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Research paper

Liposomal incorporation of *Artemisia arborescens* L. essential oil and in vitro antiviral activity☆

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Abstract

The effect of liposomal inclusion on the in vitro antiherpetic activity of *Artemisia arborescens* L. essential oil was investigated. In order to study the influence of vesicle structure and composition on the antiviral activity of the vesicle-incorporated oil, multilamellar (MLV) and unilamellar (SUV) positively charged liposomes were prepared by the film method and sonication. Liposomes were obtained from hydrogenated (P90H) and non-hydrogenated (P90) soy phosphatidylcholine. Formulations were examined for their stability for over one year, monitoring the oil leakage from vesicles and the average size distribution. The antiviral activity was studied against Herpes simplex virus type 1 (HSV-1) by a quantitative tetrazolium-based colorimetric method. Results showed that *Artemisia* essential oil can be incorporated in good amounts in the prepared vesicular dispersions. Stability studies pointed out that vesicle dispersions were very stable for at least six months and neither oil leakage nor vesicle size alteration occurred during this period. After one year of storage oil retention was still good, but vesicle fusion was present. Antiviral assays demonstrated that the liposomal incorporation of *A. arborescens* essential oil enhanced its in vitro antiherpetic activity especially when vesicles were made with P90H. On the contrary, no significant difference in antiviral activity was observed between the free and SUV-incorporated oil.

Keywords: *Artemisia arborescens* L.; Essential oil; Liposomes; Vesicles; Antiviral agents; HSV-1 virus

1. Introduction

In recent years, liposomes have been extensively studied as a carrier system which can improve the activity and safety of many drugs. Liposomes are promising drug carriers in antiviral therapy due to their capability to deliver the entrapped drugs across cell membranes because of the intracellular nature of virus action and proliferation. Herpes simplex virus is one of the most common viral diseases in humans. Herpes simplex virus 1 (HSV-1) and Herpes simplex virus 2 (HSV-2) have been distinguished by clinical manifestations, biological and serological criteria. Several drugs have proven to be useful in the treatment of many specific infections, but viral strains resistant to these drugs have been increasingly identified and several cases of toxicity have been encountered, particularly in immunocompromised patients [1]. In order to find less toxic antiviral agents much research has focused on plant products. Indeed, many plant extracts have been described as potential antiviral agents. In recent years, the antiviral and virucidal activities of several essential oils have been described in literature [2–9]. In particular, various Herpes viruses were reported to be very sensitive to the inhibitory activity of these plant extracts. Recently, we reported that *Santolina insularis* essential oil has shown a good in vitro antiviral activity and that liposomes could be a very interesting delivery system for these antiviral agents. Moreover, the incorporation in liposomes greatly improved the essential oil stability [10].

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Pursuing our interest in herbal medicinal products obtained from endemic Sardinian plants, in this work we have studied the antiviral activity of liposomal essential oil extracted from *Artemisia arborescens* L., an evergreen shrub from the Asteraceae family, which is endemic in Mediterranean regions.

Since liposomes have already demonstrated both the capability to deliver various antiviral agents in vitro and to protect their content from environmental exposure, we have carried out research into the formulation of liposomal *A. arborescens* essential oil in order to evaluate the influence of the vesicular inclusion on the in vitro antiviral activity of this plant extract. Liposomes are a very interesting delivery system for antiviral agents as they could be administered by several routes according to the type and severity of the infection: parenterally for diffused systemic infections, in drops for ocular herpetic infections and topically applied on lips and mucosae in gel or cream formulations. In particular, our study is aimed at finding new topical delivery systems for antiviral agents.

It is well known that the activity of liposome-entrapped drug may vary depending on the vesicle size, the surface charge, and lipid composition. Therefore, several vesicular formulations were prepared following different methods (i.e. film method and sonication) and using hydrogenated (P90H) or non-hydrogenated phosphatidylcholine (P90) mixtures. Moreover, as Non-ionic Surfactant Vesicles (NSVs or niosomes) have already proved to show the same properties of liposomes with several advantages (higher chemical stability, low cost, greater availability of materials) [11], we also prepared a niosomal formulation made with polyoxyethylene (4) lauryl ether (Brij® 30). All vesicular formulations also contained cholesterol. In order to improve vesicle–cell interactions we prepared positively charged liposomes by adding small amounts of stearylamine to the lipid components. Characterization of vesicles was carried out by different techniques: transmission electron microscopy (TEM) and optical microscopy for morphology, light polarized microscopy for lamellar formation, dynamic light scattering for size distribution and UV spectroscopy and gas chromatography/ion trap mass spectrometry (GC/ITMS) for incorporation efficiency. The antiviral activity of free and vesicle-incorporated essential oil was tested in vitro against HSV-1 by a quantitative tetrazolium-based colorimetric method (MTT).

