diseases and bioactive substance (Faculté de Pharmacie de Monastir).

Briefly, the protocol used to obtain primary cell culture from the biopsy was as follows, each biopsy was washed three times with phosphate buffered saline (PBS) containing 200 units/ml of penicillin, 200 µg/ml of streptomycin and 0.5 µg/ml of amphotericin B. The skin was discarded the cartilage and the subcutaneous tissue were minced. Finely, the pieces of tissue were phased in 25 cm^2 cell culture flasks with just enough growth medium, that is eagle minimum essential medium (MEM) with 2 mM L-glutamine, 1% vitamins, 1% non essential amino acids, 100 units/ml of penicillin, 100 µg/ml of streptomycin, 0.25 µg/ml of amphotericin B, and 10% of fetal bovine serum (FBS) to cover the pieces of tissue when the fibroblasts had proliferated to 30 or 40% confluence, the pieces of tissue were discarded by gently shaking with PBS and again the cells were fed with 50% of used medium and 50% of fresh medium. When 80% confluence was reached, the cells were trypsinized and cultured in 150 cm^2 flasks. Once the cells had covered about 80% of the surface, they were trypsinized, centrifuged and cryopreserved. All cells were grown in MEM supplemented with 10% (FBS), 100 units/ml of penicillin, 100 µg/ml of streptomycin (4 ml), 2 mM L-glutamine (2 ml), 0.07% $NaHCO_3$,

1% non essential amino acids and vitamin solution. The cultures were maintained at 37°C in humidified 5% CO_2 atmosphere.

Herpes Simplex (herpes Viridae family) virus Type 1 (HSV-1) was obtained from the Laboratoire des Maladies Transmissibles et Substances Biologiquement actives, Faculté de Pharmacie 5000 Monastir Tunisia. *Echovirus* (ECV-11): Type 11 (Picornaviridae family) clinical strains. *Adenovirus* (ADV): non serotyped (Adenoviridae family) clinical stain.

Titration of viral strains

The virus strains were prepared from HSV-1 infected Vero cell cultures. The infected cultures were subjected to three cycles of freezing-thawing and centrifuged at 2000 rpm for 10 min. The supernatant was collected, titrated, and stored at – 80°C in 1 ml aliquots. Viral strain was carried out on 96-well flat-bottomed plate containing confluent cells into monolayer; decimal dilutions of the initial viral suspension were made then inoculated in to deprived cells from their growth medium. Each dilution was divided up in to a column of 8 wells at a density of 100 µl/well. The last column was reserved for control cells not infected by the virus and containing only the survival medium; then the plaque was incubated at 37°C in humidified 5% CO_2 atmosphere and controlled every 24 h by taking note of each time the number of well showed a CPE, when the CPE ceased to progress (5 to 7 days).

We evaluated the infectious title of the virus according to the method of Reed and Muench (1938) (Watanabe et al., 1994): the title of a virus is expressed by the $TCID_{50}$ (infectious amount on cultured tissue), it shows the dilution of the viral suspension of which 50% of the cells degenerate. The exploitation of dilution DL_{50} or $TCID_{50}$ (in absolute value) corresponds to the dilution which is giving a degeneration immediately above 50%, increased product of the proportional distance X by the logarithm of dilution factor. The proportional distance X between two critical dilutions at which the $TCID_{50}$ is located and obtained by spearman Kàber formula: X = (D > 50% - 50)/(D > 50% to D 50%). D > 50%: degeneration immediately grater than 50%. D < 50%: degeneration immediately lesser than 50% (Lorenz and Bögel, 1973).

To titer the virus suspension, confluent monolayer Vero cells were grown in 96-well flat-bottomed plates, and were infected with 0.1 ml of serial 12-fold dilutions of the virus suspension by 0.1 ml quadruplicated for a period of 48 h. The virus titer was $10^{3.5}$ (the dilution of the virus required to $TCID_{50/0.1ml}$ lytic effect, 50% of the inoculated cultures).

