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Cycle XXXIII

DESIGN, CHARACTERIZATION AND EFFICACY EVALUATION OF NATURAL NASAL SPRAYS BASED ON PHOSPHOLIPID VESICLES LOADING PLANT-EXTRACTS

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PhD Student: Eleonora Casula

Supervisor: Prof. Maria Manconi

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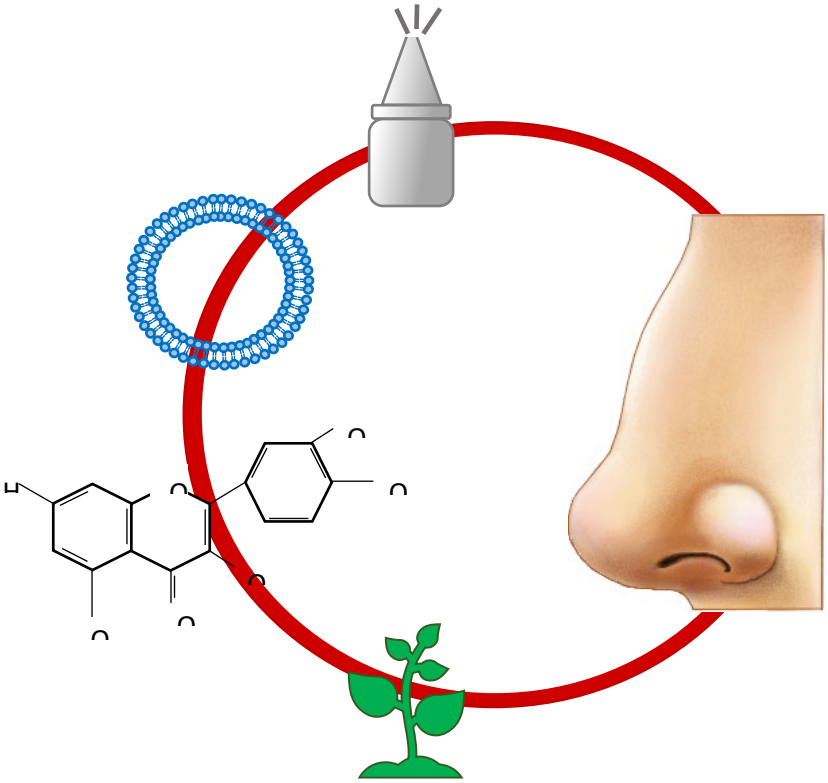
La mia Tesi di Dottorato è dedicata a tutte le Donne,
affinché un giorno tutte noi potremo accedere all'Istruzione,
valorizzare la nostra Intelligenza
e correre verso le nostre Ambizioni,
e che queste siano sempre le luci che ci guidano per arrivare ancora Oltre.

Abstract

Plants and their derivates have been used as medicines beneficial products for centuries and today is being re-discovered their usefulness for the human health is being re-discovered. The therapeutic properties of phytochemicals are re-evaluated under the light of medical and pharmacological research, pushed by a constantly growing market demand, where consumers trust more natural products than synthetic drugs. New studies are specifically focused on the effectiveness of phytochemicals against a wide range of ailments. In this thesis, the efficacy of topical formulations based on natural bioactive molecules in the treatment of nasal mucosal diseases, has been presented. Three nasal sprays based on phospholipid vesicles loading the extracts of *C. halicacabum L.*, *Z. officinalis* and co-loading *Z. officinalis* plus *citral*, were designed to counteract disorders involving the upper airways, such as rhinitis and rhinosinusitis. For each formulation, physico-chemical characteristics, spray geometry, biocompatibility and biological activity were evaluated.

Introduction



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An integrative review on the uses of plant-derived bioactives formulated in conventional and innovative dosage forms for the local treatment of damaged nasal cavity

Eleonora Casula, Maria Letizia Manca *, Maria Manconi

Dept. of Scienze della Vita e dell'Ambiente, University of Cagliari, Via Ospedale 72, 09124 Cagliari, Italy

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ABSTRACT

Plants and their derivates have been used as medicines for centuries and today is being re-discovered their usefulness for the human health. The therapeutic properties of phytochemicals are re-evaluated under the light of medical and pharmacological research, pushed by a constantly growing market demand, where consumers trust more natural products than synthetic drugs. New studies are enlightening the effectiveness of phytochemicals against a wide range of ailments, nevertheless very few evaluate the efficacy of topical formulations based on natural bioactive molecules in the treatment of nasal mucosal diseases.

This review aims at exploring this little covered topic. An overview on the properties and functionality of the nasal mucosa and the different diseases affecting it has been provided. We summarized various nasal dosage forms containing natural bioactive and explored how innovative delivery systems loading phytochemicals can improve the treatment results. Finally, the potential use of novel nanocarriers for the treatment of nasal ailments has been covered as well.

1. Introduction

Plants and derived products with therapeutic properties have been used since ancient times in traditional medicine for the treatment of different chronic diseases and minor disorders (Yuan et al., 2016). Throughout the development of human culture, the use of plants has had magical-religious significance associated with their beneficial and therapeutic activity. The 19th century saw the rise of synthetic organic chemistry, which had progressed to the point that large-scale development of non-natural drugs was economically feasible. New compounds were easily obtained, allowing structural modifications that enabled the production of potentially more active and safer drugs through carefully controlled processes (Rates, 2001). This resulted in a preference for synthetic drugs and an increase of the economic power of the pharmaceutical companies. On the other hand, the interest in natural drugs has somehow shown a decline. The length of time required to conduct efficient extraction processes, the large number of separation cycles needed to obtain pure compounds, and the difficulty to obtain commercial quantities, are factors that slowed down the advancement of natural drugs in the market. Nevertheless, the latter continue to attract interest because they provide unique structural diversity in comparison with standard combinatorial chemistry, thus providing opportunities for discovering mainly low-molecular weight lead compounds (Hoareau and DaSilva, 1999). In addition, in 2015, the Nobel Prize was awarded for the discovery of two natural drugs, avermectin and artemisinin, bringing phytotherapy into the spotlight (Tambo et al., 2015). The discovery of these compounds established new therapies to treat human diseases and inspire the scientific world to continue the search for natural drugs. They are an increasingly valuable alternative to synthetic drugs for the development of commercial products to be used not only in pharmaceutical, but also in cosmetic and food industries (Atanasov et al., 2015). Unfortunately, the pathway from the discovery of natural drugs to the manufacture of commercial products is long-lasting, costly and rather complex. It involves not only the discovery and evaluation of in vivo models of safety and clinical efficacy, but also the assessment of the eco-sustainability of natural sources (avoiding the harvest of wild biodiversity), the optimization of the extraction yield by using sustainable, efficient and cost-effective methodologies, and the improvement of their efficacy in vivo by using suitable formulations (Dias et al., 2012). Despite these limitations, natural bioactives remain an unmatched source of chemical scaffolds and possess a unique and broad chemical diversity that cannot be reached by combinatorial chemistry. They serve

* Corresponding author.
E-mail address: mlmanca@unica.it (M. Letizia Manca).

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as a source and inspiration for a large fraction of synthetic drugs and between 25 and 50% of currently marketed drugs owe their origins from natural products (Naman et al., 2017). Plants and plant-derived products still remain a valuable and safe source of health-promoting and therapeutic molecules (Shukla and Mehta, 2015). However, their pharmacological assays and phytochemical analyses have been carried out only for 6–15% of existing plant species.

Like synthetic drugs, natural bioactive molecules are usually formulated in dosage forms, even in traditional medicine, which involves the preparation of herbal formulations. Dosage forms facilitate the administration of a bioactive and the achievement of its beneficial effect. They are the products marketed for an intended use, made of a specific mixture of one or more active ingredients and inactive components (excipients or additives), which are functional to the preparation of the final products (Drumond et al., 2017). Excipients are used to facilitate the absorption of bioactives, adjust viscosity, improve stability, adjust organoleptic properties, and increase bulk or weight. They are selected and combined to prepare dosage forms in specific configurations, such as capsules, tablets, and syrups, containing a specific dose of bioactives. Dosage forms are used to: mask undesirable taste or odour of bioactives; stabilize the bioactives facilitating their storage; improve the efficacy of poorly water-soluble or insoluble bioactives; protect the bioactives from the destructive influence of gastric juice following oral administration; bypass the first-pass metabolism (Ozkan et al., 2020).

The choice of a specific dosage form is an essential parameter because it can affect the absorption and the bioavailability of the bioactives as a function of interactions that can occur between excipients and biological barriers (i.e., skin, mucosae and intestinal epithelium), and between excipients and bioactives. Different types of dosage forms and excipients can be used to maximize the beneficial effect of bioactives depending on the target, the characteristics of the bioactives, and the administration route (Garg et al., 2015).

Natural molecules are widely formulated as topical dosage forms for local applications in skin and mucosa, which are attractive application sites due to their large extension, which permit not-invasive applications (Peppin et al., 2015). In particular mucosa seems to be attractive because of its optimal accessibility, rapid repair and high permeability profile (Arora et al., 2002), especially that of nose and larynx, which are the outer surface of airways and the limit with the environmental air and its contents. In order to this, it is structured as the first line of defence system thank to the mucus and the clearance associated to the airway microbiome and protective mechanisms (Zhang et al., 2016). However, it is widely affected by moderate acute, subacute, chronic, seasonal or not-seasonal diseases, which can be limited to the nasal cavity (rhinitis) or also involve the sinonasal tract: only the paranasal sinuses (sinusitis) or both sites (rhinosinusitis). It may also be unilateral or bilateral (Barnes, 2020).

Actually, very few studies have been carried out to specifically evaluate the efficacy of topical formulations in the treatment of nasal mucosal diseases and less report the use of formulations based on natural bioactive molecules. The main results of these studies underline that the treatment of nasal mucosa with local therapies based on phytochemicals would provide important advantages. Among this literature, no review was provided about the use of phytochemicals and natural derivates for the treatment of nasal ailments. According to this, the present review aims to shed a light on this underdeveloped topic. The properties and functionality of the nasal mucosa and the different diseases affecting it, with special focus on rhinitis and rhinosinusitis have been exanimated and, a summary of tested nasal dosage forms containing natural bioactive molecules has been provided, highlighting their limitations and exploring how innovative delivery systems loading phytochemicals can improve the treatment outcomes. The potential use of promising and novel nanocarriers for the treatment of nasal diseases has been covered as well.

2. Structure and function of the nasal cavity

The human face is a complex system which undergoes to variations in its architecture as a consequence of evolution and adaptation to novel environments (Azevedo et al., 2017). The nose is part of this complex structure and represent the external door of airways, which mainly regulates the temperature and humidity from the outside, perceives olfactory stimuli, contributes to articulate sounds and words, to breathe and oxygenate body tissue (Djupesland, 2013). All these different functionalities are achieved by its complex structure and aerodynamics. Nose apparatus is articulated in cavities, valves and turbinate, covered by a filtering mucosa which is intended to counter potentially harmful external agents. Indeed, the nose is capable of filtering about 500 L/h of air, inhaling and processing more than 25 millions of particles of various nature (Bitter et al., 2011).

The nasal cavity can anatomically be segregated into five different regions: nasal vestibule, atrium, respiratory area, olfactory region and the nasopharynx (Arora et al., 2002). The external nose is formed by a pyramidal cartilage structure determined by the nasal bones shape, which at the base opens in two nasal valves, the external ones are the nostrils (Haight and Cole, 1983). Nostril's surface is covered by hairs which act as a filter for the incoming air against microorganisms, dust, pollution, and other potentially obstructive and breath inhibitors particles entering the airways. Approximately 2–3 cm from the nostril opening, nasal valves extend anterior and posterior to the head of the inferior turbinate (Cole, 2003) Beyond the nostrils opens the nasal cavity separated by the nasal septum into two cavities: the most anterior part is the nasal vestibule, which posteriorly extends to the nasopharynx. This region is a complex aerodynamic interface that works as the first line of defence against outside particles, consisting of finely tuned airfoils that direct airflow, adjust speed, generate vacuum and regulate power gas exchange between the paranasal sinuses and the lungs. In fact, inspiratory and expiratory air flow dynamics are reversing exchange direction. The shape, angle, and orientation of each turbinate is designed to facilitate the gas exchange through generation of vacuum at the contiguous sinus (Gungor, 2013). This complex aerodynamic system is divided by the 3 folds of the superior, middle and inferior turbinate, considered to be the main nasal passages. Here the airways are constricted in the region of the nasal ostium: the inhaled air accelerate because of the Bernoulli forces and then decrease during the exhalation in the preturbinate region (atrium) because air has to change direction to enter the main nasal passages of the turbinates (Alagusundaram et al., 2010; Elad et al., 2006; Kublik and Vidgren, 1998). This braking allows to maintain a positive expiratory airway pressure that keeps the pharyngeal and lower airways open and increase the duration of the expiratory phase (Djupesland, 2013; Sahin-Yilmaz and Naclerio, 2011). The respiratory region is richly supplied with blood, has a large surface area, and receives the maximum amount of nasal secretions, rendering it most suitable for the permeation of compounds (Arora et al., 2002). Therefore, the drugs, which arrive to this region, can be systemically adsorbed. The nasal epithelium is a layer of pseudostratified ciliated columnar cells interconnected via tight junctions, which regulate the diffusion of molecules through the paracellular pathway (Zhao et al., 2018) (France and Turner, 2017). Their integrity affect the epithelial barrier, its paracellular permeability and therefore the penetration of pathogenic particles and organisms, which could lead to dysbiosis and diseases development (Glorieux et al., 2011). The vestibule is lined by a non-ciliated squamous epithelium, which, in the valve and posterior region, gradually transits into ciliated epithelium (Mygind et al., n.d.). The cilia function is critical to respiratory defence and it cleans the paranasal sinus cavities and upper airways from the pathogens and debris that are continually inspired during respiration. The beating action of cilia moves the mucous blanket towards the nasopharynx at an average speed comprised between 3 and 25 mm/min. Specifically, particles larger than 3–10 μm are efficiently filtered out and trapped by the mucus blanket (Djupesland, 2013; Gudis et al., 2012). The nasal

epithelium is coated by a mucus layer, 5 μm thick, which is the first barrier to be quickly pass by the deposited molecule before the fast clearance due to the mucociliary activity. The ciliary motion can transports mucus with the flow rate of about 5 mm per minute, and the mucus layer is renewed approximately every 7–20 min (Ghadiri et al., 2019; Marttin et al., 1998; Schipper et al., 1991; Shang et al., 2019). The thickness of the mucus layer affects the drug absorption rate. Taghiloo and Halimi found that the thickness of mucus layer is in strict relationship with variation of nasal septum deviation, given by environmental or anatomical factors (Taghiloo and Halimi, 2019). In the nasal tract the mucus layer is thin and therefore highly permeable compared to other mucosal surfaces (Ozsoy et al., 2009).

3. Nasal cavity diseases

The nose is continually exposed to a myriad of infectious organisms and dangerous factors and agents, which can cause infectious or non-infectious rhinitis, the last is due to different etiological dysfunctions of the nasal mucosa. The typical symptoms of rhinitis imply mucus production, congestion, rhinorrhea, sneezing and pruritus. In general, they are not serious conditions but, if not treated properly, they can become chronic pathologies with significant negative effects on an individual's quality of life. These pathological conditions could be caused by viruses, bacteria and fungi (infectious), by allergens (allergic), by mechanical, physical or chemical causes (non-pathological). Whilst changing the etiology, they usually give similar symptoms, they are dynamic and may develop into one another (Liva et al., 2021). As a function of the duration of symptoms, rhinitis and rhinosinusitis are classified as acute (lasting up to one month), subacute (one to three months), chronic (more than three months), or recurrent (at least 4 episodes per year with asymptomatic intervals) (Zicari et al., 2020). They are usually care by local or systemic treatments with different drugs, and in recent years the efficacy of the natural molecules has been strengthened and coupled with their limited side effects. It is important to promptly care this pathology because they can facilitate the onset of more dangerous illness. Indeed, during the recent Sars-CoV-2 pandemic, World Health Organization declared rhinosinusitis as a risk factor for COVID-19 patients (Klimek et al., 2021). In addition, chronic rhinosinusitis may involve the formation of nasal polyposis, which driven nasal obstruction, anosmia, rhinorrhea, post nasal drip, and less commonly facial pain (Castillo-Bustamante et al., 2019; DeConde et al., 2017; Dykewicz and Hamilos, 2010).

3.1. Infective conditions

Infective colonization of nasal cavity can be chronic or acute involving nasal area (rhinitis), paranasal sinuses (sinusitis) or both (rhinosinusitis). Viral rhinitis is the most common form of upper respiratory infections more than the bacterial ones. It is due to rhinovirus, coronavirus, adenovirus, influenza virus, parainfluenza virus, respiratory syncytial virus, and enterovirus (Liva et al., 2021). They are dangerous since damage membranes and junctions of epithelial cells and control their metabolic activity. Influenza viruses belong to the family of Orthomyxoviridae which consists of five genera: *influenza A* and *B virus*, *influenza C virus*, *Thogotovirus*, and *Isavirus*. Only *influenza A* and *B* viruses cause clinical diseases but the primary pathogen for human infections is influenza A virus (Zhang et al., 2015). Respiratory syncytial virus, from the family *Pneumoviridae*, is the leading cause of lower respiratory tract disorders in infants and young people. This kind of infection begins in the epithelium of the upper respiratory tract and rapidly spread by intracellular transmission to the lower airways, causing the onset of various ailments such as fever, bronchiolitis, asthma, pneumonia (Drajac et al., 2017). Human rhinoviruses are small (27 nm), non-enveloped, single stranded RNA viruses of the *Picornaviridae* family genus *Enterovirus* (Ortega et al., 2021). *Rhinovirus* is the first cause of bronchiolitis and asthma in children as well as in adults (Saraya et al., 2014). *Adenoviruses* cause mild, self-limiting infections in immunocompetent people, affecting different areas of the body depending on the type, including the lower respiratory, digestive and ocular tracts, mostly children under five years, and people in close contact situation (De Oliveira et al., 2020; Mennechet et al., 2019).

Coronaviruses of humans belongs to the subfamily of the *Coronaviridae*. They are single-stranded RNA spherical viruses, sized 120–160 nm in diameter, sensitive to heat and lipid solvents (Monto et al., 2014). The novel coronavirus which arose in 2019, known as SARS-CoV-2 causes a range of specific respiratory symptoms classified as coronavirus disease, COVID-19. One of the hallmark features of COVID-19 conditions is the onset of the acute respiratory distress syndrome (ARDS), which is associated with low blood oxygenation levels and consequent result in organ failure (Geier and Geier, 2020; Marini and Gattinoni, 2020). In a research it was disclosed that the most common symptoms from COVID-19 are fever (82%), cough (61%), muscle aches/fatigue (36%), dyspnea (26%), headache (12%), sore throat (10%), and also gastrointestinal symptoms (9%) (Borges do Nascimento et al., 2020).

