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Complementary effect of Zingiber officinalis extract and citral in counteracting non allergic nasal congestion by simultaneous loading in ad hoc formulated phospholipid vesicles

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Abstract

Natural nasal spray formulations were prepared by using Zingiber officinalis (Z. officinalis) extract and citral synergically loaded into specifically designed phospholipid vesicles. Phospholipid vesicles were selected according to their stabilizing effect on the nasal mucosal barrier, and their effectiveness was further potentiated by the co-loading of Z. officinalis extract as antioxidant and anti-inflammatory agent, and citral as antibacterial molecule.

Cryo-TEM images confirmed the formation of morphologically homogeneous and small vesicles, sized around 100 nm, negatively charged (-44 mV) and highly biocompatible (viability ≥100%) as detected by using epithelial cells. The analysis of size distribution of sprayed droplets, average velocity module and spray cone angle suggested a good aptitude of the vesicles to be nebulized and their effective deposition in the nasal cavity. Moreover, vesicles were effectively capable of inhibiting some nasal pathogenic bacteria (i.e. Streptococcus pyogenes, Staphylococcus aureus, Escherichia coli) and to protect the epithelial cells against oxidative damage. The formulations are natural and safe, and all of them have shown promising technological and biological properties suggesting their possible application in the nasal cavity for the treatment of congestions and non-allergic rhinitis.

Introduction

In the last years the interest towards natural products has grown continuously especially among the new generation of consumers. The society today is more vigilant about ecological and environmental issues, and the spread of green philosophies and lifestyles has contributed to a greater awareness of consumers' health, which find natural derivatives more attractive than synthetic compounds. Given that, scientific community is focusing on the research of natural products as valid alternative to prevent degenerative or chronic pathologies or treat a wide range of diseases, especially in patients with mild and moderate symptoms [1]. Local treatments with phytochemicals offer many advantages over systemic administration, directly reaching the diseased area with minimal invasiveness, minimizing side effects and facilitating both auto medication and patient adherence to the therapeutic plan. Mucosae have been recognized as attractive application sites for local administration of therapeutics due to their enhanced permeability, high accessibility and reduced barrier function [2]. Nasal mucosa is characterized by porous epitheliums, large absorption area, and low enzyme activity, which make it an advantageous administration route for local therapy of nasal diseases [3]. Inflammation with or without bacterial infections of nasal mucosa and paranasal sinus are commonly encountered in diagnostic histopathology. They may be limited to the nasal cavity (rhinitis) or to the paranasal sinuses (sinusitis) or, as in most cases, both sites can be involved (rhinosinusitis) [4]. Their large incidence is caused by the primary and constant contact of nasal mucosa with the outside environment and, therefore, with physical, chemical and infectious agents [5]. As defense, nasal mucosa creates a physical barrier by secreting antimicrobial peptides and expelling foreign molecules through mucociliary clearance.

According to the new philosophy of safe and natural treatments, several natural-based products have been designed for the local treatment of nasal diseases. They are usually delivered by spray pumps, but to provide an effective local effect they must have adequate spray plume and droplet size distribution. These parameters can positively affect the deposition zone, improving the beneficial effect of the bioactives at local level, by enhancing their accumulation and efficacy in the anterior nose [6]. Nasal sprays containing liposomes are natural alternative mainly composed of phospholipids, which supplement the components of mucosal cells restoring the protective secretion film, whose functions are moistening, defense, and mucociliary clearance. Liposome's effectiveness can be improved by the incorporation of other natural components or extract exerting anti-inflammatory, antioxidant, and antibacterial activities.

