Antimicrobial, Cytotoxic, and Antiviral Activities of *Salvia fructicosa* Essential Oil

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Salvia fructicosa essential oil analyzed by gas chromatography/mass spectrometry showed high contents of 1,8-cineole, α- and *β*-thujone, and camphor, representing 47.48%, 11.93%, and 9.04% of the total oil, respectively. The essential oil and its isolated components thujone and 1,8-cineole exhibited antimicrobial activity against eight bacterial strains, while camphor was almost inactive against all of the bacteria tested. The essential oil was bactericidal at 1/4000 dilution, and dilutions up to 1/10000 caused considerable decrease in bacterial growth rates. The essential oil of *S. fructicosa* and the three main components exhibited cytotoxic activity against African Green Monkey kidney (Vero) cells and high levels of virucidal activity against herpes simplex virus 1, a ubiquitous human virus.

Keywords: *Salvia fructicosa; essential oil; antimicrobial activity; cytotoxicity; antiviral activity (herpes simplex virus); camphor; 1,8-cineole; thujone*

INTRODUCTION

Numerous species of the genus *Salvia* (Labiatae) have been used since ancient times in folk medicine and subjected to extensive pharmacognostic research intended to identify biologically active compounds. Compounds isolated from the roots of *Salvia miltiorrhiza* exhibited inhibitory activity against adenylate cyclase, the enzyme responsible for the production of cyclic-AMP, an important modulator of hormones and neurotransmitters (Kohda et al., 1989). In addition, salvianolic acid A, isolated from roots of the same plant species, inhibited porcine gastric H^+,K^+-ATPase activity. The inhibition of H^+,K^+-ATPase activity by salvianolic acid A is probably responsible for the antiulcer and acid antisecretory activity of salvianolic acid A observed in rats (Murakami et al., 1990). Finally, sabinyl acetate, present in *Salvia lavandulifolia* essential oil, was shown to be responsible for an abortifacient effect in rodents (Fournier et al., 1993).

Recently, many studies have focused on the biological properties, antibacterial (Janssen et al., 1986, 1987; Gonzalez et al., 1989; Darias et al., 1990), cytostatic (Darias et al., 1990), and antiviral (Tada et al., 1994), of the essential oils derived from *Salvia* species and their main components. In an attempt to identify biologically active components in the essential oil of *Salvia fructicosa* Miller (syn.: *S. triloba* L., known by the vernacular name Greek sage), widely used in folk medicine, cosmetics, and the flavoring of food products (Kokkini, 1994), we carried out a study of the antibacterial, cytotoxic, and antiviral properties of the oil and its main components.

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MATERIALS AND METHODS

Plant Material, Gas Chromatography (GC), and GC/Mass Spectroscopy (GC/MS) Analyses of Essential Oils. Aerial parts of wild growing fully flowered plants of *S. fructicosa*, collected from the island of Crete, were air-dried and grossly pulverized. The essential oil was isolated after hydrodistillation for 2 h, using a Clevenger apparatus.

The essential oil was analyzed using a Shimadzu GC-14A gas chromatograph equipped with a Supelcowax 10 (Supelco, Bellefonte, PA) capillary column (60 m × 0.25 mm i.d.). The carrier gas was helium, and the linear gas velocity was 20.4 cm/s. The injector temperature was 240 °C. The column temperature was initially 70 °C and was then gradually increased at a rate of 4 °C/min up to 220 °C. For detection, a flame ionization detector (FID) was used set at a temperature of 240 °C. GC/MS analyses were conducted using a Shimadzu GC-MS QP2000 system equipped with a Supelcowax 10 capillary column (60 m × 0.25 mm i.d.) under the same GC conditions. For GC/MS detection an electron impact (EI) quadropolar system was used with an ionization energy of 70 eV. The essential oil components were identified by comparing their relative retention times and mass spectra with those of authentic samples, the Wiley Registry of Mass Spectral data (McLafferty, 1994), and literature citations (Cornu and Massot, 1979; Masada, 1976; Jennings and Shibamoto, 1980). It should be noted that all experiments in this study were carried out with the same lot of essential oil to ensure reproducibility due to the inherent variability observed from lot to lot.