The bacterial infection of nasal mucosae is very common in young children and can persist for long periods. Aerobe and anaerobe microorganisms are usually simultaneously involved, and they form a biofilm, which strongly reinforces their pathogenesis and persistence (Drago et al., 2019). The most common bacteria found are *Streptococcus pneumoniae*, *Moraxella catarrhalis* and *Haemophilus influenzae* and they are part of the normal bacterial flora in the nasopharynx, especially in children (Kovács et al., 2020). They carriage increases during colder months of the year, since cold air stimulates increased secretion by nasal glands facilitating proliferation of these bacteria and symptomatic respiratory illness (common cold) (Hendley et al., 2005).

Other infection involving the nasal mucosa, as site of entry, is leprosy, a chronic human disease resulting from infection with *Mycobacterium leprae* (Monot et al., 2005). Its mode of transmission is not well understood yet, although it is probable that nasal droplets and aerosols are the main route (Rodrigues and Lockwood, 2011). Nasal disease may precede skin disease by several years, the incubation period range is 2–12 years (Rodrigues and Lockwood, 2011). Other nasal infection is *rhinoscleroma*, caused by the gram negative *Klebsiella rhinoscleromatis* and affecting the nasal cavities, nasopharynx, lips and oropharynx, but unusual sites such as the trachea, can be involved in rare cases (Umphress and Raparia, 2018). The disease develops in 3 phases. The first one is *catarrhal-atrophic*, lasts for weeks or months and is usually associated with rhinorrhea and sinusitis. The second one is known as *granulomatous-hypertrophic* stage, characterized by a mass formation and the consequent tissue destruction, which led to an enlargement and deformity of nasal apparatus, with the appearance of "hebra" nose. The final phase is the *sclerotic* stage, with extensive scarring, fibrosis, and chronic inflammatory condition, accompanied by marked deformity and tissue destruction (Liva et al., 2021; Shang et al., 2019; Taghiloo and Halimi, 2019).

Fungi are airborne allergens, that can cause invasive or non-invasive fungal sinusitis (Deshazo, 2009). Invasive fungal sinusitis usually occurs in immunocompromised patients with acute onset of fever, cough, nasal mucosal ulceration or eschars, epistaxis, and headache. Commonly associated conditions include malignant diseases, such as leukemia, other causes of neutropenia, diabetes, hemochromatosis, or malnutrition. More chronic forms of invasive disease may present as proptosis or orbital apex syndrome (deShazo et al., 2009). Left untreated, any of the invasive forms can lead to an acute and fulminant fungal sinusitis affecting cerebral blood vessels, with ischemic infarction or direct infection of the brain (deShazo et al., 2009; Epstein and Kern, 2008; Parikh et al., 2018; Stringer and Ryan, 2000). Non-invasive fungal diseases are caused by a colonization of mucus crusts by saprophytic fungi. This is often seen in patients after endoscopic sinus surgery and may be asymptomatic or lead to a foul smell (Aribandi et al., 2007; Helliwell, 2010). A serious non-invasive form is the allergic fungal sinusitis, an hypersensitive reaction to extra-mucosal fungal hyphae, that results in a

chronic allergic hypertrophic rhinosinusitis with the formation of nasal polyps (Helliwell, 2010), distinguishable clinically, histopathologically and prognostically from the other forms of fungal conditions (Schubert, 2012). Allergic fungal sinusitis is the most common type of sinus infection caused by fungal hyphae. It is about a robust allergic reaction to inhaled soil fungi that causes sinus inflammation. Fungal debris accumulates in the sinus cavities, causing nasal polyps, facial pain and pressure, face bone remodeling and even erosion, which may eventually lead to damage to the eyes and brain, and affect the sense of smell (Alaraj et al., 2018; Alenzi et al., 2020; Lee et al., 2020). On the other hand, eosinophilic mucin rhinosinusitis is a disorder caused by mucin in sinuses in absence of fungal hyphae. It's a disorder connected with a deregulation of the systemic immune system and associated with asthma and aspirin sensitivity, and shares some clinical, radiological and immunological characteristics with allergic fungal sinusitis (Chakrabarti et al., 2009).

3.2. Non-infective conditions

Non-infectious rhinitis could be non-pathological, when licked to mechanical, physical or chemical factors, such as trauma, radiotherapy, endoscopic surgical intervention, drugs, age. Among these, in the last years, the environmental pollution, and drugs have been mostly prominent. Indeed, environmental particulate (diameter $\leq$ 2.5 μm) can disrupt the nasal epithelial barrier, leading to oxidative stress and increased release of inflammatory cytokines such as eotaxin, and interleukins 6 and 8 in nasal epithelial cells (Zhao et al., 2018). Other non-allergic and non-infectious, which can be affected by perennial, can be subclassified in eosinophilic, vasomotor rhinitis, and local allergic rhinitis (Meng et al., 2021; Milosević et al., 2000). The first is a clinical syndrome characterized by the symptoms of allergic rhinitis with polyposis and in whom the nasal mucosa, nasal cavities and fluid contains eosinophils (Leone et al., 1997). Vasomotor rhinitis is the most common subtype of non-allergic rhinitis and is characterized by nasal symptoms like sneezing and watery rhinorrhea triggered by nonallergic irritants like changes in temperature, strong smells, or barometric pressure (Kaliner, 2009; Lal and Corey, 2004). Local allergic rhinitis is a clinical entity characterized by symptoms suggestive of allergic rhinitis owing to a localized allergic response in the nasal mucosa in the absence of systemic atopy assessed by conventional diagnostic tests such as skin prick test or determination of specific immunoglobulin E (sIgE) in serum (Altintoprak et al., 2016; Campo et al., 2016, 2015; Chang et al., 2014).

Alternatively, allergic rhinitis is due to the subjective sensitivity to specific allergens. It is a very common disease, affecting people primarily during the childhood. It is estimated that 10–25% of global population suffer from allergic rhinitis symptoms (Huang et al., 2018). Allergic rhinitis is caused by an IgE-mediated inflammatory reaction consequent to the exposure to allergen and mucosal inflammation mediated by type 2 helper T (Th2) cells (Kilic et al., 2019; Miraglia Del Giudice et al., 2014). The main cause are seasonal allergens such as tree, grass, weed pollen and fungal spores. Perennial rhinitis is typically associated with allergens found in the feces of the house dust mite. Individual susceptibility is influenced by genetic and environmental factors including cigarette smoke, infection and airborne pollutants, which depress the responses of T suppressor cells to allergens (Helliwell, 2010). The most widely used pharmacologic treatments of allergic rhinitis are nasal glucocorticoids and oral antihistamines (Pullerits et al., 2002). Arbuckle reported a case study of a severe allergic rhinitis treated with naturopathic nutritional supplements, herbal medicines, honey and a low histamine diet, resolved in an improvement of the patient's symptoms (Arbuckle, 2017).

4. Local nasal treatment

Intranasal application is a suitable option for the local treatment of upper airways, permitting an easily administration of a wide variety of therapeutics and avoiding their systemic distribution. It is a non-invasive technique, painless and highly appreciated by patients including children. The drug is easily site-targeted and deposited on a large and porous surface (Bitter et al., 2011). The efficacy of the deposition is affected by anatomical and physical factors, regarding the shape of the nose or the device characteristics (Casula et al., 2021; Djupesland, 2013). Despite few negative aspects that must be considered, such as direct contact of the tip of the spray nozzle during actuation, combined with the high-speed and localized impaction concentrated on the septum, which may create mechanical irritation and injury to the high sensitive mucosa, nasal delivery is the best choice for the local treatment of nasal disease due to the high precision and amount of doses released in a specific site (Djupesland, 2013; Husner et al., 2006; Shahi and Pardeshi, 2021). However, the permeation barriers and the rapid ciliary cleansing mechanism, limit the total amount of drug that can be absorbed. Therefore, the role of the used formulation is fundamental to reach an effective drug concentration at the target site (Bitter et al., 2011; Marttin et al., 1998; Sarkar, 1992). Moreover, unlike systemic administration, intranasal delivery can be practiced multiple times a day (Costantino et al., 2007).

4.1. Liquid dosage forms

The most common dosage forms used for intranasal administration are liquid formulations (solution, suspension or emulsion) to be sprayed. They contain one or more drugs and excipients homogeneously dispersed generally in an aqueous medium and may easily diffuse and penetrate in the nasal mucosae, thus guaranteeing the highest bioavailability. Spray formulations have a moisturizing effect and alleviate the crusting and irritation feeling associated with allergic and non-allergic rhinitis. The residence time of the sprayed formulations in the nasal cavity can affect its final effectiveness and, dependent on the delivery device, on the mode of administration and the physico-chemical characteristics of the formulation, such as droplet size and plume angle. According to the Food and Drug Administration, the ideal size of nasal droplets, to have an efficient local effect, must be comprised between 30 and 70 μm for 50% of droplets, and lower than 200 μm for 90% of them. Droplets lower than 10 μm cannot exert a local action as they may reach the lungs, therefore a very low percentage of this particles is desirable for the nasal sprays (USP et al., 2003). Plume angle is the cone generated by the aerosolized droplets. To be effective at local level the angle generated by a nasal spray must be narrow and comprised between 30° and 70°, depending also on its beneficial or therapeutic purpose. In general, an angle $< 60°$ was found to provide the maximal deposition and a good droplet size distribution of the nebulized formulations within the nasal cavity. A study performed by Kundoor and Dalby confirmed that for higher administration angles $\geq 60°$ the deposition was higher in the nasal valve region, while droplets generated by spray angles $\leq 45°$ could pass through the nasal valve and angle $\geq 75°$ led the achievement of the nasal anterior area (Kundoor and Dalby, 2011). A narrow plume $< 30°$ can favor the deposition in the turbinate region, as a function of the droplet size and velocity, since high velocity and small size improve the main deposition in the anterior region (Casula et al., 2021; Cheng et al., 2001).

Other liquid dosage forms used for local nasal applications are drops, that represent one of the most known, simplest to use and currently most available forms to treat the olfactory cleft, not only in chronic rhinosinusitis but also in olfactory dysfunction (Fujii et al., 2002; Mori et al., 2016; Trabut et al., 2020). Nasal drop formulations are generally low viscous to facilitate the administration, but, in the most suitable formulations, the viscosity increase after the deposition due to the hydration of their compounds forming a thicken system, thus decreasing the mucociliary beating (Van De Donk et al., 1982). The success of the therapy via nasal drops depends mainly by a correct head position (Karagama et al., 2001). Unlike the nasal spray, which reach the target site because pressurized inside the nose, nasal drops spread thanks to the

gravity. In this way they can reach posterior nasal areas like the olfactory cleft and middle meatus, especially when "Kaiteki" position is performed (Mori et al., 2016). Patients lie on their side with the head tilted towards the shoulder and the chin pointing upwards. In this position, the nasal drops flow into the nasal environment until they reach the posterior areas.

4.2. Nasal dry powders

Dry powders are less frequently used in local nasal treatment since their formulation is more difficult and require the involvement of complex and expensive methods and materials. They allow the delivery of high mass of active ingredients without the necessity of a vehicle because contain specific excipients able to adhere to the nasal epithelia, thus providing higher resistance against the mucociliary clearance, and prolonging the contact time of the drug with its target site (Ishikawa et al., 2002; Trenkel and Scherließ, 2021). Moreover, nasal powders are especially suitable for poorly water-soluble drugs and have better physicochemical and microbiological stability over liquid formulations (Kublik and Vidgren, 1998; Tiozzo Fasiolo et al., 2018). The deposition in the nose is determined by the aerodynamic properties of the powder (Kublik and Vidgren, 1998).

5. Local nasal treatment with phytochemicals

In the last years the interest towards the green medicine has grown exponentially. Consumers are more aware about environmental issues and the "green philosophy" is rapidly spreading among every social context. The medical and pharmacological field doesn't make an exception and are turning their attention to natural drugs, which are today recognized as more attractive, reliable and safe than the synthetic ones. Natural products have been the first therapeutics used in traditional medicine and cosmetics and today research is investigating and disclosing their pharmacological efficacy. Mainardi, Kapoor *et al.* in their article on the complementary and alternative medicine state that the number of papers published about phytochemicals "*has exploded with more than 1700 articles cited in PubMed using* "*complementary medicine" as a keyword for the year 2007, compared with only 355 in 1990*", and from the same year it has tripled in the field of allergy and immunology (Mainardi et al., 2009). Nowadays, phytochemicals are used to prevent, alleviate and care a variety of pathological conditions including, aging, cardiovascular, neuroinflammatory and neurodegenerative diseases, skin disorders, metabolic and obesity illnesses. This varied panorama also includes topical treatments for nasal mucosa. The World Health Organization support this approach affirming that it *will continue to promote the development, teaching, and application of analytical methods that can be used to evaluate the safety and efficacy of various elements of traditional medicine* (Akerele, 1984).

Phytochemicals are attractive and widely used also in the local treatment of nasal pathologies, but the actual effectiveness and mechanism of activity have not been deeply and homogenously evaluated, few literature is available and any review, which systemically analyses it. According to this, the present review examines the studies related to topical applications of phytocomplexes at nasal level for the treatment of the upper airway disorders, as it is a subject that has not been dealt with in the literature.

5.1. Conventional dosage forms based on phytochemicals

Various phytochemicals have been used in conventional topical dosage forms for the local treatment of disorders affecting the nasal cavity and upper airways. The selection of one formulation rather than another depends on the characteristics of the bioactive and the vehicle and additives used. The additive should be compatible with the bioactive used, non-irritating and organoleptically tolerable. Anyway, the formulation plays a key role in improving the bioavailability and efficacy of phytochemical and represent an innovative strategy to support their common use. Because the use of phytochemicals is lost in the mists of time, several studies evaluated the actual efficacy of traditional dosage forms used in traditional medicine, investigating by ethnopharmacological research by means of modern methodologies. The most used dosage forms are conventional spray formulations, which are optimal forms to locally administrate natural bioactive molecules, as confirmed by several studies. The therapeutic effect of *Nigella sativa* extract in patients with chronic rhinosinusitis without nasal polyp was demonstrated, being effective in reducing symptoms without causing adverse effect (Rezaeian and Amoushahi Khouzani, 2018). In adults with common cold virus infections, direct local administration of carrageenan with nasal sprays reduced the duration of the symptoms (Ludwig et al., 2013). Also, a nasal spray formulation containing an extract composed of a mixture of essential oils and flavonols from the *Artemisia abrotanum* L. genotype "Tycho", appears to be clinically useful and suitable for the prophylactic and therapeutic management of patients with allergic rhinitis and adjuvant symptoms (Remberg et al., 2004). Nasal spray based on the extract of lemon pulp combined with pure Aloe juice, soluble propolis, and essential oils of Ravensara and Niaouly caused a total reduction of eosinophils granulocytes and mast cells, suggesting that lemon-based nasal spray could be a good alternative to conventional medicine for the treatment of perennial and seasonal allergic and vasomotor rhinopathy (Ferrara et al., 2012). In their study Lv *et al.* administered intranasally 100 μl/spray of resveratrol and demonstrate that it can effectively counteract allergic rhinitis in adults (Lv et al., 2018). In another study, resveratrol and β-glucan were administered synergically via spray in children with *Parietaria* pollen-induced allergic rhinitis. Results showed that nasal symptoms (including itching, sneezing, rhinorrhea, and obstruction) were significantly improved (Miraglia Del Giudice et al., 2014). Another study provides evidence for an inhibitory effect of resveratrol on the production of several rhinoviruses-induced inflammatory mediators in nasal epithelia likely depending on the ability of resveratrol to suppress viral replication (Mastromarino et al., 2015). The potential efficacy of the *Urtica dioica* extract in inhibition of several key inflammatory events that cause the symptoms of seasonal allergies was demonstrated by Roschek *et al.* (Roschek et al., 2009). The local efficacy of onion extract in the treatment of allergic rhinitis symptoms and allergic inflammatory reaction was also confirmed in a murine allergic model (Seo et al., 2019). It can be assumed that the topical application of onion extract regulates allergic symptoms by suppressing the type-1 helper and type-2 helper responses and reducing the allergic inflammatory reaction.

Thanks to its powerful and multitargeting efficacy, quercetin has been tested in *in vitro* and in vivo experiments to treat nasal disorders (Ebihara et al., 2018; Edo et al., 2018; Sagit et al., 2017). In the work by Jafarinia *et al.* was confirmed the effectiveness of quercetin in the management and treatment of allergic asthma, allergic rhinitis, and atopic dermatitis (Jafarinia et al., 2020). Similarly, Tiboc-Schnell *et al.* found out that quercetin is effective in reducing nasal inflammatory activity, including secretion of cytokines and histopathological changes in LPS-induced acute rhinosinusitis in rats (Tiboc-Schnell et al., 2020). Recently, quercetin was recognized as a good candidate for the treatment of COVID-19 infections as well (Agrawal et al., 2020; Pan et al., 2020).

Apigenin was found effective also in the treatment of nasal mucosa. Ali *et al.* found that apigenin has potent antiallergic properties for the management of rhinitis symptoms. Apigenin regulates Th1/Th2 balance via suppression in expressions of Th2 response (IgE, histamine, ILs, GATA3, STAT6, SOCS1, and NF-kB) and activation Th1 response (IFN-g and T-bet) to exert its anti-allergic potential in a murine model of ovalbumin-induced allergic rhinitis (Chen et al., 2020). Another study by Li and Zhang described that apigenin may effectively ameliorate the progression of asthma by suppressing IL-17A, Th17 cells in an ovalbumin-induced mouse model (Li and Zhang, 2013).

Gingerols can counteract the nasal mucosal diseases as well. In

particular 6-gingerol suppresses cytokine production for T cell activation and proliferation, thereby not causing B cell and mast cell activation and resulting in prevention or alleviation of allergic rhinitis symptoms (Casula et al., 2021; Kawamoto et al., 2016). 6-Gingerol may be considered an anti-hypersecretory agents because it effectively inhibits mucus secretion of respiratory epithelial cells while maintaining normal nasal ciliary movement (Chang et al., 2010). Also, gingerol can counteract the extracellular matrix accumulation, preventing the insurgence of nasal polyps through its antioxidant effect (Park et al., 2012).

1,8-cineole the main constituent of eucalyptol, was found to be effective against bacteria-induced mucus hypersecretion in the topical application treatment against rhinosinusitis and bronchial asthma (Galan et al., 2020; Juergens, 2014; Sudhoff et al., 2015). Zhou *et al.* could demonstrate that thymol attenuates the symptoms of allergic rhinitis by reducing inflammatory cells release, such as Th2 cytokines and ovalbumin-specific IgE, ameliorating pathological changes, probably associated with its ability to block the NF-κB activation in ovalbumin-induced allergic mice (Kilic et al., 2019). Furthermore, Nabissi *et al.*, found that thymol can also modify the cilia beating thus increasing their mucociliary activity (responsible for the clearance of the airways from outside particles, debris and pathological organisms) and ameliorate the chronic obstructive pulmonary disease, a respiratory disorder characterized by a progressive and irreversible airflow limitation (Nabissi et al., 2018). Burrow *et al.* exposed 5 min a cohort of healthy patients to the inhalation of menthol, camphor and eucalyptus vapors. Results showed that the majority of subjects reported a cold sensation in the nose, due to the stimulation of cold receptors in the nose (Burrow et al., 1983). Eccles confirmed that the interaction between menthol and the specific cold menthol sensitive receptor (CMR1) on trigeminal nerve ending in the nose and upper airways, provides relief from nasal congestion associated with rhinitis and the sensation of dyspnea associated with chronic obstructive pulmonary disease (Eccles, 2003). Another study revealed that better results were obtained in the treatment of chronic rhinosinusitis through the multidrug inhalation aerosol therapy composed of essential oils (L-menthol and eucalyptus), saline, glucocorticoids (dexamethasone), and antibiotics (gentamicin), than intranasal glucocorticoid therapy with mometasone furoate and saline irrigation (Velepič et al., 2019). Regarding geraniol, an interesting study proved that chewing gums enriched with geraniol were effective against rhinitis symptoms (Arakawa T., 1992). Huang *et al.* investigated geraniol ability to reduce allergic rhinitis proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 expression in mice, and diminish levels of serum histamine, ovalbumin-specific IgE, and IL-1β levels, due to alterations in mitogen-activated protein kinase and NF-κB signaling pathways (Huang et al., 2018).