In this study, aiming at preparing a total natural and green nanotechnological formulation for the local treatment of nasal congestions, liposomes were enriched with sodium deoxycholate thus obtaining transfersomes, and citral (3,7-dimethyl-2,6-octadienal) and Z. officinalis extract were simultaneously loaded. Sodium deoxycholate is a bile surfactant, which acts by destabilizing cellular membranes, thus improving the chemical penetration and absorption in the nasal cavity [7]. Citral is a monoterpene aldehyde naturally found in herbs, plants and oils such as lemongrass (Cymbopogon citratus) with antioxidant, anti-bacterial and anti-fungal efficacy, along with expectorant and spasmolytic properties [8]. However, it is unstable and sensitive to oxygen, temperature and light, and poorly soluble in water, thus its loading into phospholipid vesicles can enhance stability, solubility, bioavailability and antimicrobial efficacy [9,10]. Z. officinalis contains various phytochemicals such as flavonoids, carbohydrates, proteins, alkaloids, glycosides, saponins, steroids, terpenoids, tannin and phenolic compounds [11]. Phenolic compounds, responsible of its peculiar taste and odor, also exert a pharmacological activity. Among all, gingerol is the most abundant phenolic compound and provides anti-bacterial, anti-oxidant, antifungal, antiviral and anti-inflammatory activities [12]. Z. officinalis extract is generally contained in products for topical application, chewable tablets and inhalable dispersions [13]. According to Stappen et al. the inhalation is the most effective administration method for the treatment of infectious diseases of the upper respiratory tract and respiratory diseases, such as cough, cold and asthma, acting as antibacterial and anti-inflammatory [13,14].

The main physicochemical properties (i.e., mean size, electrical charge, and entrapment efficiency) and the feasibility to be sprayed in the nose apparatus (i.e., droplet size distribution, average drop velocity and initial spray plume angle) of prepared vesicles were measured. The cytotoxicity of the formulations, the ability to protect epithelial cells against oxidative stress along with the antibacterial efficacy against Streptococcus pyogenes, Staphylococcus aureus, Escherichia coli were determined in vitro.

Materials and methods

Materials

Soy phosphatidylcholine Phospholipon® 90G (P90G) was purchased from AVG S.r.l (Garbagnate Milanese, Italy). Extract of *Z. officinalis Roscoe* containing 5% of gingerol was purchased by Farmalabor Srl (Milan, Italy). Citral, sodium deoxycholate and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (Milan, Italy). Reagents and plastics for cell culture were purchased from Life Technologies Europe (Monza, Italy).

Liposome preparation

Phospholipid P90G (90 mg/ml) was mixed with sodium deoxycholate (10 mg/ml) and hydrated with water. Dispersion was sonicated (15 cycles, 5 on /2 off, amplitude 13) using a Soniprep 150 sonicator (MSE Crowley, London, UK). After sonication citral (180 mg/ml) and *Z. officinalis* extract (10 mg/ml) were added and the dispersion was further sonicated (15 cycles, 5 on /2 off, amplitude 13 μ). Empty vesicles without citral and *Z. officinalis* extract were prepared as well and used as reference.

Liposome characterization

Observation of vesicles by cryogenic transmission electron microscopy (cryo-TEM) was performed using a TECNAI G2 20 TWIN (FEI), working at 200 KeV voltage in a bright-field and low-dose image mode as previously reported [15].

Average diameter, polydispersity index and zeta potential of vesicles were determined by using a Zetasizer Ultra (Malvern Instruments, Worcestershire, UK) [16]. These parameters were monitored for 1 months at 4 ◦C to evaluate the long-term stability of vesicles. Before the analyses samples were diluted (1:100) with water to be optically clear and avoid the attenuation of the laser beam by the particles and measured at 25 ◦C (Oleuropein Mo Nanomedicine).

The non-incorporated bioactives was eliminated by dialysis method against water for 2 h and replacing the water after one hour, as previously reported [15]. The antioxidant activity of samples, before and after the dialysis process, was measured by means of DPPH colorimetric test. 20 μl of each formulation was dissolved in 1980 μl of DPPH methanolic solution (40 μg/ml) and the obtained solutions were incubated in the dark for 30 min at room temperature. Then, the absorbance was measured at λ = 517 nm by means of a UV spectrophotometer (Lambda 25, Perkin Elmer, Milan, Italy). All experiments were performed in triplicate. The antioxidant activity (%) was calculated according to Casula et al. [15].

The entrapment efficiency of the vesicles was expressed as the percentage of the antioxidant activity after dialysis versus the value found before dialysis [10].