Bacterial Strains and Media. The following reference strains of bacteria, purchased from the National Collection of Industrial and Marine Bacteria (NCIMB, Aberdeen, Scotland), were used as test organisms in all antimicrobial assays: *Escherichia coli* (NCIMB 8879 and NCIMB 12210), *Pseudomonas aeruginosa* (NCIMB 12469), *Salmonella typhimurium* (NCIMB 10248), *Staphylococcus aureus* (NCIMB 9518 and NCIMB 8625), *Rhizobium leguminosarum* (NCIMB 11478), and *Bacillus subtilis* (NCIMB 3610). Bacteria were grown either in nutrient broth or in nutrient agar and incubated at 37 °C (*E. coli*, *P. aeruginosa*, and *S. typhimurium*), at 30 °C (*S. aureus* and *B. subtilis*), or at 25 °C (*R. leguminosarum*). These particular strains are standard reference strains that are routinely used for the evaluation of antimicrobial compounds.

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Antimicrobial Assay (Disk Diffusion Assay). Filter paper disks (Whatman No. 1, 5 mm diameter) containing 5 μL of the essential oil or isolated compound (authentic samples, Aldrich Chemical Co., Milwaukee, WI) were applied to the surface of agar plates that were previously seeded by spreading of 0.2 mL of a bacterial overnight culture. The plates were incubated overnight at the appropriate temperature (see above), and the resulting zone of inhibition was measured in millimeters. The results indicated in Table 2 and in the text represent the net zone of inhibition after subtraction of the diameter (5 mm) of the paper disk.

Determination of Bacterial Cell Growth. Well-isolated, single bacterial colonies from overnight plates were transferred into nutrient broth and grown overnight at the appropriate temperature with shaking (see above). Tubes of nutrient broth containing various concentrations of the essential oil or isolated compound were then inoculated with appropriate aliquots of bacterial cultures so that their initial optical densities (OD_{600}) were equal. The initial 10-fold dilution of the essential oil or isolated compound was done in ethanol, and all subsequent ones were done in nutrient broth. The growth of each culture was monitored in two ways, either by measuring its optical density at 600 nm (OD_{600}) at 30 min intervals for a total period of 8 h (total counts) or by plating at specific time intervals suitably diluted aliquots of the culture on nutrient agar (viable counts).

Cells and Cytotoxicity Assay. Vero cells (derived from the kidney of African Green Monkey) were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS). For cytotoxicity assays, cells were seeded into 12-well plates at a density of 2.1×10^5 cells/well and the appropriate dilution of the essential oil or isolated compound was then added. The essential oil was initially diluted 1:100 (v/v) in ethanol and further dilutions were made in DMEM. After the appropriate time of incubation (24, 48, or 72 h), the growth media were removed and the cells were trypsinized and counted in a hemocytometer according to the trypan blue-exclusion method (Hayashi et al., 1990).

Virus Neutralization. Tubes containing 2×10^9 plaque forming units (PFU) of herpes simplex virus 1 (HSV-1) strain F (Ejercito et al., 1968) were incubated for 30 min at 37 °C, in DMEM containing 1% (v/v) FCS and supplemented with various concentrations of the essential oil, or isolated compound. Since the initial dilution of the essential oil was done in ethanol, an additional tube containing the virus and the appropriate amount of ethanol was used as control. At the beginning and at the end of the incubation period, 100 μL of viral sample was removed from each tube and was assayed for remaining infectivity on Vero cells by plaque assay (Russell, 1962). Briefly, the samples were serially diluted in DMEM containing 1% (v/v) FBS, and aliquots of each dilution were adsorbed on Vero cell monolayers for 1 h at 37 °C. At the end of the adsorption period the virus inoculum was removed and the cells were replenished with fresh DMEM containing 1% (v/v) FBS and 0.1% human immunoglobulins. The cells were incubated at 37 °C in a 5% (v/v) CO_2 atmosphere, and viral plaques were counted after 72 h.

RESULTS AND DISCUSSION

The main components of *S. fructicosa* essential oil were 1,8-cineole, thujone, and camphor, representing 47.48%, 11.93% (α-thujone, 4.32%; and β-thujone, 7.61%), and 9.04% of the total oil, respectively (Table 1). Comparison of these results with those mentioned in the literature indicates that these compounds are in most cases the main components of the Greek sage essential oil (Katsiotis and Iconomou, 1984; Harvala et al., 1987; Karousou, 1995).