6. Innovative dosage forms with phytochemicals

Phytochemicals have enormous therapeutic potential, which can be further potentiated through the suitable use of different innovative formulations or nanocarriers like nanoparticles, phospholipid vesicles, phytosomes, micelles, microspheres, solid lipid nanoparticles, which have already contributed significantly to the enhancement of therapeutic potential of synthetic drugs and may exponentially maximize that of phytochemicals (Baishya et al., 2019; Manca et al., 2012). Indeed, they are more subjected to low instability and bioavailability, that can be overcome thanks to their delivery with these systems, especially liposomes and phytosomes (Kumar et al., 2017; Manconi et al., 2020). Nanocarriers have the unique power to tackle challenging routs, such as nasal mucosa thanks to their structure, physicochemical properties, stability, pharmacodynamics, and pharmacokinetics, thus affecting the fate of payloads. In the last years, nanocarriers and other alternative formulations have been proposed for the development of innovative dosage forms based on phytochemicals and devoted to the local treatment of nasal cavity. Dahanayake *et al.* converted a traditional decoction into a new freeze dried and spray dried formulation. They used *Tamalakyadi* decoction obtained from 12 plants and traditionally used in Sri Lankan as a remedy for nasal diseases and allergic rhinitis. Researchers compared the phytochemical constituents and antioxidant activities of traditional decoction with the modified dosage form, finding that they were similar (Dahanayake et al., 2019). A novel in situ gel system delivering of poly-herbal extracts was prepared and used for nasal application in the treatment of allergic rhinitis model. The efficacy of in situ gel-forming containing poly-herbal extract was confirmed and compared with that of conventional nasal drops (Srivastava et al., 2019). The same liposomes, prepared only using natural occurring soy lecithin without other additives, are effective in the local treatment of rhinitis. Their commercial spray formulation is available in the market and exert a hydrating and moisturizing effect on nasal mucosa (Böhm et al., 2012).

Actually, the studies reporting the loading of phytochemicals in nanocarriers for the local treatment of nasal cavity are very few and in these, the proposed delivery strategies are different, involving a high variety of carriers even if the tested natural molecules are only the most common used.

Recently, curcumin gained a great interest from the scientific community and it was tested also to alleviate the symptoms of upper airway diseases by local application, especially loaded in innovative carriers (Liang et al., 2019; Sun et al., 2010). In particular, a research found that curcumin liposomes systems have shown good encapsulation efficiency for curcumin, and the application in vitro of nano-curcumin showed the suppression of important pro-inflammatory markers and also improved patient compliance (Ng et al., 2018). In another study, the combination of curcumin and ovalbumin in free form or loaded into the innovative polylactic co-glycolic acid nanoparticles was proved to enhance the sublingual immunotherapy efficiency in mouse model of rhinitis allergic. Results from nasal lavage fluid showed significant decreased levels of total and eosinophil cell count treated with nano-formulation. Researchers concluded that using curcumin and nanoparticles with allergen (Allaw et al., 2020a,b; Balaji et al., 2016; Bartos et al., 2021; Forni et al., 2019; Howes et al., 2020; Johnson, 2007; Manca et al., 2021; Miller and Snyder, 2012; Ranjan et al., 2019; Sahab et al., 2020; Uddin et al., 2020; Vaiserman et al., 2020; Mahdavinia et al., 2016; Saad et al., 2017) can be considered as potential immune modulatory agents (Shahgordi et al., 2020).

Innovative nasal delivery systems were investigated for resveratrol as well. To overcome resveratrol rapid metabolism limitations, several ways have been explored and, among them, aerosolized suspensions like resveratrol-containing spray and co-spray dried microparticles showed beneficial effects on individual health, supporting their potential application in the complications associated with nasal and respiratory infections (Filardo et al., 2020). Solid lipid nanoparticles and nanostructured lipid carriers have been found to protect the incorporated resveratrol from rapid metabolism, to increase its physical stability and to allow a controlled release after uptake due to the in situ gel-forming (Neves et al., 2013). Exploitation of polymeric in situ nasal gels for controlled release of drug provides numerous advantages over conventional dosage forms and can be considered as reliable and non-invasive drug delivery system (Karavasili and Fatouros, 2016; Sabale et al., 2020). Salem *et al.* successfully evaluated resveratrol pharmacokinetic in vitro and in vivo loaded in nanosized nasal emulgel (Salem et al., 2019).

Quercetin is also worth of attention as part of the nasal treatments. It was formulated in the Phytosome® delivery system (Quercefit™, QFit) and administered in healthy subjects with mild-moderate asthmatic attacks and rhinitis. Its treatment prevent and reduce daily and night symptoms, maintain higher peak of expiratory flow and in decrease their variability (Cesarone et al., 2019).

Gingerol is a promising, natural occurring polyphenol traditionally used in the oriental medicine also as adjuvant in nasal disease. Singh *et al.* prepared phytosomes by blending gingerol with soy lecithin and adding them to an aqueous solution of chitosan to formulate the phytosome complexed with chitosan (Singh et al., 2018). Casula *et al.* loaded

Z. officinalis extract in nanovesicles tailored for the treatment of rhinitis and rhinosinusitis. Special phospholipid vesicles, called hyalurosomes were used and enriched with glycerol. The formulation was prepared by using a green and environmentally friendly method and natural components. *In vitro* experiments performed on cells confirmed the protective effect of the formulation on nasal epithelium. Studies of droplet size distribution and plume angle confirmed its good sprayability and deposition in the anterior part of the nasal use in the management of rhinitis and rhinosinusitis (Casula et al., 2021).

No further studies were reported on the use of natural extracts loaded into nanocarriers and used for local nasal treatment, but several plant extracts have been used to produce silver, iron or zinc nanoparticles by a green approach (Castangia et al., 2017). Silver nanoparticles were prepared using different antioxidant extracts such as *Ostericum koreanum, citrus aurantium, Fritillaria* flower, *Trifolium resupinatum*, *Laminaria japonica, Taxus baccata, Aloe vera* and *Cassia tora* (Hemmati et al., 2019; heydarzadeh and Yaghoubi, 2017; Kajani et al., 2014; Khatami et al., 2016; Kim et al., 2016; Logaranjan et al., 2016; Saravanakumar et al., 2015; Xiaoshang et al., 2021). They were used for treatment of allergic rhinitis and were recognized as a valuable strategy to successfully enhanced the allergic inflammatory reaction of the mast cell implying their use for potential treatment of allergic diseases mediated by mast cell. Morris *et al.* demonstrates that silver nanoparticles effectively reduce respiratory syncytial virus replication and production of pro-inflammatory cytokines in epithelial cell lines and in mouse lung (Morris et al., 2019).

7. Conclusion

In the last years the interest towards the green medicine has grown exponentially, and consumers find therapeutics based on natural molecules as more attractive, reliable and safe than synthetic and conventional drugs. Plants and natural products have been the first and only curatives available in past times, and several medical texts have been written by ancient medicians throughout history. Today, research is confirming the therapeutic potential of natural products for the human health, but few studies report the effectiveness of phytochemicals in the treatment of local nasal pathologies. The present review collects and sheds a light on these underdeveloped results. However, new studies are needed, specially to evaluate the efficacy of phytochemicals when loaded into innovative nanocarriers such as liposomes, which showed a great potential in local delivering of bioactive molecules and an improvement of their effectiveness.

CRediT authorship contribution statement

Eleonora Casula: Writing – original draft. **Maria Letizia Manca:** Writing – review & editing. **Maria Manconi:** Supervision.

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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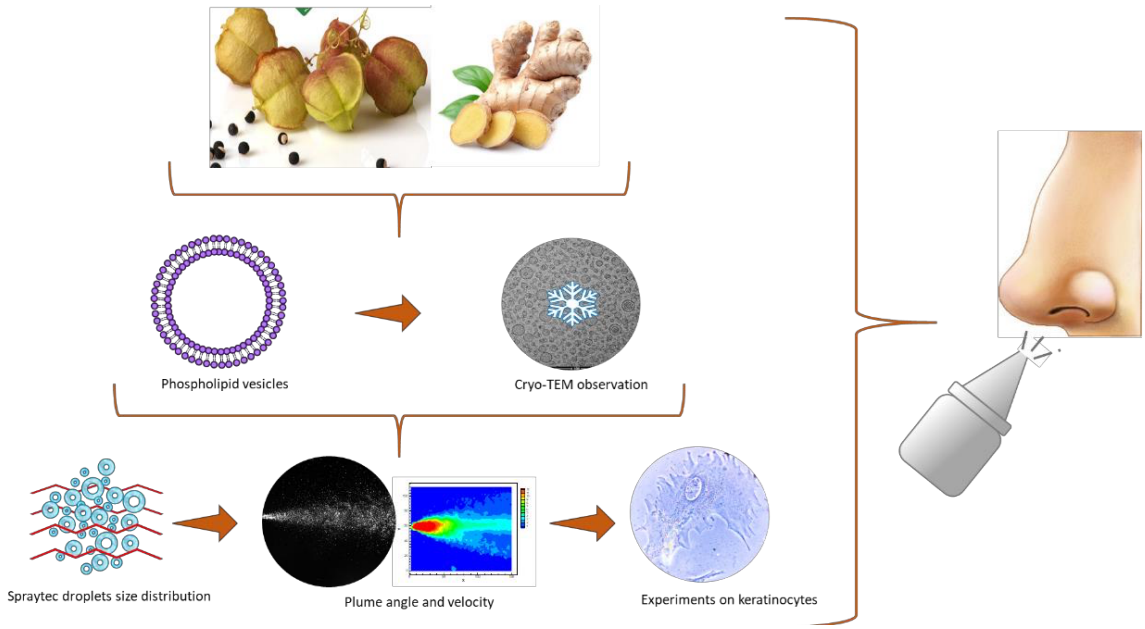
Aim of the Thesis

To prepare affordable and effective natural nasal sprays tailored for the local treatment of rhinitis and obtained using advanced phospholipid vesicles as nanocarriers to deliver natural extracts of *Cardiospermum halicacabum and Zingiber officinalis.*



Cardiospermum halicacabum

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Design of a Nasal Spray Based on *Cardiospermum halicacabum* Extract Loaded in Phospholipid Vesicles Enriched with Gelatin or Chondroitin Sulfate

Eleonora Casula [1], Maria Manconi [1,*], José Antonio Vázquez [2], Tania Belen Lopez-Mendez [3,4], José Luis Pedraz [3,4,5], Esteban Calvo [6], Antonio Lozano [6], Marco Zaru [7], Andreia Ascenso [8] and Maria Letizia Manca [1]

1 Department of Scienze della Vita e dell'Ambiente, Sezione di Scienze del Farmaco, University of Cagliari, Via Ospedale n. 72, 09124 Cagliari, Italy; e.casula@studenti.unica.it (E.C.); mlmanca@unica.it (M.L.M.)
2 Group of Recycling and Valorization of Waste Materials (REVAL), Marine Research Institute (IIM-CSIC), C/Eduardo Cabello, 6, 36208 Vigo, Spain; jvazquez@iim.csic.es
3 NanoBioCel Group, Laboratory of Pharmaceutics, School of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain; tania.lopez@ehu.eus (T.B.L.-M.); joseluis.pedraz@ehu.eus (J.L.P.)
4 Biomedical Research Networking Center in Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), 01006 Vitoria-Gasteiz, Spain
5 Bioaraba, NanoBioCel Research Group, Calle José Achotegui s/n., 01009 Vitoria-Gasteiz, Spain
6 Laboratory for Research in Fluid Dynamics and Combustion Technology (LIFTEC), Consejo Superior de Investigaciones Cientificas (CSIC)–Universidad de Zaragoza, María de Luna, 10, 50018 Zaragoza, Spain; Esteban.Calvo@unizar.es (E.C.); a.lozano@csic.es (A.L.)
7 Icnoderm S.r.l., Sardegna Ricerche Ed. 5, Pula, 09010 Cagliari, Italy; m.zaru@icnoderm.com
8 Faculty of Pharmacy, University of Lisbona, Av. Gama Pinto, 1649-003 Lisbona, Portugal; andreiaascenso@ff.ulisboa.pt
* Correspondence: manconi@unica.it; Tel.: +39-0706758542; Fax: +39-0706758553

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Abstract: The extract of *Cardiospermum halicacabum* L. (*C. halicacabum*) obtained from flower, leaf and vine was loaded into modified phospholipid vesicles aiming at obtaining sprayable, biocompatible and effective nasal spray formulations for the treatment of nasopharyngeal diseases. Penetration enhancer-containing vesicles (PEVs) and hyalurosomes were formulated, and stabilized by adding a commercial gelatin from fish (20 mg/mL) or chondroitin sulfate from catshark cartilages (*Scyliorhinus canicula*, 20 mg/mL). Cryo-TEM images confirmed the formation of spherical vesicles, while photon correlation spectroscopy analysis disclosed the formation of small and negatively-charged vesicles. PEVs were the smaller vesicles (~100 nm) along with gelatin-hyalurosomes (~120 nm), while chondroitin-PEVs and chondroitin-hyalurosomes were larger (~160 nm). Dispersions prepared with chondroitin sulfate were more homogeneous, as the polydispersity index was ~0.15. The in vitro analysis of the droplet size distribution, average velocity module and spray cone angle suggested a good spray-ability and deposition of formulations in the nasal cavity, as the mean diameter of the droplets was in the range recommended by the Food and Drug Administration for nasal targets. The spray plume analysis confirmed the ability of PEVs, gelatin-PEVs, hyalurosomes and gelatin-hyalurosomes to be atomized in fine droplets homogenously distributed in a full cone plume, with an angle ranging from 25 to 30°. Moreover, vesicles were highly biocompatible and capable of protecting the epithelial cells against oxidative damage, thus preventing the inflammatory state.

Keywords: *Cardiospermum halicacabum*; epithelial cells; droplet size distribution; plume angle; antioxidant activity; keratinocytes

1. Introduction

Cardiospermum halicacabum L. (*C. halicacabum*) belongs to the Sapindaceae family, which includes about 1200 species commonly found in India, South America and Africa. The

beneficial properties of *C. halicacabum* have been known for centuries as a natural cortisone for the treatment of rheumatism, as well as a remedy for digestive and respiratory disorders, joint and back pain and muscle sprains, and also as a product capable of contrasting the poisoning effect of snake bites [1–10]. The extract of *C. halicacabum* is still used in traditional eastern medicine as an anti-inflammatory remedy against rhinopharyngitis, but the effect has never been evaluated in scientific studies. The main bioactives contained in the plant are flavonoids, triterpenoids, glycosides, fatty acids and volatile esters [11]. They synergically exert anti-inflammatory effects, reducing the activity of phospholipases A2, with a consequent reduction of the availability of arachidonic acid, a precursor of prostaglandin biosynthesis [12]. At the same time, they may suppress an inflammatory mediator, such as nitrite oxide and cytokines such as TNF-α and free radical species, which are usually generated during the inflammatory response and in excess can potentiate and prolong the tissue damages [13]. Moreover, *C. halicacabum* counteracts free radicals, due to its content of powerful antioxidants including flavonoids, saponins, tannins, and glycosides [4,14].

Considering the promising anti-inflammatory and antioxidant power of this plant, its formulation in a nasal spray should represent an optimal alternative to effectively reach the nasal cavity for the treatment of nasal congestion and rhinitis, especially if associated with a suitable nasal preparation with a moisturizing effect, capable of counteracting the dryness and crusting usually associated with these disorders [15]. Phospholipid vesicles seem to be the ideal systems for this propose, because they are used on the inflamed nasal mucosa to alleviate the symptoms of seasonal allergic rhinitis [16,17]. Currently, a liposome-based spray is commercially available in Germany and a formulation containing hyalurosomes (phospholipid vesicles immobilized with hyaluronic acid) is present on the Italian market [18].

The combination of the extract of *C. halicacabum* with the phospholipid vesicles in a nasal spray represents a promising strategy for the local treatment of nasal congestion and rhinitis [15,19–24]. Moreover, *C. halicacabum* extract was not previous loaded in phospholipid vesicles and the positive effects provided by this association has not explored previously, along with their suitability as nasal spray. Indeed, to provide a local effect, nasal sprays must have specific features, such as adequate spray plume and droplet size distribution, which depend on the properties of the pump, the formulation, the orifice of the actuator, and the force applied [25]. These parameters can positively affect the deposition zone in the nose, improving the beneficial effect of the bioactives at a local level, by enhancing their accumulation and efficacy in the anterior nose [15,25,26]. Polymers are widely added in nasal sprays to improve the bioadhesiveness and mucosa dehydrations, as a function of their specific properties. Gelatin is widely used over other polymers in nanotechnology thanks to its high biocompatibility, biodegradability and non-irritability. Combined with liposomes, gelatin improves the stability of the final formulations because of its capacity to adsorb and retain water and reduce the water vapor permeability, slowing the release time of the drugs [27,28]. Chondroitin sulfate is a constituent of proteoglycans—safe, biocompatible and widely used to produce biomaterial and biomedical devices [29–31]. Research has highlighted various pharmacological properties of chondroitin sulfates from *Scyliorhinus canicula*, such as antithrombotic [29], anticoagulant [30], antioxidant, anti-inflammatory and neuroprotective activities [31].

Considering the lack of knowledge in the present study, the glycolic extract of *C. halicacabum* was loaded in penetration enhancer-containing vesicles (PEVs) and hyalurosomes, which were further modified by adding a commercial gelatin from cold-water fish or a biopolymer chondroitin sulfate, extracted from catshark cartilages of *Scyliorhinus canicular* [32–35]. The spray-ability, biocompatibility and effectiveness of vesicles as a nasal spray was evaluated. The mean size and zeta potential of vesicles were measured by a dynamic laser light scattering, and the aptitude to be sprayed in the nose (i.e., droplet size distribution, average droplet velocity and spray plume angle) was investigated by laser diffraction technique, laser plane visualization and particle image velocimetry. The

cytotoxicity of the formulations and their ability to protect epithelial cells (keratinocytes) against oxidative stress were determined in vitro as well.