2. Experimental methods

2.1. Materials

Enriched soya phosphatidylcholine (Phospholipon® 90, P90) and hydrogenated soya phosphatidylcholine (P90H) were kindly obtained from Nattermann Phospholipids, GmbH. Cholesterol, stearylamine and polyoxyethylene (4) lauryl ether (Brij® 30) were purchased from Sigma (Milan, Italy).

2.2. Virus and cells

African green monkey kidney cells (Vero) were obtained from the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia (Brescia, Italy). Cells were grown in RPMI 1640 (Gibco Life Technologies, Rockville, MD) supplemented with 10% foetal bovine serum (FBS, Gibco) and penicillin, streptomycin and amphotericin B (100 U/ml, 100 and 2.5 μg/ml, respectively). The strain of Herpes Simplex virus type 1 (HSV-1 strain F) was obtained from the American Type Culture Collection, Rockville, MD, and was propagated in Vero cells. Virus titer was determined by plaque assay in Vero cells and stored at −70 °C until used.

2.3. Preparation and characterization of essential oil

The aerial parts of *A. arborescens* were collected from the countryside around Usellus, Sardinia, Italy, during full blossom. Plants were identified and voucher specimens deposited in the Herbarium of the Institute of Botany and Botanical Garden, University of Cagliari, Italy. Two kilograms of fresh aerial parts were distilled in a Clevenger-type apparatus for 5 h. The essential oil was dried over anhydrous sodium sulphate and stored at 4 °C. The quali-quantitative analysis of the essential oil was carried out by GC/ITMS.

GC/ITMS analysis. A Varian CP 3800 gas chromatograph (Varian, Inc., Palo Alto, CA, USA) coupled with a Saturn 2000 ITMS detector, a Varian CP 7800 autosampler, a split-splitless injector, and a MS ChemStation, was used. The column was a fused silica capillary DB-5MS (5% phenylmethylpolysyloxane, 30 m × 0.25 mm; film thickness 0.25 μm) (J&W Scientific Fisons, Folsom, CA). The injector and interface were at 150 and 280 °C, respectively. The oven temperature was programmed as follows: from 60 to 180 °C (3 °C/min), and isothermally held for 15 min. Helium was the carrier gas at 1 ml/min; the sample (1 μl) was injected in the split mode (1:20). MS conditions were as follows: ionization mode EI from 50 to 450 amu. The oil compounds were identified by comparison of their relative retention times with those of authentic samples or by comparison of their retention index relative to the series of *n*-hydrocarbons, and computer matching against commercial library [12,13] and homemade library mass spectra made up from pure substances and component of known oils and MS literature data.

2.4. Vesicle preparation

Compositions of the tested samples are reported in Table 1. Multilamellar vesicles (MLVs) were prepared according to the thin film hydration method. The precise amount of surfactant, cholesterol, and *A. arborescens* essential oil (12.5 mg/ml) was dissolved in 10 ml of chloroform in a 50 ml round-bottomed flask. The solvent was evaporated under reduced pressure at room

Table 1
Sample composition (mg/ml)

Components	Formulations		
	1	**2**	**3**
Essential oil	12.50	12.50	12.50
P90H	19.50	–	–
P 90	–	19. 50	–
Brij® 30	–	–	5.75
Cholesterol	2.60	2.60	3.86
Stearylamine	0.28	0.28	0.28