The antimicrobial activity of the oil and of the main compounds was examined in the disk diffusion assay against a panel of eight bacteria selected on the basis of their relevance to public health. The results presented in Table 2 showed the following:

Table 1. Qualitative and Quantitative Composition of *S. fructicosa* Essential Oil

component	composition (%)	component	composition (%)
tricyclene	0.13	bornyl acetate	1.84
α-pinene	4.78	β-caryophyllene	1.69
camphene	3.80	terpinen-4-ol	0.09
β-pinene	4.56	myrtenal	0.09
sabinene	0.07	isoborneol	0.52
myrcene	1.75	α-humulene	0.14
α-terpinene	0.25	α-terpineol	2.95
limonene	1.42	borneol	1.18
1,8-cineole	47.48	geranial	0.06
γ-terpinene	0.38	neryl acetate	0.11
p-cymene	0.68	geranyl acetate	0.10
terpinolene	0.10	anethol	0.04
α-thujone	4.32	geraniol	0.05
β-thujone	7.61	β-bisabolene	0.16
1-octen-3-ol	0.30	γ-cadinene	0.20
camphor	9.04	caryophyllene oxide	0.48
cis-sabinene hydrate	1.03	thymol	0.04
linalool	0.22	carvacrol	0.34
linalyl acetate	0.71		

Table 2. Antimicrobial Activity of *S. fructicosa* Essential Oil and Its Main Componentsa

	bacterial strains according to NCIMB number							
substance	8879	12210	10248	12469	11478	8625	9518	3610
S. fructicosa	3	2	1	5	3	5	2	4
camphor	+[b]	1	+	2	1	−[c]	−	1
1,8-cineole	5	4	+	2	3	1	5	4
thujone	4	3	+	2	3	5	1	5

[a] The diameter (mm) of the inhibition zone is the mean of three independent experiments (the diameter of the paper disk, 5 mm, is not included). [b] +, very low activity. [c] −, no activity.

(i) The oil and the tested main compounds showed relatively low levels of antimicrobial activity against the bacteria tested. By comparison, essential oils derived from other plant genera, such as *Origanum* and *Mentha*, have been reported to possess much higher levels of antimicrobial activity against the same panel of bacteria (Sivropoulou et al., 1995, 1996; Janssen et al., 1986). However, the essential oil of *Artemisia afra* Jacq., which has a qualitative composition similar to that of *S. fructicosa* (α- and β-thujone, 52%; 1,8-cineole, 13%; and camphor, 15%), showed moderate antimicrobial and antifungal activities (Graven et al., 1992) comparable in magnitude to these reported here for *S. fructicosa*.

Among the bacteria tested, *S. typhimurium* was the most resistant against the oil and its main components, while *P. aeruginosa* was one of the most sensitive. This is particularly interesting since the latter bacterium has shown resistance to many antimicrobial agents and also to many diterpenes present in other *Salvia* species (Darias et al., 1990). Differences in sensitivity can be observed even between bacterial strains that belong to the same species. Thus, *S. aureus* NCIMB 8625 exhibited much higher sensitivity to the essential oil and thujone than *S. aureus* NCIMB 9518, whereas in the case of 1,8-cineole the latter was more sensitive than the former.

(ii) Among the three main compounds of *S. fructicosa* essential oil, camphor exhibited lower levels of antimicrobial activity than 1,8-cineole and α- and β-thujone, all of which showed variable degrees of antimicrobial activity against the bacteria tested.

Because the disk diffusion assay cannot differentiate between the bactericidal and bacteriostatic effects exerted by the oil, we examined the effect of the oil on the