2. Materials and Methods

2.1. Materials

Lipoid S75 (~70% of soy phosphatidylcholine, 9% phosphatidylethanolamine and 3% lysophosphatidylcholine) was purchased from Lipoid GmbH (Ludwigshafen, Germany). The 2:1 glycolic extract from flower, leaf and vine of *C. halicacabum* was kindly supplied by Sakura srl (Lonato del Garda-BS, Italy). Sodium hyaluronate with low molecular weight (200–400 kDa) and a polydispersity of 1.4 Mw/Mn was purchased from DSM Nutritional Products AG Branch Pentapharm (Rhein Elden, Switzerland). PEG400 was purchased from Galeno S.r.l. (Comeana, PO, Italy). Chondroitin sulfate was extracted from catshark cartilages (*Scyliorhinus canicula*) and kindly supplied by REVAL Institute (Vigo, Spain) [27–29]. The 2,2-diphenyl-1-picrylhydrazyl (DPPH), fish gelatin and all the other reagents of analytical grade were purchased from Sigma-Aldrich (Milan, Italy). Reagents and plastics for cell cultures were purchased from Life Technologies Europe (Monza, Italy).

2.2. Vesicle Preparation

For vesicle preparation, 90 mg of S75 and 3 mg of *C. halicacabum* extract was hydrated with 1 mL of a mixture of distilled water (900 µL/mL) and PEG400 (100 µL/mL) to obtain PEVs, or with 1 mL of dispersion of sodium hyaluronate (0.1%) in water (900 µL/mL) and PEG400 (100 µL/mL) to obtain hyalurosomes. In addition, PEVs and hyalurosomes were modified by adding gelatin (3 mg/mL, final concentration) or chondroitin sulfate (3 mg/mL, final concentration).

The dispersions were sonicated (25 cycles, 5 on/2 off) by using a Soniprep 150 sonicator (MSE Crowley, London, UK) to obtain a homogeneous dispersion with small vesicles. Empty vesicles without *C. halicacabum* extract were prepared as well and used as controls. Vesicles were stored at 4 °C.

2.3. Vesicle Characterization

The morphology of the vesicles has been evaluated by cryogenic transmission electron microscopy (cryo-TEM), by using a TECNAI G2 20 TWIN (FEI) microscope (FEI Company World Headquarters and North American Sales, 5350 NE Dawson Creek Drive Hillsboro, OR, USA), operating at an accelerating voltage of 200 KeV in a bright-field image mode and low-dose image mode. We applied 3 µL of sample dispersion to a glow-discharged 300 mesh Quantifoil TEM grid, which underwent plunge freezing into liquid ethane on a FEI Vitrobot Mark IV (Eindhoven, The Netherlands). The frozen grid was then transferred to a 626 DH Single Tilt Cryo-Holder (Gatan, France), maintained below −170 °C, and then transferred to TEM at the same temperature.

The average diameter, polydispersity index and zeta potential of each sample were determined by means of light scattering technique by using a Zetasizer Ultra (Malvern Panalytical Ltd., Malvern, UK). These parameters were also measured over a storage period of 7 months at 4 °C, to evaluate the long-term stability of vesicles.

Each sample (2 mL) was purified from the non-incorporated extract by dialysis (Spectra/Por® membranes, 12–14 kD, 3 nm pore size; Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) against water (2:1) at room temperature for 2 h and refreshing the water after 1 h. The antioxidant activity (AA) of the extract-loaded vesicles was measured by means of a 2-diphenyl-1-picrylhydrazyl (DPPH) colorimetric test, before and after the dialysis process. Twenty microlitres of each formulation was dissolved in 1980 µL of DPPH methanolic solution previously prepared (1:50). The methanolic solution of DPPH at the same dilution was used as a control (100% absorbance). Samples were incubated for 30 min in the dark at room temperature. Subsequently, the absorbance of each solution was measured at λ = 517 nm with a UV spectrophotometer (Lambda 25, Perkin Elmer,

Milan, Italy). All the experiments were performed in triplicate. The antioxidant activity was calculated according to the following Formula (1) [36]:

$$AA(\%) = \frac{[(ABS_{DPPH} - ABS_{DPPH})]}{ABS_{DPPH}} \times 100 \quad (1)$$

The entrapment efficiency (EE) of the vesicles was expressed as the percentage of the antioxidant activity before and after dialysis.

2.4. Spray Characterization

The velocity and angle of droplets generated by the nasal devices integrated with data provided by the measurement of droplet size distributions are essential parameters to evaluate the suitability of nasal spray formulations to evaluate their effective deposition in the nasal cavity and their correct administration to patients [37,38].

2.4.1. Droplet Size Distribution

The size of the particles generated by spraying the formulations was measured by means of laser diffraction technique by using a Malvern Spraytec® (Malvern Panalytical Ltd., Malvern, UK). For in vitro tests, the Food and Drug Administration (FDA) recommends determining the droplet size distribution at two distances comprised between 2 and 7 cm from the nozzle tip, with a difference of 3 cm apart [39–41]. Thus, herein the spray pattern data were obtained at two actuation distances from the laser beam, 4 and 7 cm. Twenty millilitres of each formulation were loaded into commercial spray pump devices kindly supplied by FAES Laboratories. The used devices were suitable for pharmacological applications composed of a 0.1 mL metered pump and a 30 mL spray bottle. Before each experiment, formulations were hand shaken for 10 s and then 3 actuations were shot blank to test the device. After applying a pressure on the supportive surface for fingers in the upper part of the device, the formulation inside was drawn up through a siphon tube from the bottom of the bottle and expelled through the nozzle [42].

Measurements were performed in triplicate at 25 °C, at 4 cm and 7 cm of distance and rotating the pump device 45° in relation to the laser beam. Data were reported as the D10, D50, and D90 volume diameter percentiles, expressed as 10%, 50% and 90% of the cumulative volume undersize. To characterize the width of droplet size distribution, the Span number was also reported as (D90−D10)/D50) as previously [43].

2.4.2. Spray Structure, Velocity Module and Spray Angle

The distribution of sprayed droplets and their average velocity were measured by laser plane visualization and particle image velocimetry. This technique allows the capture of two components of the velocity with high spatial resolution in a whole slice of the flow [43]. To visualize the atomization process, instantaneous images were acquired with a Hamamatsu 1,024 × 1,344 pixels 12-bit C4742-95-12 ORCA-ER charge-coupled device (CCD) camera (Hamamatsu Photonics, Shizuoka, Japan), equipped with a Nikon 50 mm F#1.2 lens. The images size were 154 mm × 117.35 mm, yielding a resolution of 114.6 µm/pixel. To freeze the motion, a PILS NdYAG laser from Quanta System (Quanta System, S.p.A., Milan, Italy) was used to illuminate a vertical plane across the center of the spray and generate 6 pulses with a selectable time interval of 30 µs. Images were processed with the CCDPIV computer code developed at the Laboratory for Turbulence Research in Aerospace and Combustion in Monash University (Melbourne, Australia) [44]. Analysis was performed in 32 × 32 pixel windows with 50% overlap, resulting in maps with 82 × 62 velocity vectors.

The mean velocity field of the spray droplets was obtained from 100 instantaneous measurements [45] that have been averaged to determine the angle of the spray cone. This angle was obtained by the ocular location of the limits of the spray in the average image. All the photographs have been taken under the same lighting conditions and have undergone the same renormalization of the light levels captured by the camera.

To eliminate the influence of the operator in the generation of the spray, a pneumatic actuator to drive the manual atomizer was assembled. The duration of the liquid exiting from the nozzle (i.e., the duration of the atomization pulse) varies depending on the pressure supplied to the pneumatic device. This pressure has been adjusted to obtain a reasonable actuation, with a pulse duration of 150 m, as measured from the images.

The spray speed and angle measurements were taken 83 m after the start of the atomization; that is, about 8 m after the mid-pulse. Finally, the commercial atomizers used were manufactured with plastic injection molding, so certain variability in the atomization of each device could be expected. To evaluate the device variability, the actuation of 3 atomizers with distilled water was measured as previously reported [43].

2.5. Biocompatibility of Formulations against Keratinocytes

Human immortalized keratinocytes (HaCaT), used as model cells, were grown as monolayers in 75 cm^2 flasks, incubated with 100% humidity and 5% CO_2 at 37 °C. They were cultured with phenol red-free Dulbecco's modified Eagle medium (DMEM) with high glucose, supplemented with 10% fetal bovine serum (FBS) and penicillin and streptomycin. The cells were seeded into 96-well plates at a density of 7.5×10^3 cells/well, and after 24 h of incubation, they were treated for 48 h with the extract in dispersion (water and PEG400) or loaded in vesicles. All the dispersions were properly diluted with the cell medium to reach the desired concentrations (0.03, 0.3, and 3 µg/mL). The possible toxic effects of the formulations towards HaCaT cells has been assessed by measuring the viability by means of the MTT (tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric test. After 48 h, the medium was removed and MTT (100 µL) was added to each well and incubated at 37 °C for 2/3 h. The formazan crystals formed in viable cells were dissolved in DMSO, and the absorbance was measured at 570 nm with a microplate reader (Synergy 4, BioTek Instruments, AHSI S.p.A, Bernareggio, Italy). All the experiments were repeated at least three times and each time in triplicate. The results are expressed as the percentage of live cells compared to untreated cells (100% viability).

2.6. Protective Effect of the Extract in Dispersion or Loaded in Vesicles against Oxidative Stress

HaCaT cells (5×10^4 cells/well) were seeded in 96-well plates with 250 µL of culture medium and incubated at 37 °C for 24 h, then stressed for 4 h with hydrogen peroxide (1:50,000 dilution) and simultaneously exposed to the extract in dispersion or incorporated into the vesicles (final concentrations 3, 0.3, 0.03 µg/mL). Unstressed cells were used as the positive control (100% viability) and hydrogen peroxide-stressed cells, treated with extract-free medium, were used as the negative control. After 4 h of incubation, the medium was removed and the viability of the cells was determined with the MTT colorimetric test, adding 100 µL of reagent in each well. After 2/3 h, the formed formazan crystals were solubilized by adding dimethyl sulfoxide, and their concentration was measured spectrophotometrically at 570 nm as above (Section 2.5).

2.7. Statistical Analysis

Results were expressed as mean value ± standard deviation. Statistically significant differences were determined employing variance analysis (ANOVA), and the Tukey's test and Student's t-test were performed to substantiate differences between groups using XL Statistics for Windows (XLSTAT BASIC+). The minimum level of significance chosen was $p < 0.05$.

3. Results

3.1. Vesicle Characterization

The actual formation, structure and morphology of *C. halicacabum* extract-loaded vesicles were evaluated by means of cryo-TEM observation. All tested vesicles were mostly small and unilamellar, but some larger vesicles were present as well (Figure 1).

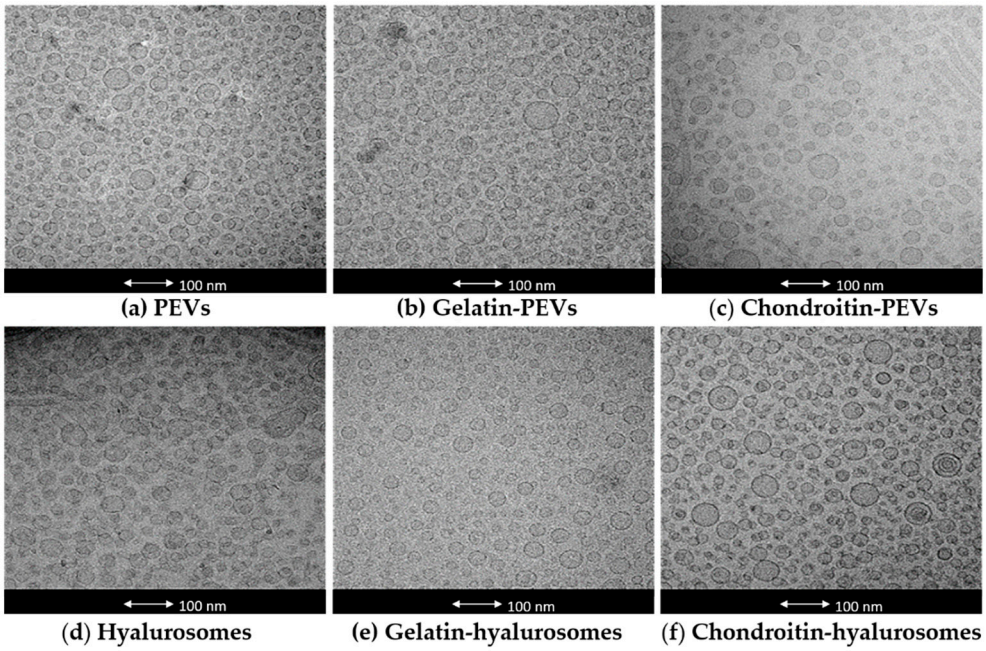

Figure 1. Representative cryo-TEM images of vesicles: (**a**) PEVs; (**b**) gelatin-PEVs; (**c**) chondroitin-PEVs; (**d**) hyalurosomes; (**e**) gelatin-hyalurosomes; (**f**) chondroitin-hyalurosomes.

The mean diameter, polydispersity index, zeta potential and entrapment efficiency of the obtained vesicles were measured (Table 1). Empty vesicles were also prepared and characterized to evaluate the effect of the extract on the vesicle assembling. Empty PEVs, chondroitin-PEVs and hyalurosomes were ~106 nm and the others were larger (~150 nm). The loading of the extract did not change the size of PEVs (~100 nm) and gelatin-hyalurosomes (~120 nm, $p > 0.05$ between the mean diameter of empty and corresponding extract-loaded vesicles). On the contrary, the loading of the extract produced an increase of mean diameter of chondroitin-PEVs and chondroitin-hyalurosomes ($p < 0.05$ versus the mean diameter of corresponding empty vesicles) and a reduction of size of the other formulations. The photon correlation spectroscopy only estimates the average size of vesicles by a cumulative intensity-based value, which is weighed according to the scattering intensity of the volume of different particle fractions. In this case, the fraction volume of few larger vesicles present in the dispersions was not clearly represented in the cumulative analysis, causing a small discordance with cryo-TEM images. In addition, the mean diameter of vesicles and its standard deviation were calculated as an average of at least six analyses, which were very repeatable as the standard deviation was very low.

Extract-loaded PEVs were slightly polydispersed (polydispersity index 0.4) and negatively charged (~48 mV). Hyalurosomes were smaller, ~65 nm ($p < 0.05$ versus the mean diameter of PEVs), less polydispersed (polydispersity index 0.3), and negatively charged (~−45 mV) like PEVs ($p > 0.05$ between the value of liposomes and hyalurosomes). The addition of gelatin did not modify the mean diameter and polydispersity index of both PEVs and hyalurosomes, but markedly affected the zeta potential, which became less negative (~−24 mV, $p < 0.01$ versus the value of PEVs and hyalurosomes) due to the positive charge of gelatin on the vesicle surface.

The behavior of chondroitin sulfate was different. Its addition led to an increase of the mean diameter of extract-loaded chondroitin-PEVs (~156 nm, $p < 0.05$ versus the mean diameter of PEVs) and chondroitin-hyalurosomes (~178 nm, $p < 0.05$ versus the mean diameter of hyalurosomes), which were the largest vesicles. On the contrary, chondroitin sulfate allowed the formation of more homogeneous dispersions, as the polydispersity index of chondroitin-PEVs was ~0.1, and that of chondroitin-hyalurosomes ~0.2. Chondroitin did not affect the zeta potential, which might be related to a strong interaction and intercalation of chondroitin within the bilayer, leading to an enlargement of the vesicle diameter.

Table 1. Mean diameter (MD), polydispersity index (PI), zeta potential (ZP) and entrapment efficiency (EE) of empty and extract-loaded PEVs, gelatin-PEVs, chondroitin-PEVs, hyalurosomes, gelatin-hyalurosomes, chondroitin-hyalurosomes. Data represent the mean ± standard deviation of at least six replicates. The same symbol (*, §, +, #, °) indicates the same value ($p < 0.05$).

	MD (nm)	PI	ZP (mV)	EE (%)
Empty PEVs	* 92 ± 2	0.23	−67 ± 4	
Empty gelatin-PEVs	+ 175 ± 25	0.31	−55 ± 7	
Empty chondroitin-PEVs	#,* 117 ± 30	0.43	−13 ± 3	
Empty hyalurosomes	* 92 ± 3	0.45	−21 ± 10	
Empty gelatin-hyalurosomes	# 129 ± 3	0.13	−30 ± 12	
Empty chondroitin-hyalurosomes	§ 154 ± 13	0.20	−44 ± 10	
PEVs	* 100 ± 20	0.43	−48 ± 14	* 99 ± 16
Gelatin-PEVs	* 98 ± 4	0.63	−25 ± 2	° 73 ± 36
Chondroitin-PEVs	§ 156 ± 23	0.23	−46 ± 1	° 75 ± 10
Hyalurosomes	° 65 ± 6	0.26	−45 ± 2	47 ± 13
Gelatin-hyalurosomes	# 118 ± 8	0.30	−23 ± 6	* 93 ± 57
Chondroitin-hyalurosomes	+ 178 ± 6	0.09	−55 ± 2	° 65 ± 9

The entrapment efficiency of the extract in PEVs was very high (~99%) and the addition of the polymers led to a decrease of this parameter (~75%, Table 1). On the contrary, the entrapment efficiency of the extract in hyalurosomes was the lowest (~47%) and the addition of the polymers allowed an increase of the loading, especially using gelatin-hyalurosomes, which reached the same value of PEVs (~90%, $p > 0.05$ versus the values of PEVs).

The long-term stability of the formulations was evaluated by storing the samples for a period of seven months at 4 °C and measuring their physicochemical properties (Figure 2). Temperature of storage is of high importance, as high temperature may promote aggregation and fusion phenomena that are slowed down at lower temperatures such as 4 °C. The mean diameter of PEVs was ~100 nm immediately after preparation, tripled (~320 nm, $p < 0.05$) after three months of storage, and became undeterminable already at the fourth month due to the phase separation and formation of a precipitate of both aggregated vesicles and free extract. The addition of the polymers further reduced the stability of PEVs as the size of gelatin-PEVs and chondroitin-PEVs reached ~950 nm after only one month of storage. After that, it was impossible to measure the size and zeta potential due to the formation of a precipitate mainly composed of aggregated vesicles. Hyalurosomes doubled their diameter after one month of storage (from ~65 nm to ~119 nm, $p < 0.01$ between the two values), and after three months the mean diameter was undetectable due to the formation of large aggregates. The addition of gelatin or chondroitin sulfate to hyalurosomes led to an improvement of the stability of vesicles in dispersion. Indeed, the mean diameter of chondroitin-hyalurosomes increased up to ~260 nm, that of gelatin-hyalurosomes reached ~141 nm after one month of storage, and both remained constant for the following six months. This could indicate that the combination of sodium hyaluronate with fish gelatin or chondroitin sulfate promoted the stability of vesicles in dispersion and facilitated the loading of *C. halicacabum* extract. The zeta potential followed the same trend, remaining coherent and strongly negative during the entire period of the stability check (Figure 2), even if some minor changes could be detected, mainly due to the aggregation and re-assembling of the vesicles in different structures.