temperature. The obtained lipid film was hydrated under mechanical stirring with distilled water working at 60 °C (sample **1**, P90H) or at room temperature (**2**–**3**, P90 and Brij 30). In order to avoid any loss of volatile essential oil components, the lipid film hydration was carried out in a two necked round-bottom flask equipped with a reflux condenser. The condenser was connected to a cold trap containing chloroform. At the end of the film hydration step, the organic solvent was analysed in GC where no signal of Artemisia oil components was detected. Sonicated unilamellar vesicles (SUV) were prepared starting with MLV dispersions, which were sonicated in a Soniprep 150 apparatus under a nitrogen stream for 30 min (6 times for 5 min). The essential oil-incorporated vesicles were separated from the unincorporated oil by ultracentrifugation. Vesicular dispersions were spun in an ultracentrifuge (Beckman L80M) at 4 °C and 30,000 rpm for 60 min. The supernatant was removed and analysed for the oil quantity by measuring the absorbance at λ 284 nm using a Hitachi-U 2000 UV/VIS spectrophotometer. This step was repeated at least three times. Vesicles were reconstituted in distilled water to obtain an essential oil concentration of 5 mg/ml.

2.5. Transmission electron microscopy

Negative stain micrographs were prepared on copper grids covered with a formvar/carbon film. The vesicle dispersion was pipetted onto the grids and stained with 1% phosphotungstic acid and were then viewed and photographed with a Zeiss EM 109 transmission electron microscope at an accelerating voltage of 80 kV.

2.6. Optical and light polarized microscopy

Optical and light polarized micrographs were obtained by an optical microscope Zeiss Axioplan 2, at 25 °C. The temperature was kept constant ± 1 °C by a hot stage device connected to a thermostatic bath.

2.7. Dynamic light scattering

Liposome size distribution was determined by dynamic light scattering (N4 Plus, Beckman Coulter) at 25 °C. Samples were scattered (633 nm) at an angle of 90°. Mean particle sizes are intensity-based data. Data were fitted by the method of inverse 'Laplace transformation' and CONTIN.

2.8. Incorporation efficiency

Incorporation efficiencies (*E%*) are expressed as a percentage of the total amount of *Artemisia* essential oil found in the studied formulations at the end of the preparation procedure. The incorporation efficiency was calculated using the following equation: $[(T-S)/T]\times 100$, where T is the total oil quantity both in the supernatant and sediment (determined after disruption of vesicles with methanol); S is the oil quantity detected in the supernatant. Essential oil recovery from supernatant and sediment accounted for more than 97% of the used dose. The concentration of *Artemisia* essential oil was determined spectrophotometrically at 284 nm using a Hitachi-U 2000 UV/Vis detector. The spectrophotometric method was previously validated by comparison of the obtained results with those from GC/ITMS (as described above). The spectrophotometric method results were also compared with those obtained by HPLC where the most important components of the oil (camphor, β-thujone and chamazulene) were used as a 'lead'. The oil content was assayed by HPLC at several wavelengths (209, 245 and 284 nm), using a Waters 2690 liquid chromatograph, equipped with a Photodiode Array detector 996. The mobile phases were methanol (solvent A) and water (solvent B). Separations were performed by the following linear gradient: 45–30% B in 15 min, 30–10% B in 25 min, at a flow rate of 1.0 ml/min. The column was a Spherisorb 5 μm ODS2 (4.6×280 mm, Waters). Appropriate standard solutions of *A. arborescens* essential oil and authentic samples of the 'lead' compounds in methanol were prepared and tested. All experiments were carried out in triplicate.

Determination of the main oil components retained (*R%*) in the vesicular formulation was carried out by GC/ITMS analysis after disruption of purified vesicles with methanol.

2.9. Stability measurements

Stability of vesicle dispersions was studied over one year. Vesicle dispersions were kept at 5 ± 1 °C, and at fixed time intervals (i.e. once a week for the first two months and then once a month) they were assayed for their content in essential oil after disruption of purified vesicles with methanol. Physical stability of vesicular dispersions was also investigated by dynamic light scattering measurements and by TEM visualization. Moreover, a stability study of the oil-loaded liposomes in RPMI medium (supplemented with 10% foetal bovine serum, penicillin, streptomycin and amphotericin B) was also carried out. Briefly, 0.1 ml of liposomal dispersion were stored in 1 ml of the medium for

24 h. Then, purified vesicles were disrupted with methanol and the methanol solution was analysed by GC/ITMS.