Solvent cytotoxicity assay

The solutions of extracts were already prepared with 100 mg/ml, a serial dilutions (1, 1/10, 1/20, 1/40, 1/80, 1/160, 1/320, 1/640, 1/1280, 1/2560 and 1/5120 mg/ml) were done in a medium, made up of RPMI 1640 supplemented with 1% L. glutamine, 2% of antibiotic and 10% (FBS) (serum of be worth fetal) (Chiang et al., 2002). Cells monolayer were trypsinized, washed with culture medium and plated in 96-well flat-bottomed plate with 2. 10^4 cells per well. After 24 h of incubation, each diluted extract was added to the appropriate wells, some wells containing only cells with the medium of dilution [Cat 2% (FBS)] were taken as a cellular control and the plate were incubated for a further 48 h at 37°C in humidified incubator with 5% CO_2. The supernatants were removed from the cells and cells viability during 5 days was evaluated by supervising the state of the cells until the appearance of cytopathic effect (CPE). That effect generally appears when cells become round and fall with repression of the cores in the cellular membranes, phenomenon observed during the cells bursting by a serial of freezing and defrosting. The results are obtained from triplicate assays with at least five extract concentrations.

Antiviral activity assay

Viral reproduction inhibitory, extract can act on the reproduction itself, or on the adsorption, or during the penetration of the virus in the cell. Three tests were carried out.

Contact between the virus and the extract before inoculation with the cells. This test allowed us to know, if the extract is able to deteriorate the integrity of the virus and so to prevent it from carrying out its infectious process. Contact between cells and extract before virus attaching, and so, the extract has the possibility of acting on the cellular receivers and can therefore prevent the adsorption, thus the penetration of the virus in the cell. In both cases, extracts will prevent the adsorption and so the penetration of virus in the cell.

Contact between cells and viral suspension then we add the extract. In this case, the virus has enough time to penetrate in the cell and to begin the replication process. The inhibition of viral multiplication after adding the extract was due to the blocking effect of the viral replication by the extract. These three tests were applied to the viruses and the two extracts (P2 and P3) of which their cytotoxicity has already being studied.

Incubation of the extract with the viral suspension

The various concentrations of the extract were contacted with the viral suspension from line 2 to 11 and from column B to G in 96-well flat-bottomed plate at a density of 10 $TCID_{50}$ /ml (v/v) equalized volumes (Grazìa et al., 2003). In line 1, non infected cells were held in their growth medium used as a negative control, line 12, columns A and H were reserved to infected cells as a positive control. The plate was incubated at 37°C for 1 h, and then inoculated on cultured Vero cells, presenting a confluent layer at a density of 200 µl/well. The cells were again incubated at 37°C and observed for 5 to 7 days until the appearance of a total CPE by the viral control (Ooi et al., 2004; Tshikalange et al., 2005).

Incubation of the cells with the extract

The incubation of the cells, which were separated from their growth medium were contacted with various concentrations of the extract at a density of 100 µl/well for 1 h at 37°C. Then, 100 µl of the viral suspension were added in each well of the plate which was again incubated at 37°C and observed for 5 to 7 days until the appearance of the viral CPE on the control as explained above (Beloin et al., 2005).

Incubation of the cells with the viral suspension

The cells were infected by the viral suspension at a density of 100 µl/well incubated for 1 h at 37°C, then 100 µl/well of various extract concentrations were added, and incubated for 5 to 7 days until the appearance of total CPE on the viral control (Cos et al., 2002). These tests were performed in triplicate for the three viral strains, each test performed used as a negative control of none infected cells held in their growth medium, and as a positive control of the cells infected by the viruses at equal concentration used for all tests (Tshikalange et al., 2005; Beloin et al., 2005). The absence of the CPE at a concentration level of the extract when the viral control expresses a CPE indicates that the extract has an antiviral activity with a specific concentration (Meyer et al., 1996).

RESULTS

GC-MS analysis

The average percentage of individual compounds of caprifig latex (Jrani variety) hexanic and hexane ethyl acetate (v/v) extracts were presented in Tables 1 and 2, respectively. GC-MS analysis of extracts resulted in the identification of 36 and 17 compounds representing 96.12 and 77.46%, respectively. Among the identified compounds, were sesquiterpens, triterpens, monoterpene (bornanone-3), coumarins and alcans, Furthermore, the most abundant compounds (> 8%) of extracts were lanosta-8 (13.17 and 30.82%), urs-12-en-24-oic acid (21.52 and 22.36%), aristolone (15.63 and 10.30%), olean-12-en-3-ol, (23.47, 3.66%), maragenin I acetate (8.78%) and A'-Neogammacer-22(29)-en-3-ol (22.06%).

Extracts cytotoxicity assay

The optical densities obtained after spectrophotometric measurement at 540 nm, were useful to determine the percentage of cellular viability. Only the concentrations having a little or no cytotoxic effect on the cells (cellular viability from 90 to 100%) were tested for antiviral activity.