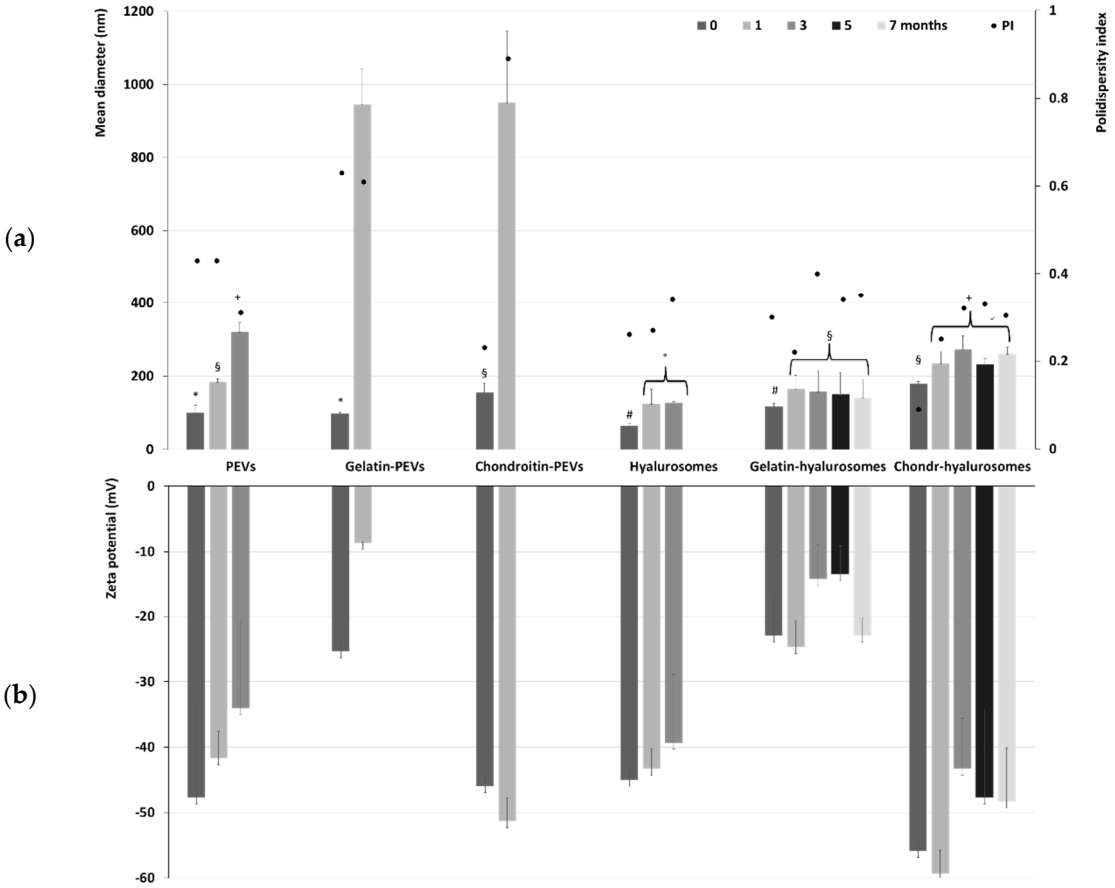

Figure 2. (**a**) Mean diameter, polydispersity index (PI) and (**b**) zeta potential of the extract-loaded PEVs, gelatin-PEVs, chondroitin-PEVs, hyalurosomes, gelatin-hyalurosomes, chondroitin-hyalurosomes, measured over seven months. Data represent the means ± standard deviations of at least six replicates. The same symbol (*, §, +, #, ◦) indicates the same value.

3.2. Determination of Size Distribution of Sprayed Droplets

The suitability of formulations to be sprayed into the nasal cavity was firstly evaluated by measuring the droplet size distribution. The Food and Drug Administration (FDA) and the European Medicines Agency (EMA) request droplet sizes of greater than 10 µm, to avoid the possibility to being inhaled and reaching the lungs [46]. It is well accepted that nasal local sprays should be composed of particles with a mean size higher than 120 µm to be mainly deposited in the anterior part of the nose [40]. In accordance with the FDA, measurements were performed at a 4 cm and 7 cm distance from the nozzle exit, rotating the pump device 45° in respect to the laser beam [41,47]. The size of 50% of droplets (D50) generated by the used vesicles, except chondroitin-hyalurosomes, were lower than 120 µm (59–89 µm at 4 cm and 47–72 µm at 7 cm), but 90% of droplets were higher than 118 µm (118–196 µm), disclosing the size suitable for the deposition in the anterior region of nose. The size of the droplets generated by chondroitin-hyalurosomes were slightly larger, ~102 µm (D50) and 220 µm (D90) at 4 cm, and 154 µm (D50) and 133 µm (D90) at 7 cm, suggesting a slightly reduced suitability of these formulations to be deposited in the anterior area of nose (Table 2).

3.3. Measurements of Spray Plume and Angle

The characteristics of the spray plumes generated by the vesicle dispersions were also measured. To assess the possible variability introduced by pump and nozzle manufacture, the behavior of distilled water sprayed with three different devices has been evaluated and used as a reference, as reported in a previous work [43]. The images of sprayed

water established the atomizing ability of the device to compare the results obtained when spraying the formulations of *C. halicacabum* extract loaded in vesicles (Figure 3).

Table 2. Average diameter of droplets generated by spraying *C. halicacabum* extract-loaded PEVs, gelatin-PEVs, chondroitin-PEVs, hyalurosomes, gelatin-hyalurosomes, and chondroitin-hyalurosomes, and measured using Spraytec®. Mean values ± standard deviations of three measurements were reported. The same symbol (*, §, +, #, ◦, @) indicates the same value.

	4 cm				7 cm			
	D10 (μm)	**D50 (μm)**	**D90 (μm)**	**SPAN (μm)**	**D10 (μm)**	**D50 (μm)**	**D90 (μm)**	**SPAN (μm)**
PEVs	29 ± 2	*,+ 63 ± 18	§ 130 ± 46	2 ± 1	29 ± 2	+ 57 ± 1	@ 173 ± 53	3 ± 1
Gelatin-PEVs	31 ± 3	# 89 ± 10	@ 185 ± 43	3 ± 1	31 ± 1	* 66 ± 6	@ 168 ± 50	4 ± 1
Chondroitin-PEVs	33 ± 2	◦ 69 ± 4	§ 131 ± 6	1 ± 0	30 ± 1	* 47 ± 20	§ 118 ± 2	1 ± 0
Hyalurosomes	27 ± 1	*,+ 59 ± 8	§ 154 ± 49	3 ± 1	33 ± 1	* 61 ± 2	@ 162 ± 76	4 ± 2
Gelatin-hyalurosomes	30 ± 1	# 81 ± 9	@ 171 ± 27	3 ± 1	33 ± 2	◦ 72 ± 2	@ 196 ± 58	3 ± 1
Chondroitin-hyalurosomes	30 ± 3	102 ± 48	220 ± 27	3 ± 1	38 ± 5	§ 154 ± 129	§ 133 ± 30	4 ± 2

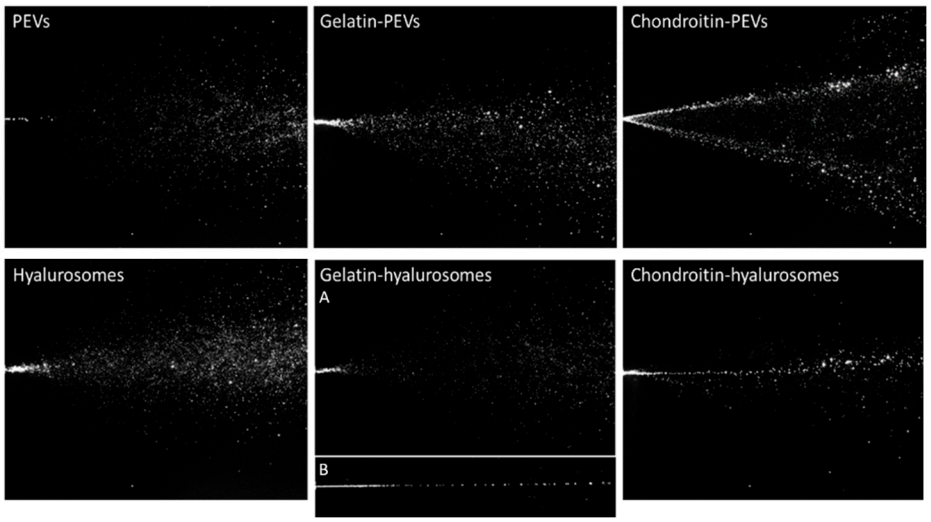

Figure 3. Representative images of instant visualization of plume generated by three consecutive sprays of PEVs; gelatin-PEVs; chondroitin-PEVs; hyalurosomes; (A, B) gelatin-hyalurosomes; and chondroitin-hyalurosomes.

Dispersions of PEVs and gelatin-PEVs produced a full cone plume, like that of water but less intense and large, while chondroitin-PEVs produced a hollow cone plume, not desirable for our purposes, where the liquid droplets were mainly located in the outer layers and sparsely populated the interior region [43] (Figure 3).

Hyalurosome and gelatin-hyalurosome dispersions generated a full cone plume. Gelatin-hyalurosomes atomization was poorer, and in some cases, a liquid column was generated (Figure 3, gelatin-hyalurosome image B). Chondroitin-hyalurosomes only generated a very narrow cone (almost a liquid column) of large droplets, according to the results obtained by Spraytec®, which underlined the formation of large particles having a mean diameter > 100 μm (D50), and containing a small number of particles >200 μm (D90).

The spray angle was calculated by the obtained images of the plume (Table 3). The spray angle of the distilled water cases was ~17.6° as reported in a previous work [37]. The spray cone angle of chondroitin-PEVs and hyalurosomes was the lowest (~19–20°) and similar to that obtained by spraying water. Gelatin-PEVs and gelatin-hyalurosomes generated a large opening plume (~25°), that further increased using PEVs. The plume of the chondroitin-hyalurosomes was not quantified due to the formation of a liquid column.

Table 3. High speed values and spray angle measured from the plume generated by *C. halicacabum* extract-loaded PEVs; gelatin-PEVs; chondroitin-PEVs; hyalurosomes; gelatin-hyalurosomes; and chondroitin-hyalurosomes.

	High-Speed (M/S)	Spray Angle (°)
PEVs	13.9	29.7
Gelatin-PEVs	8.0	24.5
Chondroitin-PEVs	13.1	19.2
Hyalurosomes	15.2	20.7
Gelatin-hyalurosomes	15.1	25.4
Chondroitin-hyalurosomes	7.5	-

Measurements of Average Velocity Module

The velocity measurements of sprayed vesicles generated, at the atomizer exit, a conical sheet of liquid with decreasing intensity (Figure 4), similar to that of water [40].

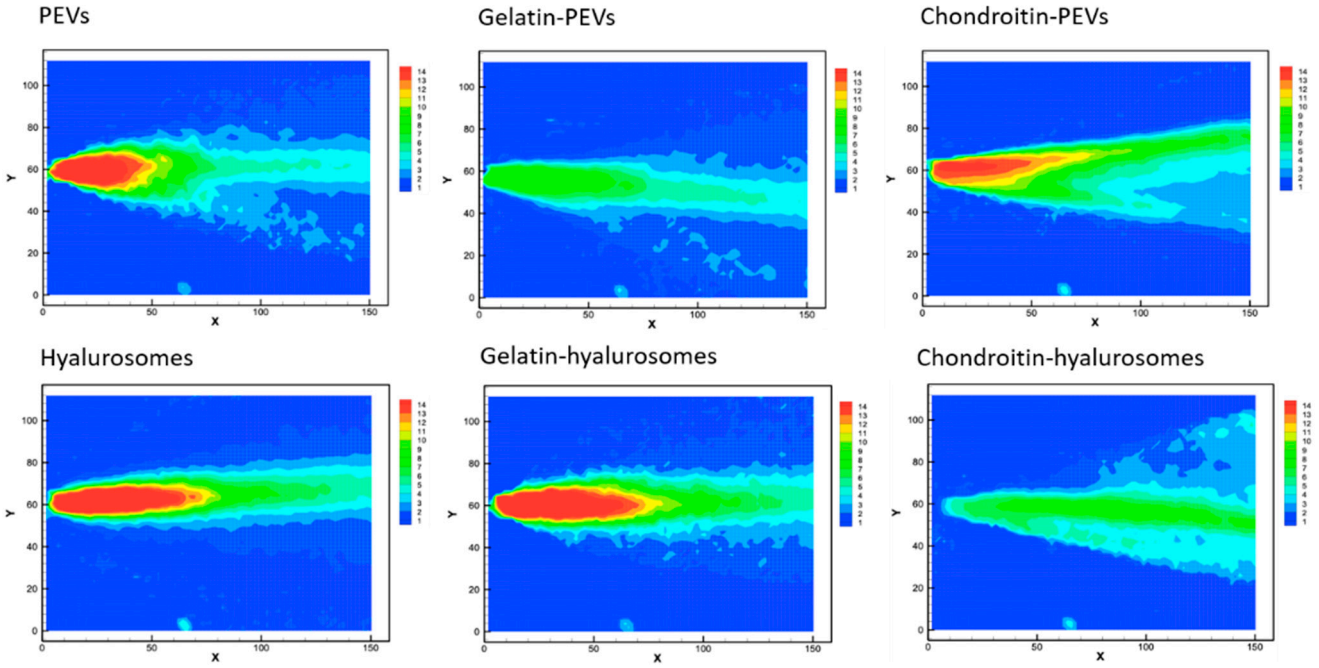

Figure 4. Representative images of average drop speed generated by the spray of the extract loaded PEVs; gelatin-PEVs; chondroitin-PEVs; hyalurosomes; gelatin-hyalurosomes; and chondroitin-hyalurosomes. Red indicates high velocity, yellow indicates intermediate velocity, and green and light-blue indicate low velocity.

The cone generated by PEVs, chondroitin-PEVs, hyalurosomes and gelatin-hyalurosomes had a central part intensely colored in red, indicating high velocity (Figure 4). Indeed, the maximum speed values for PEVs and chondroitin-PEVs were ~13.4 m/s, and those of hyalurosomes and gelatin-hyalurosomes were ~15.1 m/s, thus quite similar in structure and values to those obtained using the water [44]. The maximum velocity of gelatin-PEVs and chondroitin-hyalurosomes was the lowest (~7.7 m/s), as they did not generate a red cone core.

3.4. Biocompatibility of Vesicles and Protective Effect against Oxidative Stress Damage

Considering the promising technological properties of the obtained vesicle dispersions, the in vitro biocompatibility and antioxidant activity against keratinocytes were evaluated as well, and compared with that of the extract in dispersion [2,4,5,7–10,12,48,49]. Firstly, the biocompatibility was monitored incubating the cells with the extract in dispersion or incorporated in the vesicles for 48 h and measuring their viability (Figure 5). The dispersion

of the extract was highly biocompatible and even capable of stimulating cell proliferation (viability ~118%, $p < 0.05$ versus the viability of cells treated with chondroitin-hyalurosomes at lower concentrations). The loading of the extract in vesicles differently affected the cell viability as a function of the used carriers and the dilution tested. More specifically, when PEVs were used at the lower concentrations (0.03 and 0.3 μg /mL), the viability was ~115% ($p > 0.05$ versus the viability of cells treated with the dispersion), as found using the extract dispersion. At the highest concentration (3 μg /mL), the cell viability strongly decreased (~80%, $p < 0.05$ versus the values provided by lower concentrations), denoting a low toxicity at this concentration. Using hyalurosomes and gelatin-hyalurosomes, the cell viability was ~80% ($p < 0.05$ versus the viability found using the dispersion), irrespective of the used concentrations. Using gelatin-PEVs and chondroitin-PEVs, the cell viability was ~112% ($p > 0.05$ versus the viability of cells treated with the dispersion or the PEVs at lower concentrations), irrespective of the carrier and concentration used. The addition of chondroitin sulfate in hyalurosomes seems to stimulate cell proliferation, as the viability approached ~150% ($p < 0.05$ versus the other values) when the lower concentrations (0.03 and 0.3 μg/mL) were used. The cell proliferation can be related to the dual effect of sodium hyaluronate and chondroitin, associated with the extract, which can favor the proliferation of keratinocytes, thus suggesting a positive contribution of this formulation in accelerating epithelial regeneration processes [50]. Using these vesicles at the highest concentration (3 μg/mL), the cell viability was lower (~110%) ($p < 0.05$).

The protective effect of extract-loaded vesicles (0.3 and 3 μg/mL) against oxidative stress damage was evaluated using keratinocytes (Figure 6). The exposure of cells to hydrogen peroxide (untreated cells) reduced the viability up to ~55%, while the addition of the extract in dispersion or loaded in vesicles successfully prevented the cell mortality caused by hydrogen peroxide, thus restoring the healthy conditions. Indeed, the cell viability increased up to ~110% ($p < 0.01$ versus the viability of untreated cells), except when chondroitin-hyalurosomes were used, as these formulations only partially protected the cells, allowing a cell viability of ~85% ($p < 0.05$ versus other values).

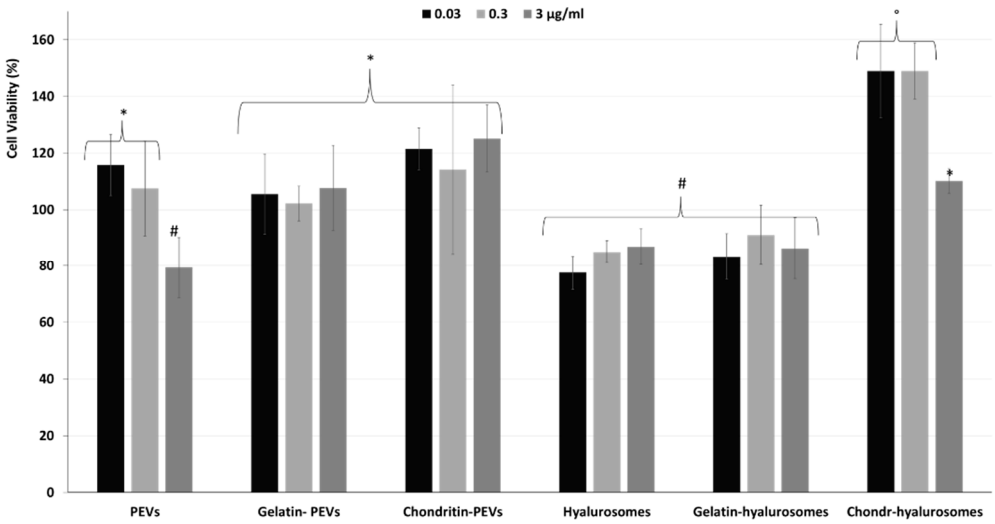

Figure 5. *Cont.*

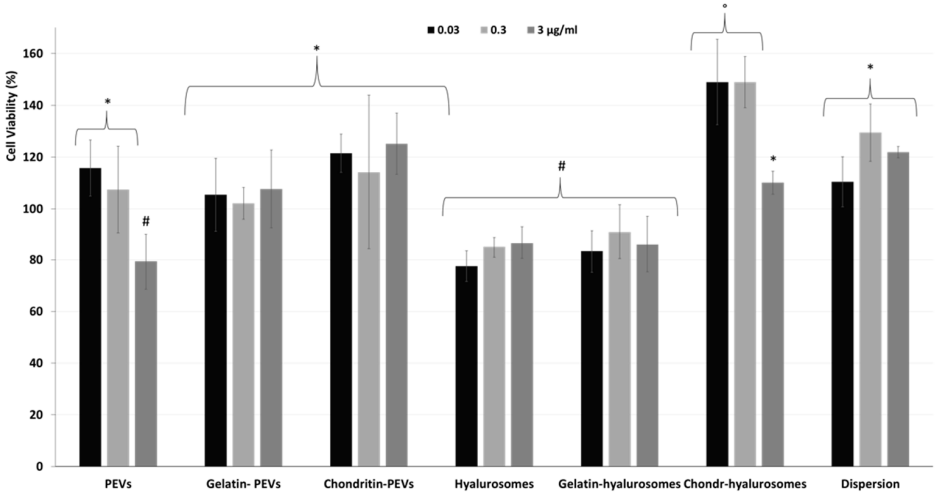

Figure 5. Viability of keratinocytes treated with *C. halicacabum* extract in dispersion or loaded in PEVs; gelatin-PEVs; chondroitin-PEVs; hyalurosomes; gelatin-hyalurosomes; and chondroitin-hyalurosomes, at different dilutions (extract 0.03, 0.3, 3 µg/mL). Data represent the means ± standard deviations of at least three experimental determinations. Each symbol (*, #, °) indicates the same value.