2.10. Antiviral activity

The antiviral activity of *A. arborescens* free and liposomal essential oil was evaluated against HSV-1. Vero cells 5×10^4 in 50 μl of RPMI 1640 containing FBS 5% were seeded in 96 multiwell microtiter plates (Falcon 353872, Becton Dickinson, Franklin Lakes, NJ). The essential oil or liposomal formulations in 50 μl of RPMI 1640 were added to cells and infected with 20 μl of HSV-1 1×10^4 pfu/ml to obtain final concentrations of essential oil ranging between 100 and 0.39 μg/ml (100.00–50.00–25.00–12.48–6.24–3.12–1.56–0.78–0.39 μg/ml) and a multiplicity of infection (MOI) of 0.02. For an appropriate comparison, vesicles without essential oil were also tested. Plates were incubated at 37 °C in 5% CO_2 incubator until the viral cytopathic effect (CPE) was observed in untreated virus control wells (usually 48–72 h). The reduction of the viral CPE in the essential oil containing wells in relation to cell controls and virus controls was determined by a quantitative tetrazolium-based colorimetric method. Thirty microliters of a 5 mg/ml solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma, St Louis, MO) in phosphate-buffered saline were added to each well to obtain a final concentration of MTT of 1 mg/ml. Plates were incubated at 37 °C for 4 h and the formazan product was dissolved with a mix solution consisting of 0.1 N HCl and 5% Nonidet P40 (Fluka Chemie GmbH, Buchs, Switzerland) in isopropanol. After a few minutes at room temperature to ensure that all crystals were dissolved, the plates were read using an automatic plate reader (Sunrise Tecan, Grödig/Salzburg, Austria) at 570 nm test wavelength with a reference wavelength at 690 nm. The 50% inhibitory dose values (ID_{50}), defined as the concentration of essential oil inhibiting 50% of the viral CPE, were calculated by regression analysis of the dose–response curves generated from the data.

2.11. Cellular toxicity assay

Cellular toxicity of free, SUV- and MLV-incorporated essential oil of *A. arborescens* was tested in vitro by a colorimetric cell viability assay [14]. In brief, monolayers of Vero cells in 96 multiwell plates were incubated with free and vesicular essential oil at concentrations ranging between 100 and 0.39 μg/ml in RPMI 1640 for 48 h and the medium replaced with 50 μl of 1 mg/ml of MTT in RPMI without phenol red. Cells were incubated at 37 °C for 3 h, the untransformed MTT was removed and 50 μl of acid-isopropanol (0.04 N HCl in isopropanol) were added to each well. The plates were then read using an automatic plate reader with a 570 nm test wavelength and a 690 nm reference wavelength.

3. Results and discussion

Distillation of the aerial part of *A. arborescens* in a steam apparatus (Clevenger-type) gave a blue essential oil (EO) in good yield (0.8%). The oil slowly became green when stored in the presence of air and/or light. The analysis of EO confirmed the literature data [15], that is that the oil composition is a complex mixture of organic compounds among which monoterpene ketones β-thujone and camphor represent more than 50% of the essential oil.

Chamazulene, which is responsible for the blue colour of the volatile oil, is also one of the main components. In Table 2, the composition of the most abundant molecules of the essential oil is given, while the complete characterization of the EO from *A. arborescens* will be discussed in another paper. Three different vesicular formulations were prepared using the essential oil. Their composition (Table 1) is the result of a formulation study during which several ratios of essential oil to lipids were evaluated. Best results in terms of incorporation yields and stability were obtained using compositions of Table 1. In particular, liposomes were prepared using hydrogenated soy phosphatidylcholine (P90H, **1**) or soy phosphatidylcholine (P90, **2**), while niosomal formulations were made with polyoxyethylene (4) lauryl ether (Brij® 30, **3**). In order to exclude the presence of emulsion droplets we purified vesicles from the unincorporated material by ultracentrifugation [16]. Moreover, the morphology of the prepared vesicles was evaluated using TEM, light polarization and optical microscopy. As an example, Fig. 1 shows a photomicrograph of the prepared multilamellar niosomes where it can be seen that the external multilayered membrane is very clear. As can be seen in Fig. 2a, a further confirmation of vesicle formation was achieved by light polarization microscopy that revealed Maltese crosses, which are typical for lamellar phases, while optical microscopy excluded the presence of oil droplets (Fig. 2b). Dynamic light scattering analyses showed that vesicle sizes are strongly dependent on bilayer composition and drug load. In fact some differences in size among the studied formulations can be observed (Table 3). Firstly, empty vesicles are always larger than the essential oil-loaded counterparts. This behaviour has already been described in the literature for liposomes incorporating essential oil as well as lipophile drugs, as a consequence of the higher cohesion and packing among the apolar chains in the vesicular membrane [10]. The same behaviour was