Droplet size distribution

Droplet size distribution was evaluated using a Malvern Spraytec® laser diffractometer (Malvern Panalytical Ltd., Malvern, U.K.). Formulation (6 ml) was loaded in commercial devices (20 ml) kindly supplied by FAES laboratories. According to Food and Drug Administration (FDA) [17], experiments were performed at room temperature in triplicate at 4 and 7 cm of distance from the nozzle exit [15]. The device was turned at a 45◦ angle respect to the laser beam. The focal length of the lens was 300 mm; the dispersant refractive index was 1.00 + 0.000i and the particulate refractive index was 1.33 + 0.000i (water droplets in air). Data were reported as volume diameter expressed as 10%, 50% and 90% of the ejected volume. Span was also reported as correlation value among D10, D50 and D90, as (D90-D10)/D50.

Spray structure, drop average velocity module and spray angle

The average velocities of the drops and the initial spray opening angle of vesicles were determined by means of Particle Image Velocimetry and by laser plane visualization [15]. Instantaneous images of the atomization process were obtained with a Hamamatsu (1024 × 1344 pixels 12-bit C4742-95-12 ORCA-ER) charge-coupled device camera (Hamamatsu Photonics, Shizuoka, Japan) equipped with a Nikon 50 mm F#1.2 lens. To freeze the motion, a Quanta System model PILS Nd: YAG laser (Quanta System, S.p.A., Milan, Italy), was used illuminating a vertical plane across the center of the spray. PIV image pairs were acquired with a time interval of 30 µs and processed with the CCDPIV computer code (Laboratory for Turbulence Research in Aerospace and Combustion (LTRAC) in Monash University, Melbourne, Australia).

The spray cone angle was determined tracing the limits of the spray in an image obtained averaging 100 instantaneous frames [15]. To actuate the atomizers avoiding human errors, a pneumatic device was used, with an atomization pulse duration set to 150 ms. The instantaneous images were acquired 83 ms after the start of the atomization.

Biocompatibility of extract loaded vesicles in keratinocytes

Human epithelial cells (HaCaT) were grown as monolayers in 75 cm^2 flasks, incubated with 100% humidity and 5% CO2 at 37°C. Cells were cultured with Dulbecco's Modified Eagle Medium (DMEM) with high glucose, supplemented with 10% of fetal bovine serum and penicillin/streptomycin. The cells were seeded into 96-well plates at a density of 7.5 × 103 cells/well and after 24 h of incubation, were exposed for 48 h to the formulation properly diluted to reach different concentrations of citral (18, 1.8, 0.18 µg/ml) and *Z. officinalis* (1, 0.1, 0.01 µg/ml). As comparison, citral and *Z. officinalis* dispersed in water at the same dilutions were tested. The possible toxic effect of the formulations towards HaCaT cells was assessed by measuring cell viability by means of the MTT (tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test as previously reported [18]. All the experiments were repeated at least three times and each time in triplicate. The results are expressed as the percentage of viable cells compared to untreated cells (100% viability).

Protective ability of extract loaded vesicles against oxidative damage induced in cells

HaCaT cells ($5x10^4$ cells/well) were seeded in 96-well plates with 200 µl of culture medium and incubated at 37°C for 24 h, then stressed for 4 h with hydrogen peroxide (1:50,000) and simultaneously treated with the vesicular formulations (citral 18, 1.8, 0.18 µg/ml and *Z. officinalis* 1, 0.1, 0.01 µg/ml). Unstressed cells were used as positive control (100% viability); cells stressed with hydrogen peroxide and untreated were used as negative control. After 4 h of incubation the medium was removed and the viability of the cells was determined by the MTT colorimetric test, as reported above.

Antibacterial Assay

The antibacterial activity of vesicles was measured by Kirby Bauer agar diffusion method. The experimental procedures were performed by using three different Gram-positive and Gram-negative bacterial species, already described in nasal infections [19]: Staphylococcus aureus ATCC 6538 (American Type Culture Collection), Streptococcus pyogenes clinical isolate NC4, Escherichia coli ATCC 7075. The antimicrobial susceptibility testing was done by a modified procedure derived from the CLSI guidelines (https://clsi.org/) [20]. Briefly, a suspension containing 1·107 bacterial cells/ml was inoculated onto the surface of a 90 Ø Petri plate containing an agarized growth media: (i) Muller-Hinton agar was used for S. aureus and E. coli, while Schaedler agar for S. pyogenes (Microbiol Uta, Cagliari). 100 ml of each undiluted formulation was put inside a well performed on the growth medium’s surface. The antimicrobial activity was expressed as mm of inhibition diameter around the well after the microbial growth at 37 ◦C in 5% CO2 for S. pyogenes and at 37 ◦C in the air for other strains. All experiments were performed in triplicate.