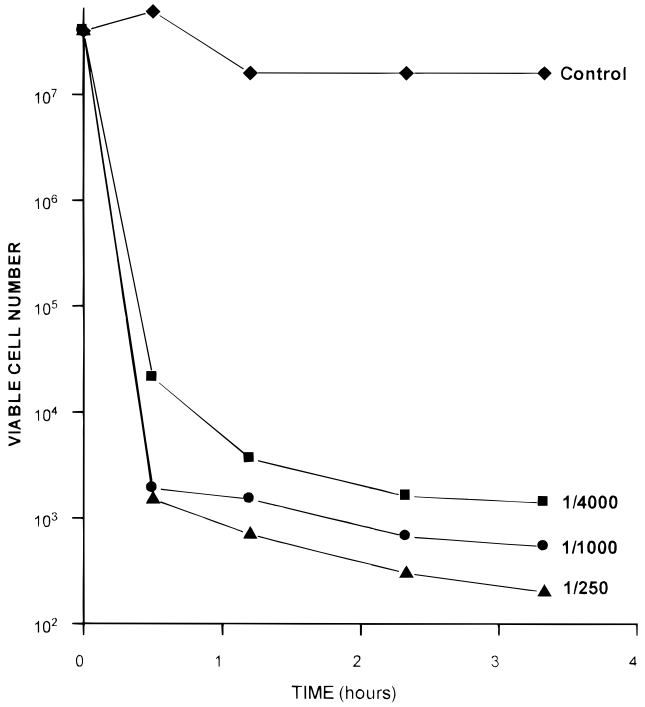

Figure 1. Time-dependent effect of *S. fructicosa* essential oil on the viability of *S. aureus* (NCIMB 8625). Equal aliquots of overnight bacterial cultures were inoculated in equal amounts of nutrient broth supplemented with *S. fructicosa* essential oil at 1/4000, 1/1000, or 1/250 dilutions or with diluent alone. At specific time intervals, suitably diluted aliquots of the cultures were plated on nutrient agar and the viable cell numbers were counted. These experiments were repeated independently two times and yielded essentially the same results.

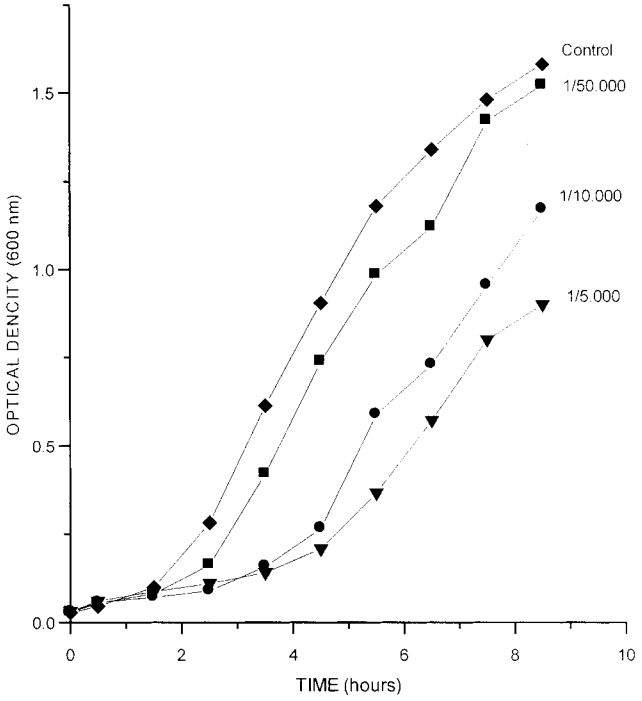

Figure 2. Time- and concentration-dependent effect of *S. fructicosa* essential oil on the rates of growth of *S. aureus* (NCIMB 8625). Equal aliquots of overnight bacterial cultures were incubated in equal amounts of nutrient broth supplemented with or without various concentrations of the essential oil (1/5000, 1/10000, and 1/50000), and cell growth was monitored spectrophotometrically at 600 nm at specific time intervals. These experiments were repeated independently two times and yielded essentially the same results.

bacteria in relation to cumulative time of exposure. From the panel of eight bacteria used, we selected *S. aureus* (NCIMB 8625) on the basis of its relevance to public health and its high sensitivity to the oil. The oil at dilutions up to 1/4000 reduced the number of viable cells by almost 3 $\log_{10}$ values within 30 min (Figure 1) of exposure.

The high bactericidal activity of the oil prompted us to examine its effect on bacterial growth rates at higher oil dilutions (1/5000, 1/10000, and 1/50000) and over longer periods of exposure. The results (Figure 2) showed that the oil at dilutions up to 1/10000 caused considerable decrease in the growth rate of *S. aureus* over the 8 h incubation period of the experiment.

Recently, compounds isolated from *S. broussonettii Benth.* and *Salvia canariensis* L. species have been reported to exhibit cytostatic activity against cultured human HeLa 229 cells (Darias et al., 1990). More recently, Tada et al. (1994) reported the presence of antiviral activity in *Salvia officinalis* crude extracts. We examined the effect of *S. fructicosa* essential oil and of its main components on the growth of eukaryotic cells and on the neutralization of herpes simplex virus 1 (HSV-1), a ubiquitous human pathogen. For the cytotoxicity studies Vero cells were chosen since they are routinely used for the growth and assay of HSV-1. Monolayer cultures of Vero cells were exposed to various concentrations of the oil, and cell viability was evaluated by counting the live cells after 24, 48, and 72 h of exposure (Figure 3). The oil caused complete cell death at dilutions up to 1/500 within 24 h of exposure. At 1/1000 dilution cell mortality gradually increased and was almost complete after 48 h of exposure. At 1/2000 dilution a transient decrease in cell viability was