Table 2
Main components of *Artemisia arborescens* essential oil as determined by GC and GC–ITMS

Component	Retention time, R_t	Area%
α-Pinene	5.25	3.17
β-Thujone	11.32	23.97
Camphor	11.50	35.73
Terpinen-4-ol	13.85	2.20
Chamazulene	35.70	7.66

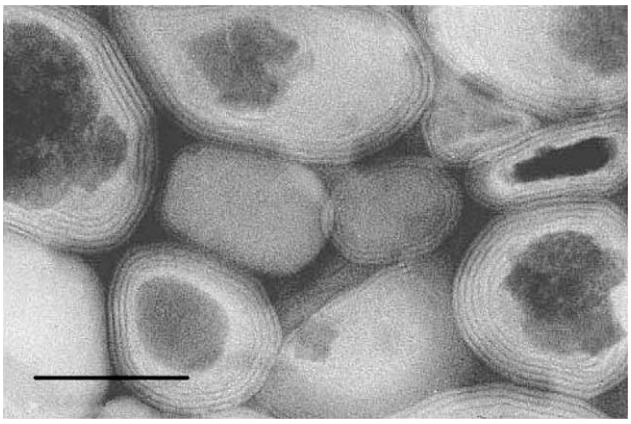

Fig. 1. Negative stain electron micrograph of *Artemisia arborescens* essential oil-loaded multilamellar niosomes made with Brij® 30; bar = 100 nm.

also found when the essential oil was incorporated in non-ionic surfactant vesicles made from Brij 30 (Table 3).

As known, sonication generally gives small unilamellar vesicles (SUVs, size less than 100 nm) whose size depends on experimental conditions, bilayer composition and drug load. In this study, mean size of sonicated oil-loaded vesicles ranged from about 70 to about 150 nm, while empty vesicles were larger (134–207 nm). We still call them SUVs because they are unilamellar and are prepared by sonication.

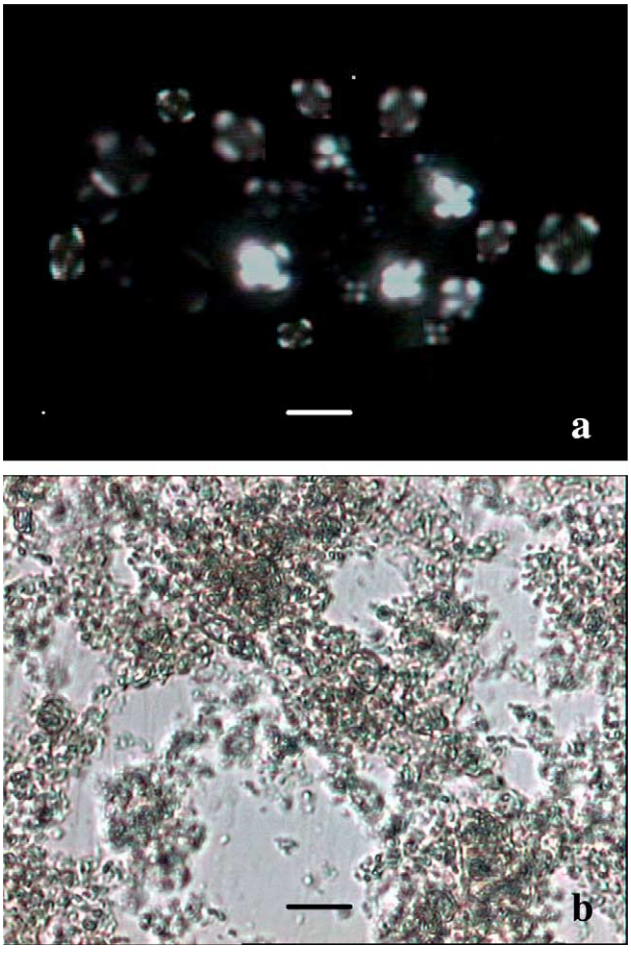

Fig. 2. Optical micrographs of *Artemisia arborescens* essential oil-loaded multilamellar liposomes made with P90H. (a) Light polarization microscopic image showing Maltese crosses; (b) optical microscopic image; bar = 5 μm.