Statistical analysis

Statistical analysis was performed by processing the collected data by ANOVA, T-Test and F-Test. Results were expressed as mean value ± standard deviation. The minimum level of significance chosen was $p < 0.05$.

Results

Aiming at obtaining phospholipid vesicles with a powerful antioxidant, antiinflammatory and antibacterial effect, several vesicular formulations were prepared by using different preparation methods, phospholipids, surfactants, and water co-solvents and loading increasing amount of both citral and *Z. officinalis* extract. After several attempts, citral (180 mg/ml) and *Z. officinalis extract* (10 mg/ml) were simultaneously loaded adding sodium deoxycholate (10 mg/ml) to phospholipid and obtaining transfersomes.

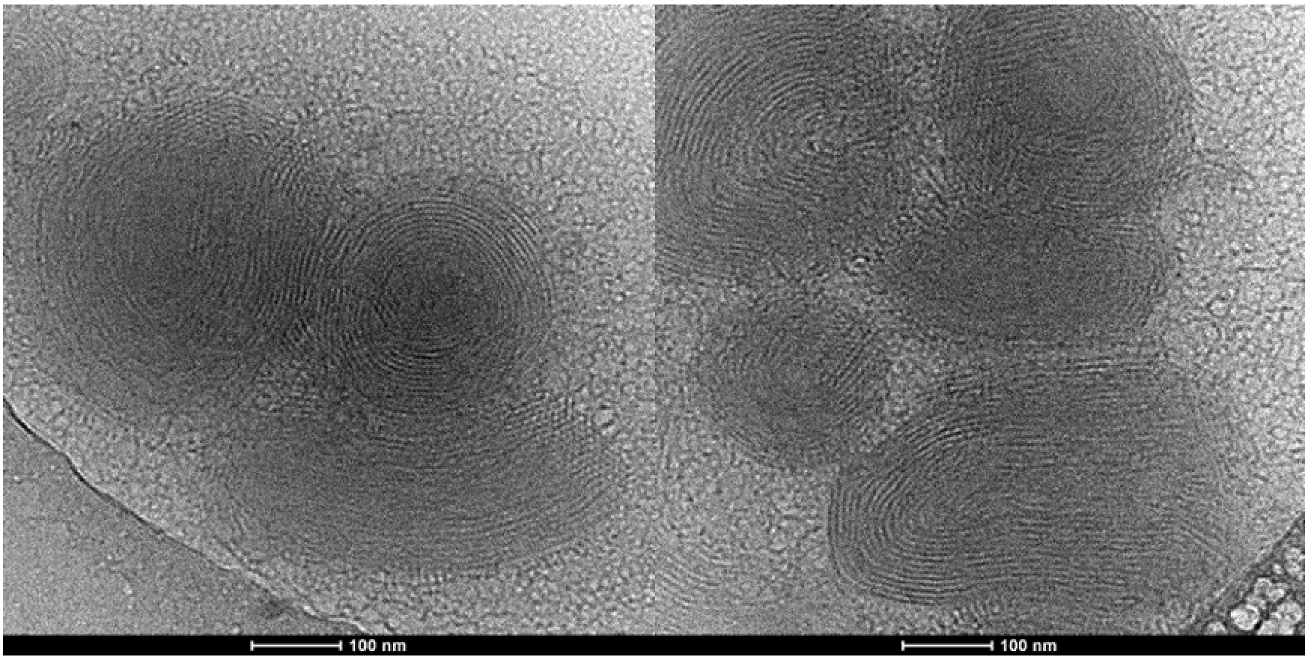

Fig. 1. Representative cryo-TEM images of transfersomes loading citral and *Z. officinalis* extract

Vesicle characterization

Cryo-TEM images disclosed the formation of multilamellar and spherical transfersomes (Fig. 1). The mean diameter and zeta potential of transfersomes loading citral and *Z. officinalis* extract were measured along with that of empty vesicles, used as comparison (Table 1).Transfersomes loading citral and *Z. officinalis* extract were slightlylarger than the empty vesicles, indicating a contribution of both bioactivesin the bilayer assembling. The zeta potential was highly negative and the low polydispersity index (0.21) indicated a monodispersed sample.