Effect of hexanic extract (P2)

The observation of the cells state under a microscope after 48 h of contact with the extract and incubated at 37°C did not show any CPE. We note that the percentage of cellular viability is higher than 90% at all concentrations of the extract. We can deduce that P2 does not have any cytotoxic effect on the cells. All tested concentrations were used thereafter for antiviral activity.

Effects of ethyl acetate-hexane (v/v) extract (P3)

We note that this extract does not have any cytotoxic effect on the Vero cells. Thus, all the concentrations were tested for antiviral activity.

We could demonstrate for these tested extracts that all concentration range of the pure extract and the different dilutions were tolerable by the cells and gave a percentage of viability higher than 90%. From the cytotoxicity results of the tested extracts towards the Vero cells, we understand that a cellular fall, in the presence of an extract and of a virus was really due to the cytopathic effect CPE of the virus and not to the cytotoxic effect of the extract.

Activity against HSV-1, ECV-11 and ADV

The results of the three antiviral tests which were carried out against HSV-1, ECV-11 and ADV are presented in Table 3 and proved that P2 and P3 extracts had an antiviral activity by deteriorating the integrity of the virus and to prevent it, from carrying out its infectious course. These extracts acted on the cellular receivers and prevented the adsorption and the virus penetration in the cell and blocked the viral replication in the infected cell. It is noticed, that these positive tests which were obtained in all concentration range starting from the pure extracts to the highest dilution (1/5120).

DISCUSSION

Infectious diseases are leading cause of death world wide due to multidrug resistant strains of viruses. Many medicinal plants remain unexplored; screening of antiviral resistance modifying compounds from plants sources are expected to provide the basis for identifying leads for the isolation of therapeutically useful compounds. This study will definitely open scope for future utilization of the waste products for therapeutic purpose.

The contact type of the first antiviral activity test showed the competition between extracts and viruses on the cellular receivers. If an extract is able to stick to the cellular receivers, it can prevent the adsorption and thus the penetration of the virus on the cells. In this case, the viral CPE will be absent and the antiviral activity of the extract in question will be proved. Among the studied latex extracts P2 and P3 proved to be active against three viral strains (HSV-1, ECV-11 and ADV). The sensitivity of HSV-1 against natural substances was also showed by Beloin et al. (2005) by testing various extracts of *Momordica charantia* (Cucurbitaceae).

In the second test the virus had sufficient time to be adsorbed and penetrated in the cell. Thus, the absence of the viral CPE proved that the extract acted to prevent viral replication progress within the cell therefore, proving the antiviral activity of this extract. Both extracts P2 and P3 expressed an antiviral activity against these three tested strains. Thereafter, these extracts were able to inhibit simultaneously, the DNA viruses HSV-1 and ADV and ARN viruses ECV-11 replication. Cos et al. (2002) studied the antiviral activity of *Colenrs kilimandschari* and *Leonotis neptaeflia* extracts witch belongs to Lamiacées family. Only *C. kilimandschari* leaves extract showed an anti-HSV-1 capacity. The type contact of the third test showed no viral CPE, means that viruses were already inhibited, and showed the antiviral activity.

These tests demonstrate that, ECV-11, HSV-1 and ADV were sensitive to both extracts. Grazia et al. (2003) studied the antiviral activity of essential oils from eight (Lamiaceae) plants of which *Hyptes mutabilis* did not detect any anti-HSV-1 activity. Therefore the studied extracts P2 and P3, were able to stop the replication cycles of HSV-1, ECV-11 and ADV by preventing their adsorption and their penetrations in the cells (by interaction with the cellular receivers designed for these viruses), or by inhibition of their intracellular replication, or by virus inhibition after a direct contact. These results

Table 1. Chemical composition of hexane extract obtained by GC/MS.