Both multi- and unilamellar vesicles showed a good capability (60–74%) of entrapping the essential oil. Little differences in incorporation efficiency related to the composition of vesicle formulation were observed. In fact, non-ionic surfactant vesicles always showed a lower incorporation capability than phospholipids as a consequence of the higher hydrophilicity of the niosomal bilayer in comparison with the liposomal membrane. SUV dispersions always gave a lower content in incorporated essential oil than MLVs because of their smaller size and unilamellar structure.

All prepared formulations showed a good stability for more than one year when stored at 4–5 °C. Stability was checked by measuring size distribution and essential oil content of the different formulations at time intervals (Table 4). Results showed that formulations were very stable for the first six months. Indeed after six months oil leakage was very low and at least 95% of the incorporated oil was already associated with liposomes while vesicle sizes increased slightly (5–10%). TEM analyses also confirmed the bilayer integrity. On the contrary, after one year at 4–5 °C, oil retention was still good (90–92%) while vesicle size had increased by about 40–45%. These results as well as TEM analyses, which still did not show any bilayer deterioration, suggest that fusion of vesicles had occurred (Fig. 3).

Cellular toxicity studies carried out on Vero cells showed that niosomal (Brij® 30) vesicles incorporating oil were toxic at the studied concentrations. Therefore, the in vitro antiviral activity of *A. arborescens* essential oil was evaluated only using the liposomal carriers.

The in vitro activities of the free and liposomal *A. arborescens* EO against HSV-1 were determined by reduction of the viral CPE in Vero cells using a MTT colorimetric method. The evaluation of the antiviral activity using the CPE inhibition assay, based on the reduction of tetrazolium salts such as MTT, is a well-established procedure. This method has been used for the assay of various antiviral agents against several viruses as well as for cytotoxicity assay [17,18]. For an appropriate comparison, the antiviral activity of free and liposome-loaded essential oil was compared with that of 'empty' liposomes. Performed experiments indicated that empty liposomes (MLVs or SUVs) did not show any detectable antiviral activity.

Results are shown in Fig. 4. Using this method, the free essential oil showed a poor activity against HSV-1. At the highest concentration employed (100 μg/ml) the free essential oil induced a 22.86% inhibition. No significant differences in the antiviral activity were observed by including the essential oil in P90H SUVs (21.1% inhibition at 100 μg/ml), while the inclusion in P90 SUVs led to

Table 3
Characterization of the prepared formulations: mean size, polydispersivity index (PI) and incorporation efficiency

Formulations and main lipid component	Vesicle structure	Mean size (nm ± SD)	PI	Incorporation efficiency (% essential oil)
1 (P90)	MLV empty	457 ± 42	0.521	–
1 (P90)	MLV essential oil-loaded	252 ± 29	0.363	74.15 ± 0.35
1 (P90)	SUV empty	207 ± 30	0.523	–
1 (P90)	SUV essential oil-loaded	123 ± 21	0.346	65.90 ± 0.65
2 (P90H)	MLV empty	327 ± 46	0.346	–
2 (P90H)	MLV essential oil-loaded	232 ± 25	0.351	71.42 ± 0.58
2 (P90H)	SUV empty	134 ± 21	0.524	–
2 (P90H)	SUV essential oil-loaded	78 ± 11	0.579	66.21 ± 0.85
3 (Brij® 30)	MLV empty	402 ± 18	0.521	–
3 (Brij® 30)	MLV essential oil-loaded	304 ± 21	0.363	66.09 ± 0.48
3 (Brij® 30)	SUV empty	174 ± 23	0.523	–
3 (Brij® 30)	SUV essential oil-loaded	104 ± 19	0.346	60.14 ± 0.91

Table 4
Average mean size and oil retention during conservation at 4–5 °C for one year of the multilamellar vesicular formulations