Table I. Mean diameter (MD), polydispersity index (PI), zeta potential (ZP) of empty and bioactive loaded vesicles, and entrapment efficiency (EE) of the empty and extract loaded vesicles. Data represent the means ± SD of at least six replicates. Each symbol (*, +, #, °) indicates a value statistically different from the others (p>0.05).

	MD (nm)	PI	ZP (mV)	EE (%)
Empty PEVs	*90±2	0.2	°-54±5	
Citral EO+Z. officinalis liposomes	+110±1	0.21	#-44±3	74±10

Droplet size distribution

Vesicle formulation were designed for nasal administration and the key parameters to reach the anterior cavity of nose (droplet size distribution, velocity and plume angle) were evaluated. The aptitude of citral and *Z. officinalis* extract in aqueous dispersion with sodium deoxycholate to be sprayed was measured as well, mainly aiming at evaluating the improvement due to vesicle loading. Sprayed particles size must be comprised between 30 and 70 µm for D50, and lower than 200 µm for D90 as recommended by the FDA. The size of droplets generated by transfersomes were very similar to those recommended, only slightly higher, confirming their suitability to reach the anterior cavities of nose (Table II)

Tab II. Distribution of droplet size generated by citral and Z. officinalis extract in dispersion or loaded in transfersomes, at 4 and 7 cm from laser beam. Mean values of three measurements ± standard deviations were reported. The same symbol ($^{\$}$, $^{\wedge}$, $^{@}$, *, °, $^{\#}$) indicates the same value.

		D10 (µm)	D50 (µm)	D90 (µm)	SPAN (µm)
4 cm	Dispersion	$^{@}$22 ± 0.5	°46 ± 1	$^{\#}$101 ± 11	2 ± 0.2
	Transfersomes	$^{\$}$27 ± 0.5	*49 ± 1.5	$^{\#}$94 ± 3	1 ± 0.05
7 cm	Dispersion	^28 ± 2	°46 ± 2	$^{\$}$78 ± 6	1 ± 0.1
	Transfersomes	^30 ± 0.5	*50 ± 1.5	$^{\#}$101 ± 13	1 ± 0.2

Measurements of spray plume morphology and angle

When sprayed, the aqueous dispersion of citral and extract produced a large plume with low droplet consistency, where the droplets were not uniformly distributed but split in the external areas. On the contrary, transfersomes generated a plume with a spatial structure initially corresponding to a hollow cone spray whose core is filled with drops (perhaps from the smallest fractions) downstream. Their plume was larger, and the outlet jet appears split into two more consistent parts, upper and lower, generating almost a hollow cone (Fig. 2).

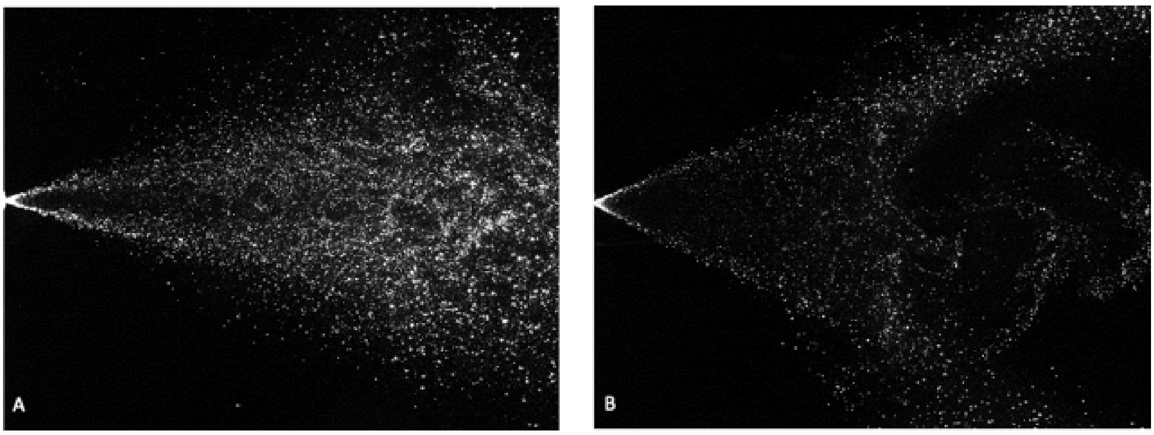

Figure 2. Representative images of instant visualization of sprays generated transfersomes (A) and water dispersion (B).