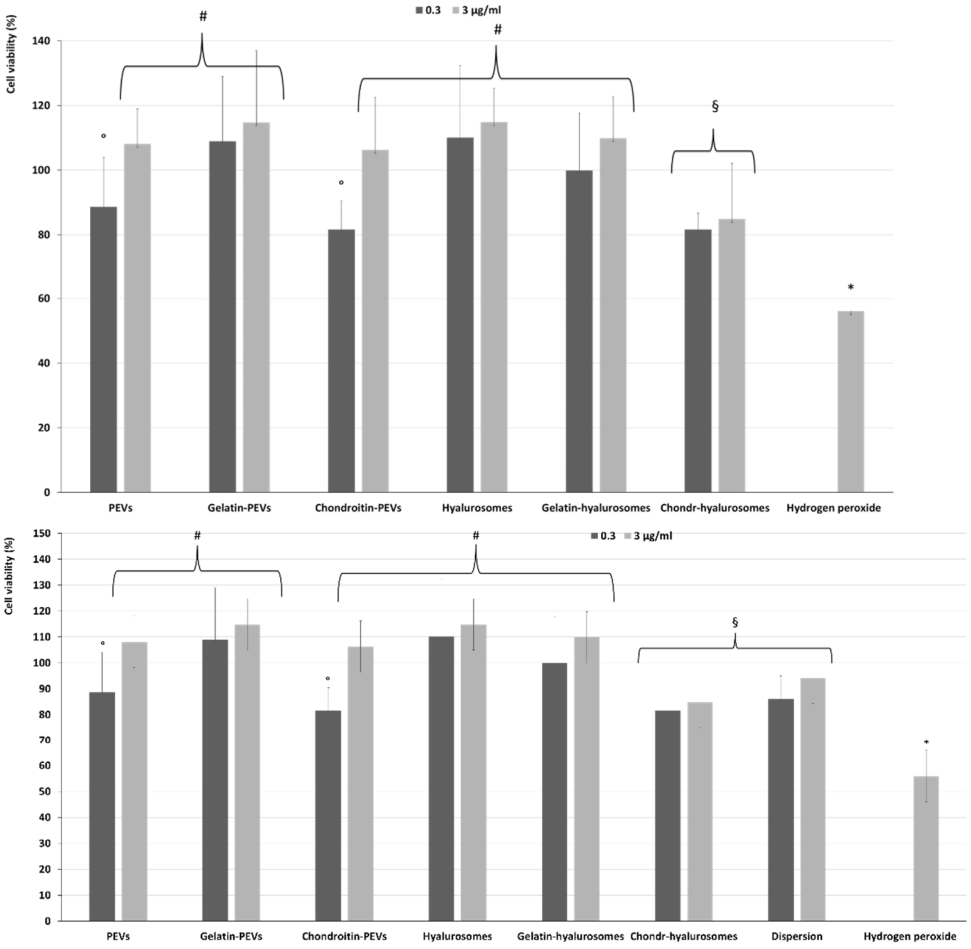

Figure 6. Viability of keratinocytes stressed with hydrogen peroxide and treated with *C. halicacabum* extract-loaded PEVs; gelatin-PEVs; chondroitin-PEVs; hyalurosomes; gelatin-hyalurosomes; chondroitin-hyalurosomes, at different dilutions (extract 0.3, 3 µg/mL). Data represent the means ± standard deviations of cell viability expressed as the percentage of the negative control (100%). Each symbol (*, #, °, §) indicates the same value.

4. Discussion

Aiming at developing an effective nasal spray based on natural components and capable of reaching the anterior part of nasal mucosa to attenuate nasopharyngeal congestion and rhinitis, *C. halicacabum* extract was loaded into phospholipid vesicles specifically tailored for nasal delivery. *C. halicacabum* extract was selected based on its rich phytocomplex, which contains apigenin, apigenin-7-O-glucuronide, chrysoeriol-7-O-glucuronide, luteolin, luteolin-7-O-glucuronide, saponin, quebrachitol, proanthocyanin, beta-sitosterol and stigmasterol, and its beneficial properties, well known for centuries [1,2,51]. It was loaded in phospholipid vesicles due to their effectiveness in the treatment of rhinitis. Indeed, the simple empty phospholipid vesicles (without bioactives) are currently used to counteract nasal congestion and inflammation, which generally occur in rhinitis, and formulations based on liposomes and hyalurosomes are already available on the market in Europe [16,17]. Definitely, the association of phospholipid vesicles with the extract of *C. halicacabum*, which has anti-inflammatory and antioxidant activities, is expected to provide a synergic effect [19]. To load the highest amount of extract, in a pre-formulation study, several formulations were prepared, testing different amounts and types of phospholipids, water co-solvents, polymers and surfactants. Finally, small and stable vesicles were obtained using 3 mg/mL of the extract of *C. halicacabum* and 92 mg/mL of a commercial mixture of soy phosphatidylcholine (Lipoid S75). The former were hydrated with a mixture of water and PEG400 to prepare PEVs, and with a dispersion of sodium hyaluronate in water and PEG400 to obtain hyalurosomes. PEVs and hyalurosomes were further modified using a commercial cold-water fish gelatin or chondroitin sulfate extracted from catshark cartilages (*Scyliorhinus canicula*) to stabilize the vesicles. Gelatin is widely used in cosmetic, biomedical and pharmaceutical applications [52]. Chondroitin sulfate, similarly to hyaluronic acid, is an eco-sustainable biopolymer that can be obtained from animal wastes and by-products [53].

The obtained vesicles were small sized and negatively charged; the addition of gelatin markedly affected the zeta potential, which became less negative due to the positive charge of gelatin, which is a polyampholyte molecule (~13% positively-charged amino acids, ~12% negatively-charged amino acids and ~11% is hydrophobic amino acids) with cationic and anionic nature as a function of pH and temperature, that may modify its behavior in solution [51,54,55]. Fish gelatin is a cationic type A gelatin, whose isoelectric point is between 7.0 and 9.0 [52,55]. Elzoghby et al. disclosed that the positive charge of gelatin-based nanoparticles is related to the prevalence of NH_3^+ groups [50]. In the vesicle surface, gelatin interacted with the negatively-charged phospholipids, leading to an increase in the surface zeta potential, which became less negative.

The addition of chondroitin sulfate mostly affected the mean diameter, forming larger vesicles while the zeta potential remained strongly negative due to its carboxyl groups and sulfo groups, which are probably mainly located on the vesicle surface [56]. The presence of gelatin or chondroitin was not capable to stabilize the PEVs in dispersion, but improved the stability of hyalurosomes, which kept a small size and negative zeta potential for up to seven months.

Considering the small size of the obtained formulations, which are suitable for skin and mucosal delivery, their spray-ability into the nasal cavity was firstly evaluated in vitro by measuring the droplet size distribution, spray angle and droplet velocity [50,57]. Indeed, nasal spray devices commonly generate a cone of aerosolized droplets, called a plume, having a mean size in the range of 10–200 μm, which are recognized as a fine mist able to well deposit in the nasal cavity [37,57–61]. It has been reported that spray pumps that produce droplets >10 μm can ensure its deposition in the nose, and if the droplets are >120 μm, they mostly deposit in the anterior part of the nose, exerting a local effect [40,62]. According to the FDA recommendations, nasal sprays must generate droplets <10 μm to avoid their lung inhalation. In the present study, the size distribution of sprayed droplets was within these parameters, and 90% of droplets (D90) were >118 μm, confirming the suitability of these formulations to be sprayed in the nose, reach the anterior nasal cavity,

and exert a local effect. Among all, chondroitin-hyalurosomes were slightly less suitable to deposit in the anterior zone.

The spray plume analysis confirmed the ability of PEVs, gelatin-PEVs, hyalurosomes and gelatin-hyalurosomes to be atomized in fine droplets homogenously distributed in a full cone plume, with an angle ranging from 25 to 30°. Generally, significant changes in the area of deposition have also been reported as a function of plume angle [63]. As the plume angle decreases (narrower plume), an increased deposition in the turbinate region has been reported, but it was also dependent on the droplet size and velocity [62,64]. Indeed, high-speed droplets are mostly deposited in the anterior part of the nose [62]. In this study, PEVs, chondroitin-PEVs, hyalurosomes and gelatin-hyalurosomes generated droplets with maximum speed ~14 m/s, which was equal to that of water, and double if compared to that of gelatin-PEVs and chondroitin-hyalurosomes.

Thus, overall results on sprayed plume underlined that PEVs, hyalurosomes and gelatin-hyalurosomes have the most suitable characteristics for nasal local delivery thanks to their ability to form a full cone of homogeneous droplets having a size of always <196 μm, a cone angle ~27° and high-speed ~14 m/s. The formed droplets can be well deposited in the nasal cavity, which serves as a target site for the treatment of local diseases such as nasal allergic conditions and nasal congestions [65].

In this study, the analysis of technological properties of vesicle dispersions has been associated to the evaluation of their biological efficacy. The biocompatibility of *C. halicacabum* extract in dispersion or loaded in vesicles confirmed that all PEVs and chondroitin-hyalurosomes, along with the extract in dispersion, at lower concentrations (0.03 and 0.3 μg /mL), were not toxic and favored cell proliferation, as the viability of epithelial cells after 48 h of incubation was ≥100%. Differently, using hyalurosomes and gelatin-hyalurosomes the cell viability was ~80%, indicating a weak toxicity of these formulations as previously observed using phospholipid vesicles [66]. Considering that the anti-inflammatory effect of *Cardiospermum halicacabum* extract and phospholipid vesicles were previously demonstrated, to perform a preliminary biological evaluation of formulations, their ability to protect the tissues from the oxidative damages was also evaluated. Indeed, oxidative stress can activate a variety of transcription factors, which lead to the differential expression of some genes involved in inflammatory pathways. The inflammation triggered by oxidative stress is the cause of many chronic diseases [67]. All the formulations, irrespective to their composition, inhibited the damaging effect of hydrogen peroxide in the cells at the intermediate non-toxic concentration used (0.3 μg/mL), counteracting their damages and death [68]. Their effectiveness can be mainly connected to the ability of vesicles to interact with cells favoring the internalization of the bioactives contained in the extract, which are mainly flavonoids and polyphenols capable of chelating iron and scavenging free radicals [69,70]. The beneficial effect of extract loaded in chondroitin-hyalurosomes was lower than that of extract in dispersion or loaded in other vesicles, such as PEVs, hyalurosomes and gelatin-hyalurosomes, which also disclosed the better spray-ability suggesting an effective deposition in the nasal cavity and improved biological performances. Among these formulations, considering the overall results comprehensive of physicochemical, spray-ability and biological parameters, gelatin-hyalurosomes seemed to be the most promising and effective dispersion for the treatment of nasal disorders connected with oxidative stress.

5. Conclusions

The evaluation of physicochemical, technological and biological properties of *C. halicacabum* extract-loaded vesicles indicated that the prepared formulations, especially PEVs, hyalurosomes and gelatin-hyalurosomes, are suitable to be atomized and deposited in the anterior nasal cavity, where they can exert local activity against congestion and rhinitis. In addition, they are able to protect the epithelial cells from oxidative stress, avoiding local tissue damages. Gelatin-hyalurosomes showed long-term stability with storage and seemed to be the most promising formulation for nasal delivery, thus encouraging their

possible application for the treatment of nasopharyngeal congestion and rhinitis associated with oxidative stress. Alternatively, the other formulations, like PEVs and hyalurosomes, which could not keep their physicochemical properties during storage, could be further stabilized by using other additives.

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Conflicts of Interest: The authors declare no conflict of interest.

Sample Availability: Samples of the compounds are not available from the authors.

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Zingiber officinalis

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Nasal Spray Formulations Based on Combined Hyalurosomes and Glycerosomes Loading *Zingiber officinalis* Extract as Green and Natural Strategy for the Treatment of Rhinitis and Rhinosinusitis

Eleonora Casula [1], Maria Letizia Manca [1,*], Matteo Perra [1], Jose Luis Pedraz [2,3,4], Tania Belen Lopez-Mendez [2,3], Antonio Lozano [5], Esteban Calvo [5], Marco Zaru [6] and Maria Manconi [1]

1 Department of Scienze della Vita e dell'Ambiente, University of Cagliari, via Ospedale 72, 09124 Cagliari, Italy; e.casula@studenti.unica.it (E.C.); Matteo.perra@unica.it (M.P.); manconi@unica.it (M.M.)
2 NanoBioCel Group, Laboratory of Pharmaceutics, School of Pharmacy, University of the Basque Country (UPV/EHU), 01006 Vitoria-Gasteiz, Spain; joseluis.pedraz@ehu.eus (J.L.P.); tblopez01@gmail.com (T.B.L.-M.)
3 Biomedical Research Networking Center in Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), 01006 Vitoria-Gasteiz, Spain
4 Bioaraba, NanoBioCel Research Group, 01006 Vitoria-Gasteiz, Spain
5 LIFTEC, CSIC—Universidad de Zaragoza, María de Luna 10, 50018 Zaragoza, Spain; a.lozano@csic.es (A.L.); calvober@unizar.es (E.C.)
6 Icnoderm Srl, Sardegna Ricerche Ed. 5, Pula, 09010 Cagliari, Italy; m.zaru@icnoderm.com
* Correspondence: mlmanca@unica.it; Tel.: +39-07-0675-8582; Fax: +39-07-0675-8553

Abstract: A total green nanotechnological nasal spray has been manufactured and proposed as an alternative treatment of rhinitis and rhinosinusitis. It was obtained by combining the strengthening effect of liposomes on barrier function, the hydrating and lubricating properties of sodium hyaluronan and the anti-inflammatory and antioxidant activities of the extract of *Zingiber officinalis*. To this purpose, the extract was loaded in special phospholipid vesicles immobilized with hyaluronic acid (hyalurosomes), which were further enriched with glycerol in the water phase. Liposomes and glycerosomes were prepared as well and used as reference. Vesicles were oligolamellar and multicompartment, as confirmed by cryogenic transmission electron microscopy (cryo-TEM) observation, small in size (~140 nm) and negatively charged (~−23 mV). Spray characteristics were evaluated by using the Spraytec® and instant images, from which the plume angle was measured. The range of the droplet size distribution and the narrow spray angle obtained suggest a good nebulization and a possible local deposition in the nasal cavity. In vitro studies performed by using human keratinocytes confirmed the high biocompatibility of vesicles and their ability to effectively counteract oxidative damage on cells induced by hydrogen peroxide. The overall collected data suggest that our vesicles are suitable as nasal spray.

Keywords: *Zingiber officinalis*; traditional medicine; phospholipid vesicles; antioxidant; epithelial cells; nasal spray; spray angle; droplet size

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1. Introduction

Rhinitis and rhinosinusitis are the two major clinical pathologies involving the upper airways [1]. They differ in their atopic status, symptom severity, duration, medical history and aetiology but have some common clinical presentations such as mucosa inflammation, sneezing, pruritus, purulent rhinorrhoea, nasal congestion, facial pressure and loss of smell. In addition, hyperresponsiveness to nonallergic stimuli, such as cold air and irritants is usually present due to the inflammation on the sensory nerves in the upper airway mucosa [2]. Rhinosinusitis involves inflammation of paranasal sinuses, other than the nasal cavity mucosa. Allergic rhinitis is commonly assumed to have a cause and effect

relationship with chronic rhinosinusitis [3]. They are serious common disorders that affects between 15% and 45% of the population worldwide negatively influencing the daily life of patients, especially those with severe symptoms and major disabilities [4].

The recommended treatment requires a combination of oral therapy and intranasal administration of corticosteroids, anti-leukotrienes and antihistamines to reduce mucosal oedema and promote sinus drainage, and eradicate infections in chronic rhinosinusitis [5]. In addition, nasal irrigation has been proven to be an effective additional treatment. It can reduce the associated symptoms and the frequency of acute exacerbation [6,7]. This treatment directly enhances the movement of the mucus toward the nasopharynx, removes inflammatory mediators such as histamine, prostaglandins and leukotrienes, and increases the ciliary beating frequency and consequently the mucociliary clearance [8]. Hyaluronic acid is widely used in allergic or non-allergic rhinitis and acute or moderate rhinosinusitis to reduce disease symptoms, neutrophil count and improve mucociliary transport [9]. It is a typical component of normal airway secretions, which hydrates and lubricates the mucosae of the upper respiratory tract by osmotic effect. It is also involved in the regulation of vasomotor tone and gland secretion and stimulates mucociliary clearance. In addition, hyaluronic acid can regulate inflammatory response by immune-suppressing role [10].

Liposomes are also used as nasal spray in rhinitis to support the cleansing, lubrication and hydration of nasal mucosa. Several studies demonstrated their effectiveness in reducing the symptoms of rhinitis probably because phospholipid are natural occurring surfactants, which reinforce the protective nasal layer [11]. Liposomal nasal sprays exert an effect comparable to that of antihistamine and glucocorticosteroid sprays [12,13]. Indeed, in the German pharmaceutical market, liposomal nasal sprays have been available since 2007 for the treatment of inflamed nasal mucosa [14]. The exact mechanism of action of liposomes is still unknown. It was hypothesized that they stabilise the nasal mucosal barrier by integrating in the damaged cell membrane and strengthening their barrier function [15]. Other studies disclosed that liposomes can absorb and inactivate allergens in allergic rhinitis [16,17].

Several herbal sprays have been used in the local treatment of rhinitis or rhinosinusitis due to their anti-inflammatory and anti-oxidant properties [18]. Ginger (*Z. officinalis*) is widely used as a spice in the world but it is also known for its beneficial properties [19]. Historical evidences attest that the therapeutic efficacy of ginger inhalation was already known since 4000 years in India where it was used for the treatment of respiratory diseases [20,21]. At the dawn of medical and pharmacological science, the administration of chemicals by local inhalation is consistent, because it is the most patient-friendly, natural, spontaneous and painless rout of administration. Today, the inhalation of ginger-based drugs is still used, mainly to counteract nausea and vomiting in post-surgery patients [22–24]. According to Stappen et al., the main effect of ginger inhalation is achieved by the odour [25]: the inhalation combines the effect given by the ginger's active compounds and its pungent smell, making it as a good alternative for the treatment of infectious diseases of the upper respiratory tract as well. Recent research proved that ginger is an effective remedy against respiratory diseases, such as cough, cold and asthma [26,27], acting as antibacterial [28,29], anti-inflammatory [30,31] and muscle relaxant [32] and in vivo experiments confirmed the bronchodilator effect of ginger on histamine-induced bronchospasms [33–35].