Days after preparation	Average mean size (nm ± SD) MLV			Percentage of oil retained MLV		
	1	**2**	**3**	**1**	**2**	**3**
7	252 ± 38	232 ± 25	306 ± 29	100 ± 0.20	100 ± 0.30	100 ± 0.40
30	265 ± 25	235 ± 30	300 ± 30	99 ± 0.90	98 ± 0.88	99 ± 0.95
90	272 ± 24	240 ± 22	324 ± 23	98 ± 1.00	98 ± 0.12	97 ± 1.20
180	275 ± 20	248 ± 35	320 ± 20	97 ± 1.20	96 ± 1.10	95 ± 1.65
360	352 ± 48	335 ± 42	408 ± 35	93 ± 3.38	92 ± 2.35	91 ± 1.80

a further decrease in the activity (8.1% inhibition at 100 μg/ml). As a consequence of their poor antiviral activity, the EC_{50} values of the free essential oil, P90H and P90 SUVs against HSV-1 were not determined since they demonstrated toxicity at concentrations higher than 100 μg/ml.

When the EO was entrapped in MLVs, a significant increase in the antiviral activity of *A. arborescens* was observed with respect to the free oil. EC_{50} values of 18.5 and 43.6 μg/ml were determined for P90H MLV and P90 MLV, respectively. A 100% reduction of viral CPE was observed employing P90 MLV 100 μg/ml and P90H MLV 50 μg/ml and the reduction of 22.86% of viral CPE induced by 100 μg/ml of free essential oil was determined at 5.95 μg/ml employing P90H MLV.

The differences in antiviral activity found between MLV and SUV liposomes could be due to a better settling of the largest multilamellar vesicles next to the cells in culture, as reported by other researchers [19]. However, it could also be due to a higher leakage of the essential oil components from the smallest and unilamellar vesicles in the presence of the culture medium RPMI 1640 supplemented with 10% foetal bovine serum and penicillin, streptomycin and amphotericin B and therefore containing several compounds capable of interacting with the liposomal bilayers. In order to verify this hypothesis, we studied the stability of both multilamellar and unilamellar vesicles when dispersed in the culture medium for 24 h. The study was carried out by analysing the retention (*R*%) of the main essential oil components in GC/ITMS. Obtained results are shown in Table 5, where the percentage of the main essential oil components retained in the vesicular formulation after 24 h of treatment with RPMI medium are reported in comparison with that of freshly prepared formulations. It is evident from these data that after 24 h of treatment with the cell culture medium, SUVs made either with P90 or P90H

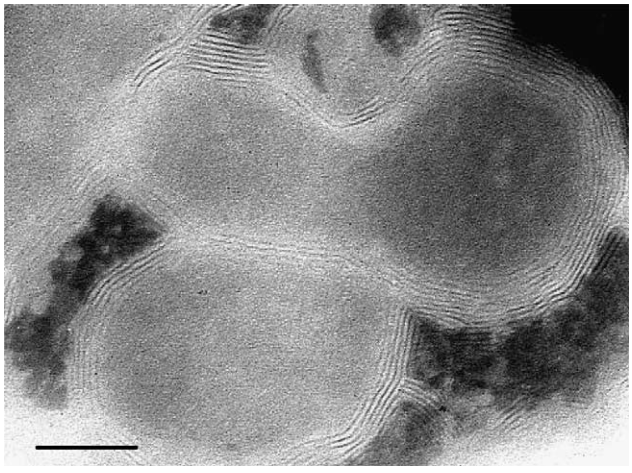

Fig. 3. Negative stain transmission electron micrograph of *Artemisia arborescens* essential oil-loaded P90H multilamellar liposomes after one year of storage at 5 °C (bar = 100 nm).