Measurements of average velocity module

The analysis of average velocity modules was performed by using citral and extract dispersed in water or loaded in vesicles (Fig. 3). The bioactive dispersion formed a plume shaped like a scissor with separate flows where the velocity developed. Instead, in the middle of the cone, a light and dark blue zone was presented indicating that the velocity of sprayed droplets decreased sharply (Fig. 3-A). On the contrary, the spray plume generated by transfersomes appeared more homogeneous in shape and velocity trend, that decrease in a coherent way from the nozzle (Fig. 3-B).

The high-speed and the angle obtained during the spray were measured (Table 3). The speed of transfersomes droplets was slightly lower (~13 m/s) than that of dispersion droplets (~15 m/s) angle of the spray generated by transfersomes was almost the half (~27°) than that of the dispersion (~48°).

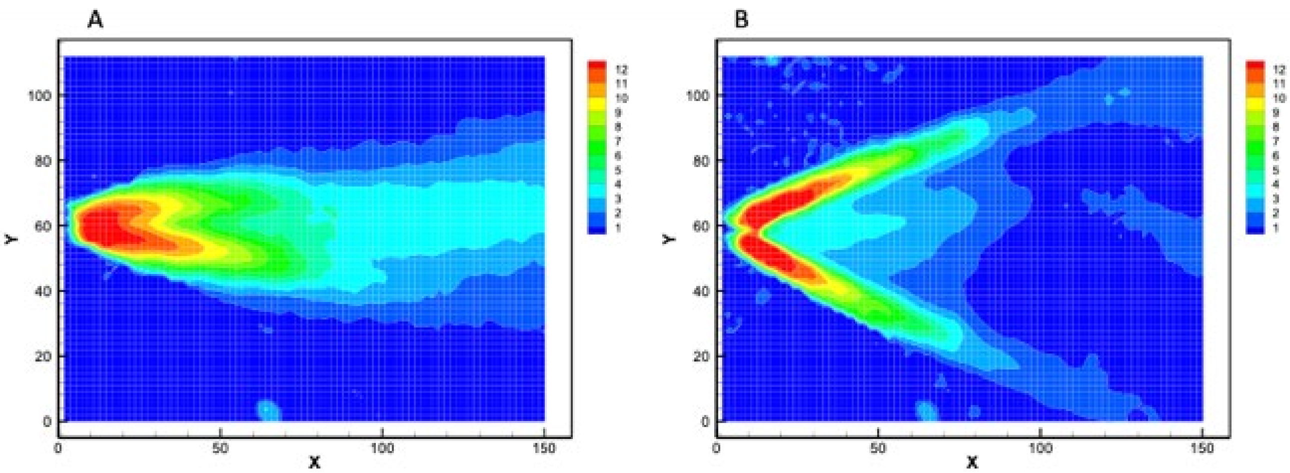

Figure 3. Images of distribution of average velocity of drops generated by transfersomes (A) and water dispersion (B).

Table III. High-speed values and spray angle measured from the plume generated by water dispersion and transfersomes.

	High-speed (m/s)	Spray angle (°)
Dispersion	15	48
Transfersomes	13	27

Antibacterial Assay

The combination of citral and *Z. officinalis* extract disclosed to be synergically efficient in counteracting the growth of S. pyogenes, S. aureus and E. coli (Table 4). Indeed, when combined in dispersion or in vesicles, they inhibited the proliferation of the three strains and the inhibition zone was ~ 30 mm, irrespective to the tested strain. The effectiveness of citral and Z. officinalis extract was the same in dispersion or in transfersomes, except against E. coli as the loading in transfersomes slightly improved the inhibition zone from ~27 to ~32 mm, probably thank to the structural architecture of the microorganism, which is Gram-negative, while S. pyogenes, S. aureus are Gram-positive. The hydrophobic lipopolysaccharide outer membrane of E. coli can interact with the liposomes prepared with phosphatidylcholine.