In this work we combined the effectiveness of liposomes and hyaluronic acid with the anti-inflammatory and antioxidant properties of ginger to manufacture nanotechnological nasal sprays suitable for the treatment of both rhinitis and rhinosinusitis. Indeed, the extract was loaded in special phospholipid vesicles immobilized with hyaluronic acid (hyalurosomes) and further improved with glycerol. Liposomes and glycerosomes were prepared as well and used as reference. The vesicle formation and morphology along with their size and zeta potential were measured. The ability of sprayed dispersions to reach the anterior nasal cavity was fully evaluated along with the biocompatibility of these systems towards keratinocytes and the ability to counteract oxidative damage induced in cells by using hydrogen peroxide.

2. Materials and Methods

2.1. Materials and Reagents

Lipoid S75 (consisting of ~70% of soy phosphatidylcholine, 9% phosphatidylethanolamine and 3% lysophosphatidylcholine) was purchased from Lipoid GmbH (Ludwigshafen, Germany). Powder extract containing 5% of *Z. officinalis* was purchased by Farmalabor Srl (Italy). Sodium hyaluronate with low molecular weight (200–400 kDa) and a polydispersity of 1.4 Mw/Mn, was purchased from DSM Nutritional Products AG Branch Pentapharm (Switzerland). Glycerol, DPPH radical (2,2-diphenyl-1-picrylhydrazyl), mucin from porcine stomach and all other reagents of analytical grade were purchased by Sigma-Aldrich (Milan, Italy).

2.2. Vesicle Preparation

The extract of *Z. officinalis* (30 mg/mL) was blended with phospholipid S75 (90 mg/mL) and dispersed in different aqueous mixtures. To obtain liposomes, a blend of water (900 μL) and PEG400 (100 μL) was used as hydrating medium; to prepare hyalurosomes, sodium hyaluronate (0.1%) dispersed in water (900 μL) and PEG400 (100 μL) were used; to obtain glycerosomes, a blend of water (800 μL), PEG400 (100 μL) and glycerol (100 μL) was used; to prepare glyhyalurosomes, a dispersion of sodium hyaluronate (0.1%) in water (800 μL), PEG400 (100 μL) and glycerol (100 μL) were used. The dispersions were sonicated (25 cycles 5 on/2 off, amplitude 13 μ) using a Soniprep 150 sonicator (MSE Crowley, London, UK) to obtain homogeneous dispersions with small vesicles. Empty vesicles without *Z. officinalis* extract were prepared as well and used as reference. After preparation, vesicles were stored at 4 °C.

2.3. Morphological Analysis Using Cryo-TEM

The vesicle morphology was observed by cryo-TEM using a Tecnai G2 20 Twin (FEI), operating at an accelerating voltage of 200 KeV in a bright-field image mode and low-dose image mode. An aliquot of sample (3 μL) was applied to glow-discharged 300 mesh Quantifoil TEM grids and the excess of water was removed with a filter paper. The prepared sample was frozen with a plunge freezing into liquid ethane on a FEI Vitrobot Mark IV (Eindhoven, The Netherlands) to preserve the sample in a frozen solid state. The frozen grids were then transferred to a 626 DH Single Tilt Cryo-Holder (Gatan, France), where it was maintained below −170 °C and then transferred to TEM at liquid nitrogen temperature (−196 °C).

2.4. Vesicle Characterization

Light scattering technology was used to determine the average diameter, polydispersity index and zeta potential of designed vesicles by using a Zetasizer Ultra (Malvern Instruments, Worcestershire, UK). These parameters were estimated over a storage period of 10 months at 4 °C to evaluate vesicle long-term stability.

Each sample (2 mL) was purified from the non-incorporated extract by dialysis (Spectra/Por® membranes, 3 nm pore size; Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) in water (2:1) at room temperature for 2 h (refreshing the water after 1 h). The dialysis method has been chosen as the main components of the extract are polyphenols having a molecular weight lower than the cut-off of the membrane (Spectrapor, 12–14 kD), thus they can pass through the membrane.

The antioxidant activity of formulations was measured by the DPPH (2,2-diphenyl-1-picrylhydrazyl) colorimetric test, before and after the dialysis process. Each formulation (20 μL) was dissolved in 1980 μL of DPPH methanolic solution (40 μg/mL). The methanolic solution of DPPH at the same dilution (1:50) was used as a control (100% absorbance). Samples were incubated for 30 min at ~25 °C in the dark. Then, the absorbance of each solution was measured at λ = 517 nm by using a UV spectrophotometer (Lambda 25, Perkin

Elmer, Milan, Italy). All the experiments were performed in triplicate. The antioxidant activity was calculated as percentage according to the following formula [36,37]:

$$\text{Antioxidant activity } (\%) = \frac{\left[\left(\text{ABS}_{\text{DPPH}} - \text{ABS}_{\text{sample}}\right)\right]}{\text{ABS}_{\text{DPPH}}} \times 100 \quad (1)$$

The entrapment efficiency of the vesicles was expressed as the percentage of the antioxidant activity after dialysis versus the value obtained before dialysis.

To evaluate the concentration of the extract before and after dialysis, a calibration curve has been built by reporting their antioxidant activity as a function of the concentration (from 1 to 40 mg/mL) of the extract (Figure 1).

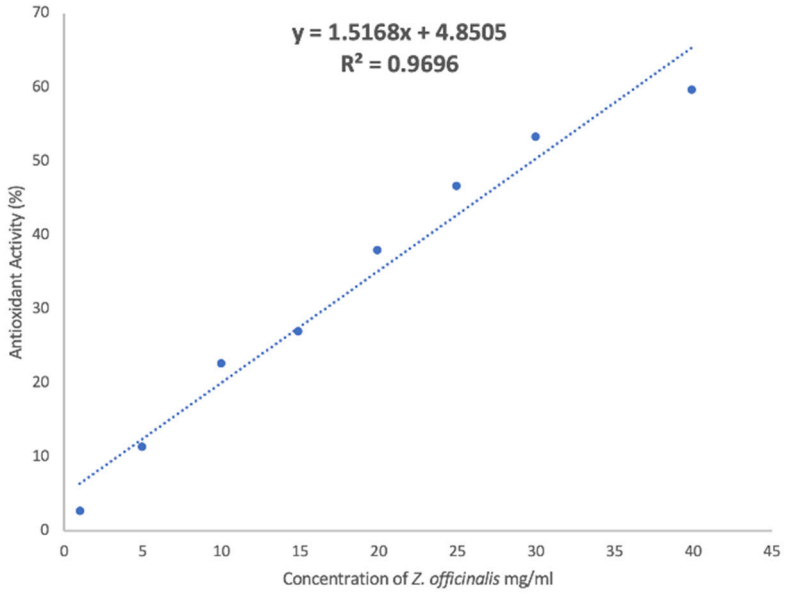

Figure 1. Calibration curve of the antioxidant activity of *Z. officinalis* extract as a function of its concentration.

2.5. Determination of Droplet Size Distribution

Droplet size distribution was evaluated by laser diffraction using a Malvern Spraytec® (Malvern Panalytical Ltd., Malvern, UK). Formulations (6 mL) were loaded in commercial pump devices (20 mL) kindly supplied by FAES laboratories. In accordance to FDA recommendations (laser distance range must be comprised between 2 and 7 cm with a difference of 3 cm between each different experiment) [38,39], measurements were performed in triplicate at 25 °C, at 4 cm and 7 cm of distance from the nozzle exit, rotating the pump device 45° respect to the laser beam [40].

Data were reported as the D10, D50, D90 volume diameter percentiles, i.e., 10%, 50% and 90% of the cumulative volume undersize. The distribution Span defined as (D90-D10)/D50 was also reported to characterize its width.

2.6. Spray Structure, Drop Average Velocity Module and Spray Angle Measurements

The average velocities of the sprayed droplets were determined by Particle Image Velocimetry and the spray opening angle by laser plane visualization. Particle Image Velocimetry method allows to capture two components of the velocity with high spatial resolution in a whole slice of the flow measuring the displacement of the droplets between two sequential images acquired with a known time interval (Δt). Instantaneous images were acquired with a Hamamatsu 1024 × 1344 pixels 12-bit C4742-95-12 ORCA-ER charge-coupled device camera (Hamamatsu Photonics, Shizuoka, Japan). To freeze the motion, a PILS Nd:YAG laser from Quanta System (Quanta System, S.p.A., Milan, Italy) capable

of generating 6 ns pulses with a maximum energy of 80 mJ at 532 nm was used as a light source capable of illuminating a vertical plane across the center of the spray. The camera was located perpendicular to the illuminated plane, to avoid geometrical distortions. The PILS system has two laser oscillators, so it can generate pairs of pulses with a selectable interval between them. The time interval between the two images in each pair was set at 30 μs. Image pairs were processed with the CCDPIV computer code developed at the Laboratory for Turbulence Research in Aerospace and Combustion (LTRAC) in Monash University (Melbourne, Australia) as described in [41]. Analysis was performed in 32 × 32 pixel windows with 50% overlap resulting in maps with 82 × 62 velocity vectors. 100 instantaneous velocity measurements were performed to obtain the mean velocity field and they were averaged to determine the angle of the spray cone. This angle was obtained by the ocular location of the limits of the spray in the average image. All photographs were taken under the same light conditions and underwent the same renormalization of the light levels captured by the camera. To eliminate the influence of the operator in the generation of the spray, a pneumatic device for driving the manual atomizer was constructed. The duration of the atomization pulse varies according to the supply pressure of the pneumatic device. This pressure has been adjusted to obtain a reasonable actuation, with a pulse duration of 150 ms as measured from the images. The spray speed and angle measurements were taken 83 ms after the start of the atomization, that is, about 8 ms after the mid-pulse.

Finally, the commercial atomizers used were manufactured with plastic injection molding. This technique does not guarantee a high dimensional quality, so a certain variability in the atomization of each device could be expected. To evaluate this variability, distilled water was atomized in triplicate (w1, w2 and w3) as reference under the same conditions used for the pharmacological formulations. Commercial atomizers manufactured with plastic injection molding were used. To evaluate the variability in the atomization of each device, distilled water was atomized in triplicate (w1, w2 and w3) as reference, under the same condition of the samples.

2.7. Biocompatibility of Extract Loaded Vesicles against Keratinocytes

Human epidermal cells (HaCaT) were grown as monolayers in 75 cm^2 flasks, incubated with 100% humidity and 5% CO_2 at 37 °C. Dulbecco's Modified Eagle Medium (DMEM) with high glucose, supplemented with 10% fetal bovine serum and penicillin/streptomycin, was used to culture keratinocytes. The cells were seeded into 96-well plates at a density of 7.5×10^3 cells/well and after 24 h of incubation, were exposed for 24 h to the extract loaded vesicles properly diluted to reach different concentrations (0.3, and 3 μg/mL). The extract dispersed in water at the same dilutions was used as reference. The possible toxic effect of the formulations towards HaCaT cells was assessed by measuring cell viability by the MTT (tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide) colorimetric test. The reduction of MTT by mitochondrial dehydrogenase leads to the formation of crystals of a blue-violet formazan and allows the estimation of the number of living cells in culture.

MTT (100 μL) was added to each well and incubated at 37 °C for 2/3 h. The formazan crystals formed in viable cells were dissolved in DMSO, and the absorbance measured at λ = 570 nm by using a microplate reader (Synergy 4, BioTek Instruments, AHSI S.p.A, Bernareggio, Italy). 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).

2.8. Protective Effect of the Extract Loaded Vesicles against Oxidative Damages Induced in Cells

HaCaT cells (5×10^4 cells/well) were seeded in 96-well plates with 250 μL of culture medium and incubated at 37 °C for 24 h. Then, cells were stressed with hydrogen peroxide (1:50.000) and immediately treated with different concentrations of the extract in dispersion or loaded into vesicles (3, 0.3 μg/mL). Unstressed cells were used as negative control (100% viability); cells stressed with hydrogen peroxide and treated with medium without extract

were used as positive controls. After 4 h of incubation the medium was removed and the viability of the cells was determined with the MTT colorimetric test, adding 100 µL of reagent in each well. After 2/3 h, the formed formazan crystals were solubilized by adding DMSO and their absorbance was measured spectrophotometrically at λ = 570 nm.

2.9. Statistical Analysis

The results were expressed as mean value ± standard deviation. Statistically significant differences were determined using variance analysis, ANOVA, Test T and Test F. The minimum level of significance chosen was $p < 0.05$.

3. Results

3.1. Vesicle Characterizations

The actual formation of lamellar vesicles and their morphology and structure were evaluated by means of cryo-TEM observation (Figure 2). Vesicles were spherical and not highly homogeneous in size. Indeed, a large amount of very small oligolamellar vesicles was observed along with some larger multilamellar and sometimes multicompartment vesicles.

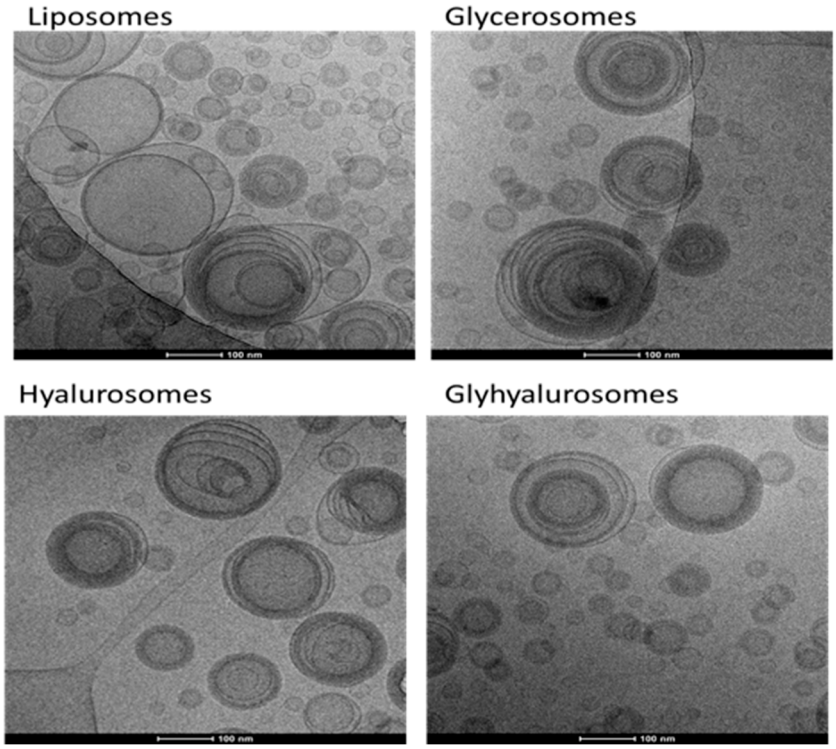

Figure 2. Representative images of liposomes, glycerosomes, hyalurosomes and glyhyalurosomes.

To complete the vesicle characterization, mean diameter, polydispersity index, zeta potential and entrapment efficiency were measured (Table 1). Empty vesicles were also prepared and characterized as well to evaluate the effect of the extract loading on the vesicle assembling. Empty liposomes were the smallest (~92 nm) while empty hyalurosomes the largest (~175 nm) and both disclosed the more negative values of zeta potential (~−60 mV). The loading of the *Z. officinalis* extract allowed an increase of the size of liposomes (~151 nm, $p < 0.05$ versus the size of corresponding empty vesicles) probably due to the interaction of extract components with the surface of the phospholipid bilayer, which in turn change the curvature radius of vesicle membrane. Result was confirmed by their zeta potential, which became less negative (~−18 mV), when the extract was loaded. The mean diameter of empty glycerosomes and glyhyalurosomes were smaller (~130 nm) and the zeta potential was less negative (~−16 mV) than that of liposomes and hyalurosomes due to the presence

of glycerol. The last has a different dielectric constant respect to that of water and can cause a reorganization of both bilayer and vesicle surface. The extract loading led a decrease of the hyalurosome mean diameter in comparison to that of the empty ones (~145 nm, $p < 0.05$ versus the size of corresponding empty vesicles), as hyaluronate may favour a better dispersion of the different components of the extract in the hydrophilic medium of vesicles and cause a different packing of the phospholipid. Indeed, vesicles were smaller and their zeta potential was slightly less negative. The mean diameter of glyhyalurosomes was not affected by the loading of the extract (~135 nm, $p > 0.05$ versus the size of corresponding empty) and the zeta potential was slightly less negative.

Table 1. Mean diameter (MD), polydispersity index (PI), zeta potential (ZP), and entrapment efficiency (EE) of the empty and extract loaded vesicles. Data represent the means ± SD of at least six replicates. Each symbol (a, b, c, d, e, f) indicates the same value of mean diameter ($p > 0.05$).

	MD (nm ± SD)	PI	ZP (mV ± SD)	EE%
Empty liposomes	^{c} 92 ± 2	0.28	−67 ± 4	
Empty glycerosomes	^{a} 128 ± 13	0.39	−15 ± 4	
Empty hyalurosomes	^{d} 175 ± 25	0.35	−55 ± 7	
Empty glyhyalurosomes	^{a} 132 ± 10	0.39	−18 ± 1	
Ginger liposomes	^{a} 151 ± 1	0.33	−15 ± 1	^{b} 59 ± 2
Ginger glycerosomes	^{d} 185 ± 8	0.30	−24 ± 3	^{e} 88 ± 4
Ginger hyalurosomes	^{f} 145 ± 1	0.26	−23 ± 1	^{e} 67 ± 13
Ginger glyhyalurosomes	^{a} 135 ± 9	0.21	−28 ± 2	^{e} 72 ± 10

Z. officinalis loaded vesicles were slightly polydispersed, confirming the cryo-TEM observation. Zeta potential was affected in different ways: the charge of liposomes and hyalurosomes were less negative after the incorporation of *Z. officinalis* extract, changing from ~−67 and ~−55 mV to ~−15 and ~−23 mV, respectively. On the other side, surface charge of glycerosomes and glyhyalurosomes, was more negative, changing from ~−16 mV to ~−26 mV. The entrapment efficiency of liposomes was the lowest (~59%) and significantly improved for the other vesicles without significant differences among these samples (~75%, $p > 0.05$ among the values of glycerosomes, hyalurosomes and glyhyalurosomes).

The long-term stability of the formulations was evaluated by storing the samples for a period of 10 months at 4 °C and measuring their physicochemical characteristics (Figure 3). After 3 months the vesicle formulations kept their characteristics as their main parameters tested were almost unchanged. Glycerosomes underwent a significant decreased in size and polidispersity index in comparison to the initial time ($p < 0.05$). Only glyhyalurosomes preserved the physicochemical characteristics for all the storage period, maintaining their physico-chemical characteristics up to 10 months (~170 nm, polydispersity index ~0.16, $p < 0.05$). On the contrary, the mean diameter and zeta potential of liposomes, glycerosomes and hyalurosomes became undeterminable after 3 months. This could indicate that the combination of PEG, glycerol and sodium hyaluronate promoted the formation of a stable vesicle dispersions, where *Z. officinalis* extract is effectively incorporated (Figure 3).