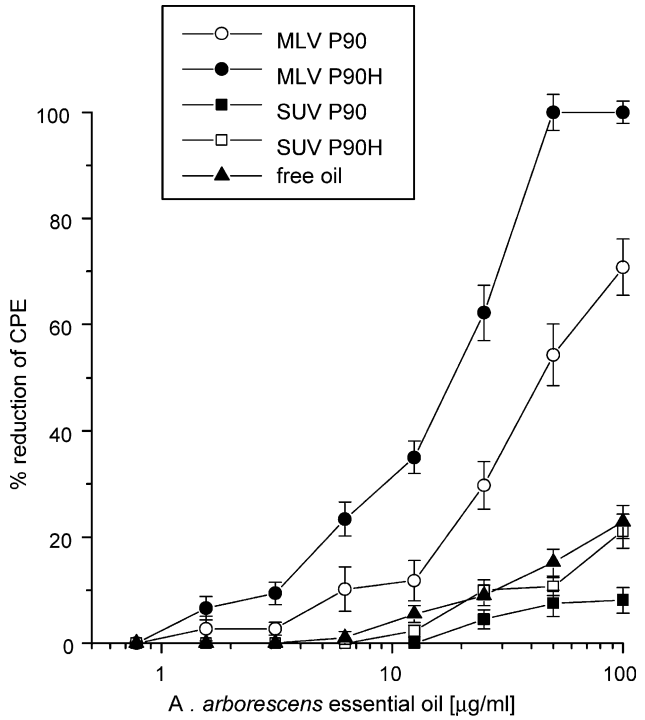

Fig. 4. Antiviral activity of *A. arborescens* essential oil as determined by the reduction of viral CPE. Vero cells were infected with HSV-1 (MOI 0.02) and incubated in the presence of serial dilutions of free essential oil (upward triangle), P90H MLV (solid circle), P90 MLV (open circle), P90H SUV (solid square), P90 SUV (open square) in RPMI 1640 until the viral cytopathic effect was observed in untreated virus control wells and then processed as described. The data represent the mean for six replicates of four separate experiments.

Table 5
Main essential oil components retained (*R*%) in the studied vesicular formulations (freshly prepared and after 24 h of treatment with RPMI medium) in comparison with free essential oil (100%)

Formulation, main component and vesicle structure	β-Thujon (*R*%)	Camphor (*R*%)	Chamazulene (*R*%)
1 P90 MLV freshly prepared	73.45	71.25	72.65
1 P90 MLV (RPMI medium)	54.34	52.67	71.99
1 P90 SUV freshly prepared	64.18	63.98	65.23
1 P90 SUV (RPMI medium)	–	–	52.35
2 P90H MLV freshly prepared	70.09	68.93	68.45
2 P90H MLV (RPMI medium)	63.21	62.13	63.83
2 P90H SUV freshly prepared	64.78	62. 44	65.03
2 P90H SUV (RPMI medium)	Traces	–	59.00

presented a high leakage of the main essential oil components with the only exception of the azulene derivatives. On the contrary, P90 and P90H MLVs showed a good retention of camphor, β-thujon and azulene derivatives whose concentrations were still similar to those of the freshly prepared liposomal formulations. Therefore, the results of this study suggest that larger and multilayered liposomes are better carriers than smaller and unilamellar vesicles for in vitro antiviral assay because they are more stable in the presence of the complex mixture used as the cell culture medium.

The liposome approach has been used successfully in the treatment of specific diseases in vivo to enhance drug targeting to cells [19,20]. The liposomal drug entrapment has also been applied in order to improve pharmacokinetics and therapeutic efficacy [21] and to reduce the toxicity of antiviral drugs [22]. The aim of this work was to determine whether liposomal incorporation of *A. arborescens* essential oil affects its antiviral properties in vitro. We found that liposomal incorporation in MLVs enhanced the antiviral activity against HSV-1 as determined by the reduction of viral CPE. P90H MLV showed a higher activity than P90 MLV (EC_{50} values of 18.3 and 43.6 µg/ml for P90H MLV and P90MLV, respectively), while no significant differences of the antiviral activity were observed between the free essential oil and SUV vesicles. These results are particularly interesting since the free essential oil showed a poor activity as determined by both the reduction of viral CPE assay and plaque reduction assay, while a virucidal activity against extracellular HSV-1 was detected at concentrations as low as 2 µg/ml (manuscript in preparation, data not shown). However, the virucidal activity was not affected when the essential oil was incorporated in MLVs or SUVs. These observations are consistent with findings of other investigators and confirm our previous data obtained with essential oil containing liposomes for which the virucidal activity did not increase [10]. Our results showed that the incorporation of *A. arborescens* essential oil in multilamellar liposomes greatly improved its activity against intracellular HSV-1.

Before our study on liposomal *S. insularis* essential oil [10], vesicular systems had never been studied as a vehicle for these natural products, which are usually formulated in emulsions [23–25]. Results obtained during this work clearly indicate that liposomes have several advantages over traditional vehicles. In particular, the liposomal approach can be useful both in enhancing the targeting of essential oils to cells and in delivering antiviral agents that cannot cross or cross with difficulty the cytoplasmatic barrier.

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