The effectiveness of citral dispersion or Z. officinalis extract in dispersion was evaluated as well to evaluate their contribution in counteracting the bacterial growth. Citral dispersion was less effective against S. pyogenes (15 ± 1 mm) and E. coli (11 ± 1 mm) than the dispersion containing both bioactives and was ineffective against S. aureus, indicating the contribution of Z. officinalis extract. The dispersion of extract alone did not disclose any activity (Table 4).

Results confirmed the synergic antibacterial performances of both citral and Z. officinalis extract.

Tab IV. Inhibition zone of *S. pyogenes*, *S. aureus* and *E. coli* provided by citral and *Z. officinalis* extract loaded in transfersomes or dispersed in water alone or in association. Mean values of three measurements ± standard deviations were reported.

	S. pyogenes (mm)	**S. aureus (mm)**	**E. coli (mm)**
Citral and extract dispersion	32±1	31±1	32±1
Citral dispersion	32±1	30±5	27±6
Extract dispersion	15±1	-	11±1
Citral and extract transfersomes	-	-	-

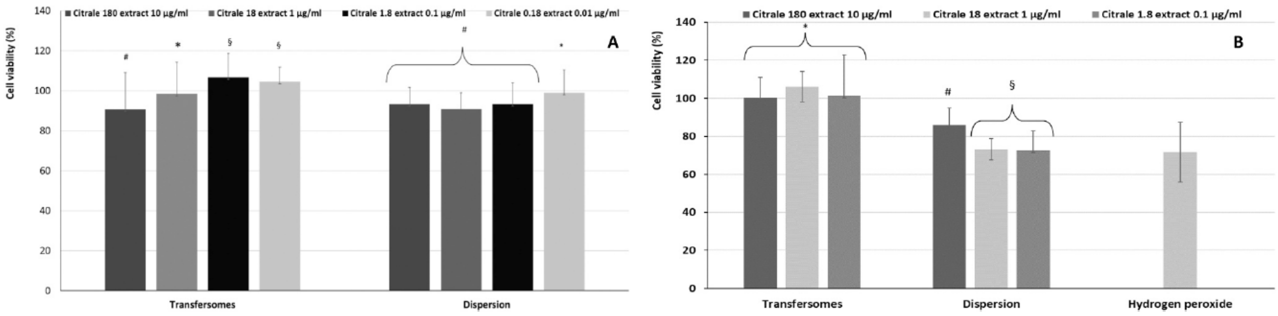

Fig. 4. A) Viability of keratinocytes treated with citral and *Z. officinalis* extract in water dispersion or loaded in transfersomes at different concentrations (citral 18,1.8, 0.18 µg/ml and *Z. officinalis* 1, 0.1, 0.01 µg/ml). Data represent the means±standard deviations of at least three experimental determinations. Each symbol (§, #) indicates the same value. B) Viability of cells stressed with hydrogen peroxide and treated with citral and *Z. officinalis* extract in dispersion or loaded in vesicles. Data are reported as mean values ± standard deviations of cell viability. Each symbol (*, #, §) indicates the same value.