Retention time (min)	Area (%)	Constituent	Wiley library Reference No	CAS No	Quality
4.70	0.06	GERMACRENE-D	121792	023986-74-5	98
5.04	0.01	Delta-Cadinene	121465	000483-76-1	97
7.03	0.33	2, 6, 10-Dodecatrien-1-ol	148283	004602-84-0	83
8.88	0.05	Dodecan-2-on	93122	006175-49-1	72
9.09	0.38	Hexadecanoic acid	213894	000112-39-0	98
10.73	0.33	CIS-LINOLEIC ACID METHYL ESTER	243137	000112-63-0	99
11.69	0.08	Pentadecane	134011	000629-62-9	96
11.88	0.04	1, E-8, z-10-Hexadecatriene	145418	080625-33-8	87
12.76	0.03	Tricosane	275685	000638-67-5	96
13.90	0.13	Triacontane	34922	000638-68-6	91
14.51	0.02	8-dimethoxynaphthalene	120716	105372-17-6	90
15.76	0.51	Bis (2-ethylhexyl) phthalate	326908	000117-81-7	91
16.30	0.05	Hexacosane	311168	000630-01-3	94
16.72	0.06	7-Pentadecyne	128099	022089-89-0	64
17.55	0.13	Heptacosane	32679	000593-49-7	95
18.79	0.10	Octacosane	329269	000630-02-4	96
19.23	0.33	Squalene	337959	007683-64-9	93
20.05	0.13	Nanocosane	337002	000630-03-5	97
20.61	0.37	Oxirane	345735	007200-26-2	89
21.73	0.29	1-ethyl-3-acetyl-5	182541	112482-88-9	86
22.51	0.72	4-methoxycarbonyl-2, 6-diphenylpyridine	237065	069209-39-8	74
22.98	1.04	Bornanone-3	49880	013854-85-8	70
23.38	0.19	1-ethyl-3-acetyl-5	182541	112482-88-9	78
23.54	0.15	1, 6, 10, 14, 18, 22-Tetracosahexaen-3-ol	345737	054159-46-5	64
23.67	1.02	[3.2] metacyclophane-10-ene	145464	121733-15-1	91
24.79	0.77	5-HYDROXY-6	236998	063955-63-5	78
27.96	0.98	9, 19-Cyclolanost-24-en-3-ol	345634	000469-38-5	93
28.96	0.94	Beta.-Amyrin	345611	000559-70-6	83
29.47	13.17	Lanosta-8	360770	002671-68-3	96
30.20	0.24	6-Aza-B-homo-5.alpha.-cholestane	345497	066233-39-4	92
30.25	0.37	Lupeol	345599	000545-47-1	87
32.33	21.52	Urs-12-en-24-oic acid	360707	020475-86-9	91
32.75	15.63	Aristolone	141923	006831-17-0	86
32.95	23.47	Olean-12-en-3-ol	360750	001616-93-9	93
33.38	8.78	Maragenin I acetate	360711	071545-16-9	89
33.62	2.32	alpha.-amyrenyl acetate	360746	000863-76-3	64

Table 2. Chemical composition of ethyl acetate extract obtained by GC/MS.

Retention time (min)	Area (%)	Constituent	Wiley library Reference No	CAS No	Quality
8.424	0.10	(+)-Aromadendrene	121608	000489-39-4	96
8.767	0.19	delta.-Cadinene	121454	000483-76-1	99
9.174	1.08	1H-Cycloprop[e]azulen-7-ol	145016	006750-60-3	95
10.398	0.72	Tetradecanal	133817	000124-25-4	81
10.867	0.30	Hexadecanoic acid	213911	000112-39-0	93
11.297	1.24	1H-Naphtho[2,1-b]pyran	238701	001227-93-6	94
11.531	1.06	Heptadecene-(8)-carbonic acid-(1)	228686	000000-00-0	90

Table 2. Contd.

19.616	30.75	Lanosta-8	360770	002671-68-3	95
19.879	1.17	Silicone grease	392047	000000-00-0	58
20.331	10.30	Olean-12-en-3-ol	360750	001616-93-9	93
21.024	19.80	alpha.-Amyrenyl acetate	360746	000863-76-3	76
22.597	10.82	A'-Neogammacer-22(29)-en-3-ol	360754	002085-25-8	86

Total area: 77.5%.

Table 3. Antiviral activity of *Ficus carica* extracts.

Extracts		Antiviral activity (%)	
		P2	P3
Extracts last dilution		**1/5120**	**1/2560**
	HSV	100	100
(E+V) + C	ECV	100	100
	ADV	100	100
	HSV	100	100
(C+E) + V	ECV	100	100
	ADV	100	100
	HSV	100	100
(C+V) + E	ECV	100	100
	ADV	100	100

P2: hexanic extract; P3: (v/v) ethyl acetate-hexane extract; (E+V) + C: extract effect against virus; (C+E) + V: extract effect against Vero cells; (C+V) + E: extract effect against viral cycle after adsorption.

confirmed the evidence that, P2 and P3 extracts had an interesting antiviral activity.

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