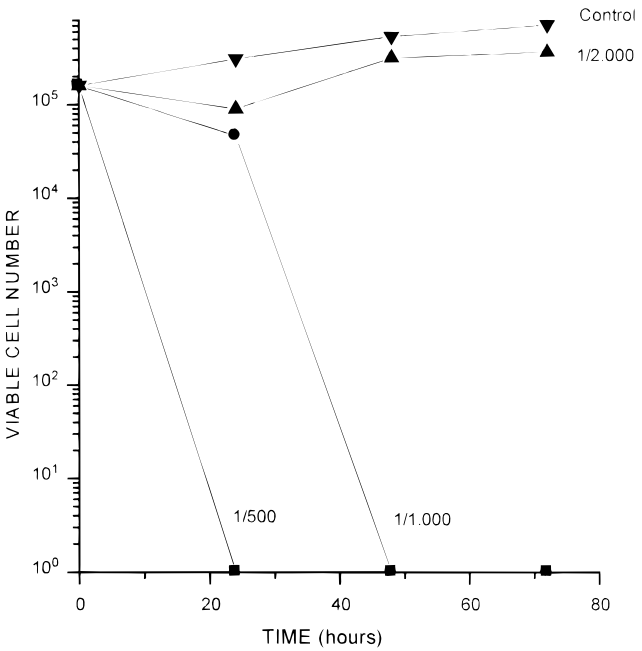

Figure 3. Time- and concentration-dependent cytotoxicity of *S. fructicosa* essential oil on Vero cells. Equal numbers of Vero cells were incubated with various concentrations of the essential oil (1/500, 1/1000, and 1/2000) or with the appropriate amount of ethanol. At specific time intervals the viable cell number was counted with the trypan blue-exclusion method. These experiments were repeated independently two times and yielded essentially the same results.

initially observed, which was overcome after 48 h of exposure, resulting in a small overall decrease in the growth rate after 72 h of exposure. A similar effect was observed in yeast cells, where prolonged exposure of the

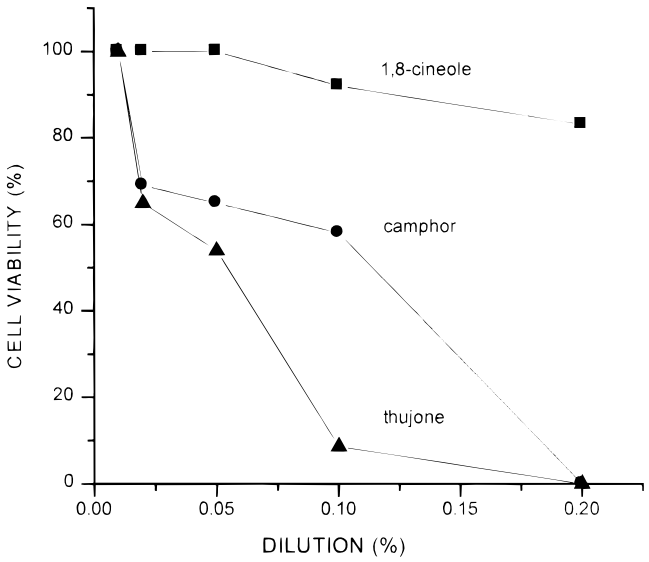

Figure 4. Concentration-dependent cytotoxicity of α- and *β*-thujone, 1,8-cineole, and camphor on Vero cells. Equal numbers of Vero cells were incubated with various concentrations of α- and *β*-thujone, 1,8-cineole, or camphor or with the appropriate amount of ethanol. After 24 h of incubation, the viable cell number was counted from each case with the trypan blue-exclusion method. These experiments were repeated independently two times and yielded essentially the same results.