Discussion

Previous studies disclosed that liposomes dispersions locally sprayed on nasal cavity are a valuable alternative to corticoids or antihistaminic drugs with an appreciable potential to reduce and care allergic and notallergic rhinitis. In addition, the loading of an antioxidant extract can improve the protective and regenerative effect of phospholipid vesicles [15]. Given that, in this work the effectiveness of phospholipid vesicles against rhinitis was improved by loading a natural antibacterial molecule (citral) and an effective antioxidant phytocomplex *(Z. officinalis* extract). The pre-formulation study performed testing different phospholipids, polymers, surfactants and cosolvents, allowed to select transfersomes, containing sodium deoxycholate as edge activator and loading 180 mg/ml of citral and 10 mg/ml of extract, as best formulation. Sodium deoxycholate was used thanks to its ability to destabilize phospholipid membranes enhancing the delivery performances of vesicles, especially in skin and mucosa [7]. Citral is an acyclic monoterpene with antibacterial activity, particularly against *Staphylococcus* genera and it is considered a promising candidate for the treatment of *S. aureus* infections resistant to other drugs [21]. To reinforce the antibacterial activity of citral and simultaneously provide a protection against oxidative damages, *Z. officinalis* extract was co-loaded in transfersomes. Indeed, this phytocomplex has been proposed as alternative system in drug resistant microbial diseases and to prevent the damages caused by reactive oxygen species [22]. The obtained transferosomes were small, monodispersed and negatively charged with features suitable for their deposition in the nasal mucosa of the anterior cavity of nose. Transfersome dispersion was sprayed in small droplets having a mean diameter ~50 µm, which may effectively deposit in the anterior part of the nose according to the FDA recommendations [15]. The plume angle of the bioactives in aqueous dispersion was almost double respect to that of transfersome dispersion. Moreover, the plume of the dispersion shaped like a scissor with separate flows in the peripheral areas and low droplets in the middle, while that generated by the transfersomes appeared narrower with high droplet concentration in the middle. This behavior is predictive of a prevalent deposition in the anterior region of the nose because the breadth of the plume is a key parameter affecting the area of deposition [23]. Thus, overall technological results confirmed that citral and Z. officinalis extract loaded transfersomes had suitable properties for the local delivery in the nose as after spraying a narrow (cone angle ~27°) and full cone of homogeneous droplets sized ~50 µm, having high-speed (~13 m/s) was formed. When deposited in the target site, the transferosomes may favor the accumulation of payloads inside the mucosa where they can exert the antioxidant, anti-inflammatory and antibacterial activities. The selected formulation has a protective effect on keratinocytes and avoid their death caused by the formation of free radicals generated by hydrogen peroxide. The effect was due to the antioxidant components of the Z. officinalis extract such as gingerol, pyrogallol p-hydroxybenzoic acid, ferulic acid and p-coumaric acid [24]. Citral is not a strong antioxidant but can synergically potentiate the antioxidant activity of other molecules.

The co-loading of citral and Z. officinalis extract led to reach a synergic activity, which potentiated the antimicrobial efficacy of formulation against three bacterial strains, *S. pyogenes*, *S. aureus*, *E. coli*, which typically colonize the first airways [20,25,26]. Indeed, *Z. officinalis* extract alone did not have any antimicrobial activity but when combined with citral increased the efficacy of this terpene also against S. aureus, as previously reported for the combination of citral and quercetin [27].

Finally, the antimicrobial and antioxidant effect of both payloads is associated to that of phospholipid vesicles, which are able to supplement phospholipid to the mucosal cells restoring the protective secretion film [28].

Conclusion

The overall results based on technological and physicochemical properties of citral and Z. officinalis extract loaded into transfersomes suggest that they are suitable to be used as nasal spray because they with the liposomes prepared with phosphatidylcholine.

The effectiveness of citral dispersion or Z. officinalis extract in dispersion was evaluated as well to evaluate their contribution in counteracting the bacterial growth. Citral dispersion was less effective against *S. pyogenes* (15 ± 1 mm) and *E. coli* (11 ± 1 mm) than the dispersion containing both bioactives and was ineffective against S. aureus, indicating the contribution of *Z. officinalis* extract. The dispersion of extract alone did not disclose any activity (Table 4).

Results confirmed the synergic antibacterial performances of both citral and *Z. officinalis* extract.

CRediT authorship contribution statement

Eleonora Casula: Investigation, Formal analysis, Data curation, Writing – original draft. Maria Manconi: Supervision, Project administration, Data curation, Writing – original draft. Tania Belen Lopez-Mendez: Investigation, Writing – review & editing. Jose Luis Pedraz: Supervision, Methodology, Validation, Writing – review & editing. Esteban Calvo: Methodology, Validation, Writing – review & editing. Antonio Lozano: Methodology, Validation, Writing – review & editing. Germano Orrù: Methodology, Investigation, Data curation. Sara Fais: Methodology, Investigation, Data curation. Marco Zaru: Resources, Writing – review & editing. Ines Castangia: Writing – review & editing. Maria Letizia Manca: Supervision, Methodology, Validation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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