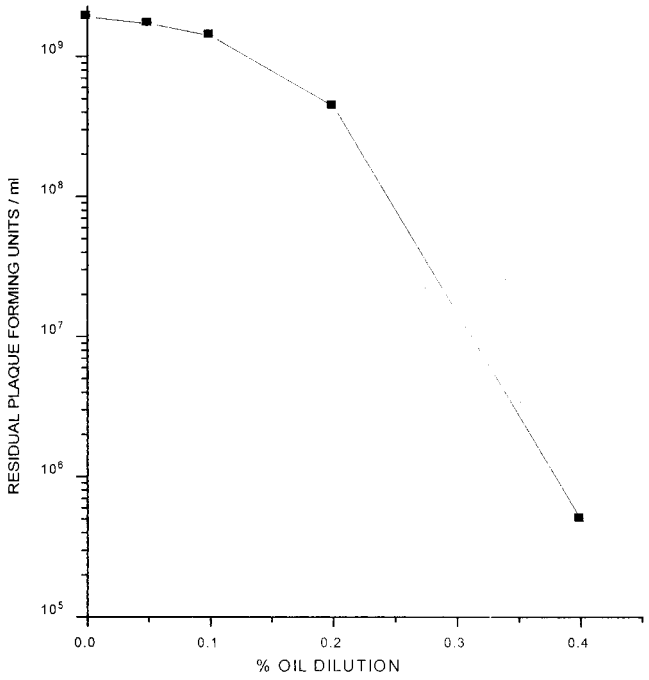

Figure 5. Concentration-dependent effect of *S. fructicosa* essential oil on neutralization of HSV-1. 2×10^9 PFU of HSV-1 was incubated for 30 min at 37 °C, in DMEM supplemented with various concentrations of the essential oil or with the appropriate amount of ethanol as control. At the beginning and at the end of the incubation period, 100 μL of viral sample was removed from each case and titrated on Vero cells.

cells to marginal concentrations of essential oils resulted in nonheritable resistance probably due to adaptation (Steinmetz et al., 1988). Among the three main components of the oil (1,8-cineole, α- and *β*-thujone, and camphor), thujone was the most cytotoxic, causing 95% reduction in viability at 1/1000 dilution, whereas camphor showed higher levels of cytotoxicity than 1,8-cineole (Figure 4).

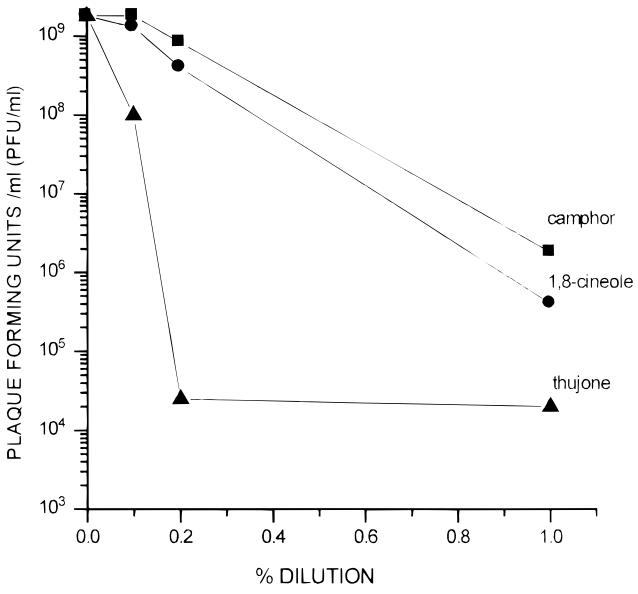

Figure 6. Concentration-dependent effect of the main compounds of *S. fructicosa* essential oil on neutralization of HSV-1. 2×10^9 PFU of HSV-1 was incubated for 30 min at 37 °C, in DMEM supplemented with various concentrations of thujone, 1,8-cineole, or camphor or in only DMEM, which served as control. At the beginning and at the end of the incubation period, 100 μL of viral sample was removed from each case and titrated on Vero cells.

The virucidal action of the oil and of its main components was evaluated against HSV-1, a ubiquitous human pathogen. The virus was exposed for 30 min to various concentrations of the oil (Figure 5) or its main components (Figure 6), and the residual plaque forming units were calculated from each case. The oil at a concentration of 0.2% inactivates 80% of infectious viruses within 30 min, while at higher concentration (0.4%) this effect is accelerated by almost 4 $\log_{10}$ values. All of the main components showed high virucidal action, but thujone was the most active. Thujone at a dilution of 0.1% inactivates 95% of infectious virus particles, while the percentage of virus inactivation by 1,8-cineole or camphor is low (35% and 0%, respectively). The virucidal action of thujone is accelerated by almost 5 $\log_{10}$ values at higher concentrations (0.2%). In addition, other monoterpenes such as borneol, bornyl acetate, and isoborneol, present in the essential oil of *S. fructicosa*, exhibited considerable antiviral (against HSV-1) and cytotoxic (against Vero cells) activities (data not shown). Other diterpenoids isolated from *S. officinalis* have been reported to possess antiviral activities against vesicular stomatitis virus (Tada et al., 1994).

In conclusion we show that the *S. fructicosa* essential oil possesses antibacterial, cytotoxic, and antiviral properties. Thujone was the most biologically active compound of the oil, since it exhibited high levels of antibacterial, cytotoxic, and antiviral activities.

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