3.2. Determination of Size Distribution of Sprayed Droplets

The feasibility of our formulations as nasal spray was evaluated by the analysis of the droplet size distribution by laser diffraction technology (Table 2). The behaviour of vesicle dispersions was very similar: at a distance of 4 cm, the average droplet size of liposomes and hyalurosomes was ~58 μm for D50 and ~121 and ~146 μm for D90 respectively, and that of glycerosomes and glyhyalurosomes was ~78 or ~70 μm for D50 and ~177 or ~160 μm for D90 respectively. At 7 cm, the droplet size decreased slightly, liposomes, hyalurosomes and glyhyalurosomes were ~60 μm for D50 and ~109 μm for D90, glycerosomes was higher, ~68 μm for D50 and ~140 μm for D90. The obtained values of the tested dispersions were comprised between 30–70 μm for D50, and <200 μm for D90, confirming their suitability

for nasal local delivery in accordance with the FDA recommendations [38]. Their efficacy was also suggested by the almost complete absence of droplet sizes <10 µm.

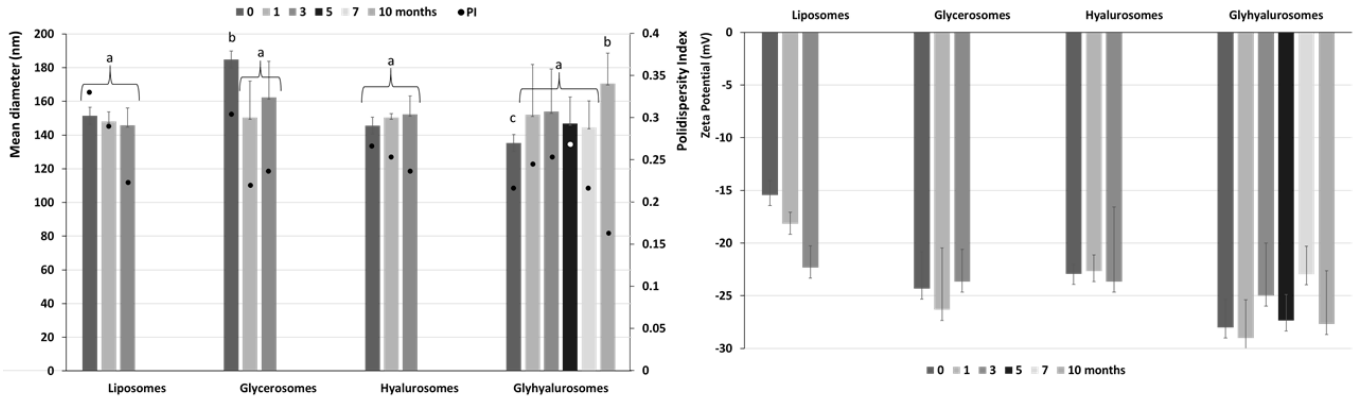

Figure 3. Mean diameter (bars), polydispersity index (dots) and zeta potential, of the extract loaded vesicles stored at 4 °C for 10 months. Data represent the means ± SD of at least six replicates. Each symbol (a, b, c) indicates the same value of mean diameter.

Table 2. Droplet size analysis of *Z. officinalis* vesicles sprayed from a distance of 4 and 7 cm from the laser beam. Standard deviation (±) is expressed as the mean of 3 measurements. Each symbol (a, b, c, d, e, f) indicates the same value.

	4 cm				7 cm			
	D10 (µm)	**D50 (µm)**	**D90 (µm)**	**SPAN (µm)**	**D10 (µm)**	**D50 (µm)**	**D90 (µm)**	**SPAN (µm)**
Liposomes	26 ± 1	^{a} 57 ± 4	^{c} 121 ± 14	1 ± 0.1	32 ± 2	^{a} 58 ± 2	^{c} 109 ± 3	1 ± 0.05
Glycerosomes	30 ± 0.7	^{b} 78 ± 2	^{d} 177 ± 3	2 ± 0.00	35 ± 1	^{f} 68 ± 2	^{e} 141 ± 3	1 ± 0.1
Hyalurosomes	24 ± 1	^{a} 59 ± 4	^{e} 146 ± 16	2 ± 0.1	36 ± 2	^{a} 61 ± 3	^{c} 109 ± 3	1 ± 0.1
Glyhyalurosomes	27 ± 0.3	^{f} 70 ± 4	^{e} 160 ± 11	2 ± 0.04	36 ± 1	^{a} 60 ± 1	^{c} 103 ± 9	1 ± 0.1

3.3. Measurements of Spray Plume Morphology and Angle

Instant Views

The spatial distribution of the sprays was determined from instantaneous images. To assess the possible variability introduced by pump and nozzle manufacture, the behaviour of distilled water sprayed different times by the same device was evaluated as well, and used as reference (Figure 4). The sprays of water generated full narrow cones of drops (plume) remarkably similar in dimension and geometry and only break up length was larger for sprays W2 and W3.

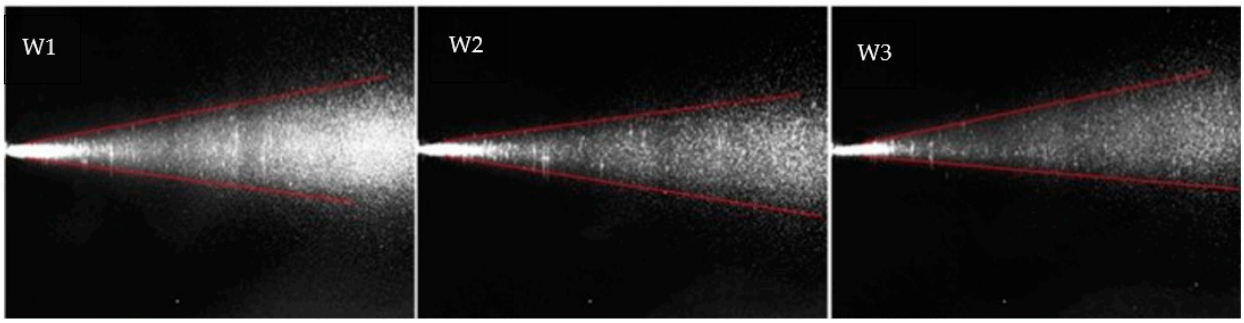

Figure 4. Representative images of instant visualization of the subsequent three water sprays.

Observation confirmed that the three sprays are nearly identical, and the only visually appreciable difference was that the cone angle of spray W1 was slightly wider in comparison with those for sprays W2 and W3. So, appreciable differences were due to the atomizer pump can be neglected and all the obtained results for the different formulations are reliable.

The atomization cone of vesicle dispersions was larger than that of water and the drops were homogenously distributed on the generated cone sprays (Figure 5).

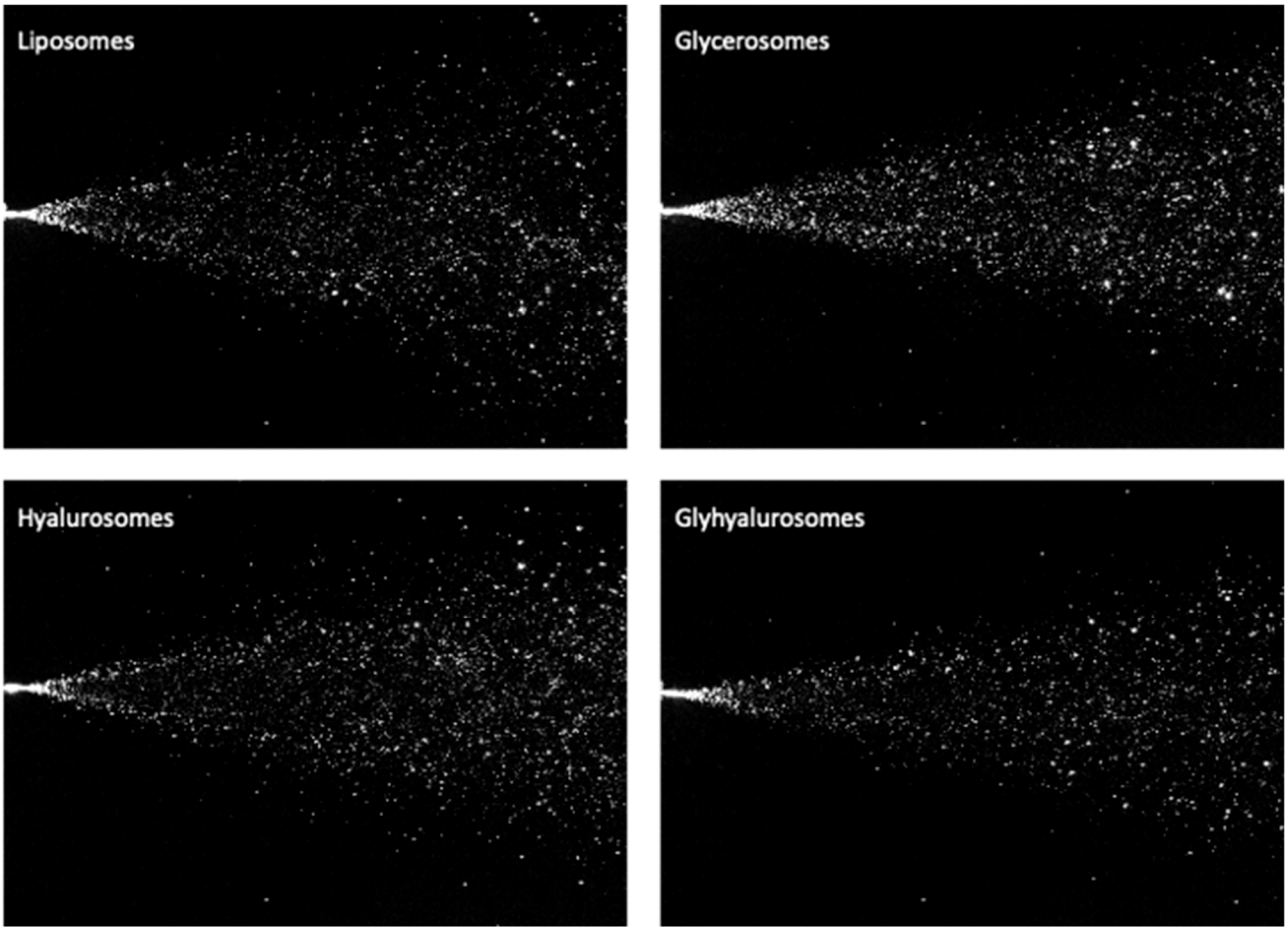

Figure 5. Representative images of instant visualization of sprays generated by liposomes, glycerosomes, hyalurosomes and glyhyalurosomes.

In particular liposomes and hyalurosomes generated a more dispersed nebulization, were droplets spread around the generated cone, which was wider and less dense in drops. The plume of glycerosomes and glyhyalurosomes was more compact and homogeneous, with a narrower plume angle respect to the other formulations. The spray angle was measured to better evaluate the differences among the formulations (Table 3). The angle of the cone generated by glycerosomes and glyhyalurosomes was around 22° while that of liposomes and hyalurosomes was around 30°, indicating the contribution of glycerol on the formation of homogeneous and narrow cone probably due to the increased viscosity of the dispersion. The small angle can facilitate the deposition of drops in the anterior part of the nasal cavity [42,43].

3.4. Average Velocity Module

The velocity modulus of sprayed formulations was calculated averaging 100 instantaneous measurements. The resulting images of velocity modulus provide information of the structure of the semi-hollow cone spray.

Figure 6 shows the mean velocity of the droplets along the midplane of the spray. The spray speed of drops of hyalurosomes was the lowest (~10.1 m/s) while that of liposomes was higher (~12.2 m/s) followed by that of glycerosomes and glyhyalurosomes (~13.15 m/s). All the atomizers generate hollow-cone sprays, but the spray angle was diminished when glycerol was added, most likely due to an increase of the liquid viscosity, as already pointed out. In addition, the area of large absolute velocity (in red in the figures) becomes thinner and larger for the formulations with glycerol (see also Table 3). These data suggest that the increase of the viscosity worsen the performance of the atomization device, which is a usual behaviour of almost all atomizer families. However, the maximum velocity level (Table 3) is similar for all formulations except when the device atomized just hyalurosomes. A possible explanation to this exception could be a slightly defective atomizer device. Although the present atomizers can atomize reasonably well the liquids, such viscous formulations are not easy to be atomized and they require devices without any fault, even the slightest.

Table 3. Average maximum velocities and spray angle generated by liposomes, glycerosomes, hyalurosomes and glyhyalurosomes.

	Liposomes	Glycerosomes	Hyalurosomes	Glyhyalurosomes
Max velocity (m/s)	12.2	13.2	10.1	13.1
Spray angle (°)	31.1	20.0	27.7	24.4

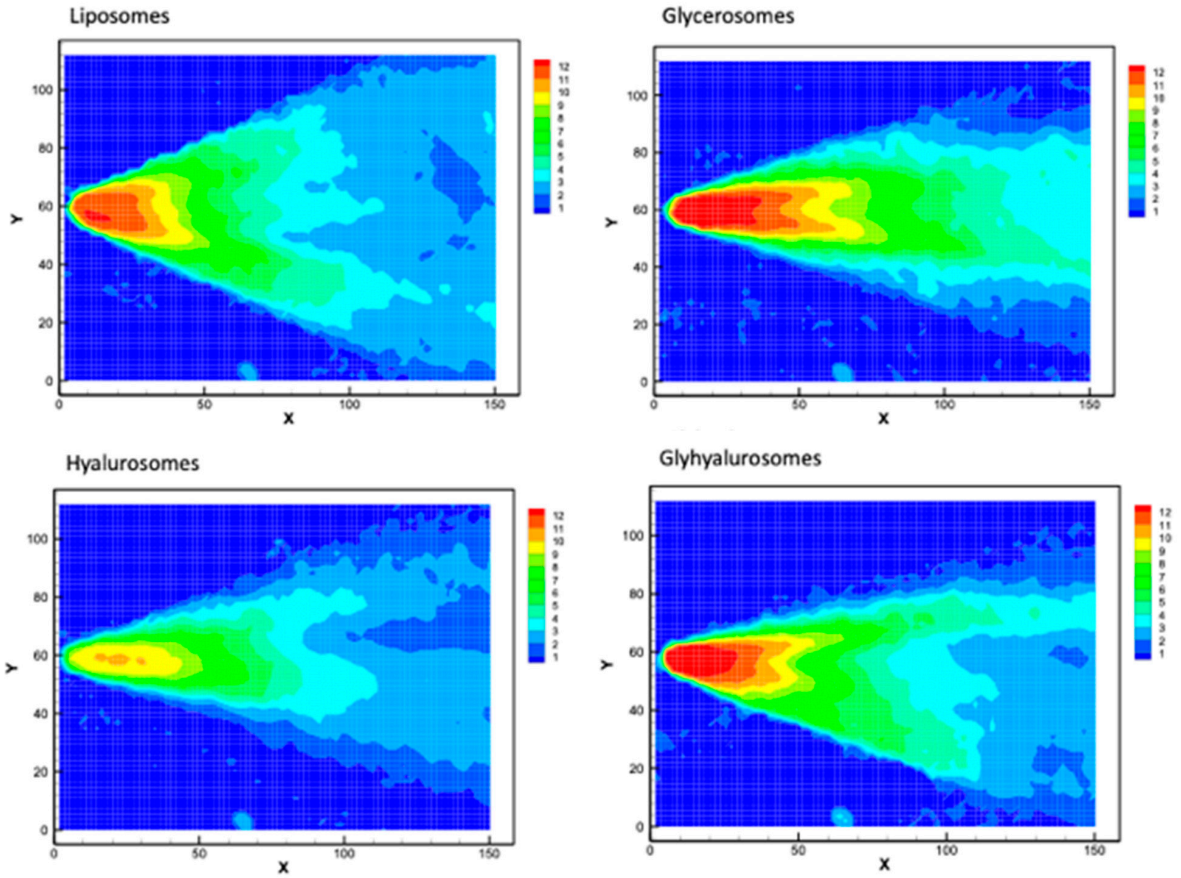

Figure 6. Images of modulus of average velocity of sprayed liposomes, glycerosomes, hyalurosomes and glyhyalurosomes.

3.5. Biocompatibility of Vesicles and Protective Effect against Oxidative Stress Damage

The biocompatibility of *Z. officinalis* loaded vesicles was evaluated using human epithelial cells. The cells were incubated with the formulations for 48 h and their viability was measured (Figure 7). The dispersion of the *Z. officinalis* extract in water was used as reference. The cell viability after incubation with dispersion and vesicle formulations was >90% irrespective of the used concentration (0.3, 3 µg/mL) ($p > 0.05$ among all the values).

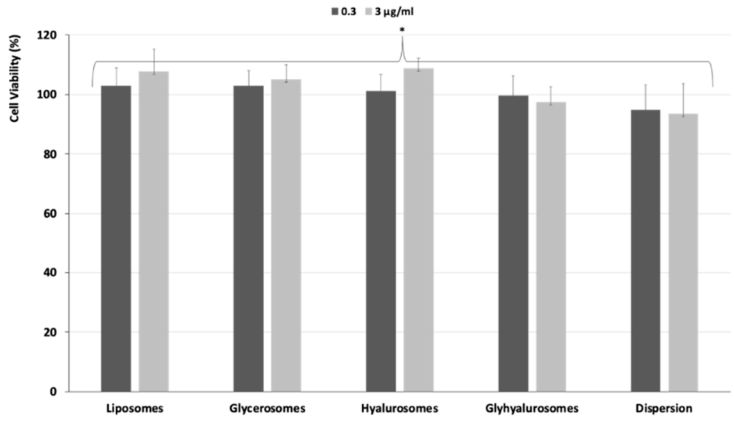

Figure 7. Viability of keratinocytes treated with *Z. officinalis* extract in dispersion or loaded in liposomes, glycerosomes, hyalurosomes and glyhyalurosomes and properly diluted to reach two different concentrations (0.3, 3 µg/mL of extract). Data represent the means ± standard deviation of at least three experimental determinations. The symbol (*) indicates the same value ($p > 0.05$).

The ability of *Z. officinalis* extract loaded vesicles to scavenge the peroxide radicals generated by hydrogen peroxide, thus protecting the cells from damage and death, was also evaluated (Figure 8). The stressing with hydrogen peroxide decreased the cell viability up to 70%. The treatment with extract in dispersion using the higher concentration of extract (3 µg/mL) was not effective and cell viability further decreased (<60%, $p < 0.05$ versus the viability of cells stressed with hydrogen peroxide). On the contrary, the treatment with extract loaded in vesicles provided an effective protection from oxidative damages avoiding or reducing the cell death. Indeed, the viability of cells treated with liposomes, glycerosomes, hyalurosomes and glyhyalurosomes was ≥100% irrespective to the used dilution. Only cells treated with liposomes loading 3 µg/mL showed a slightly lower viability (~90%). These data suggest an effective ability of vesicles to improve the efficacy of the extract and to counteract the damages induced in cells by oxidative stress.

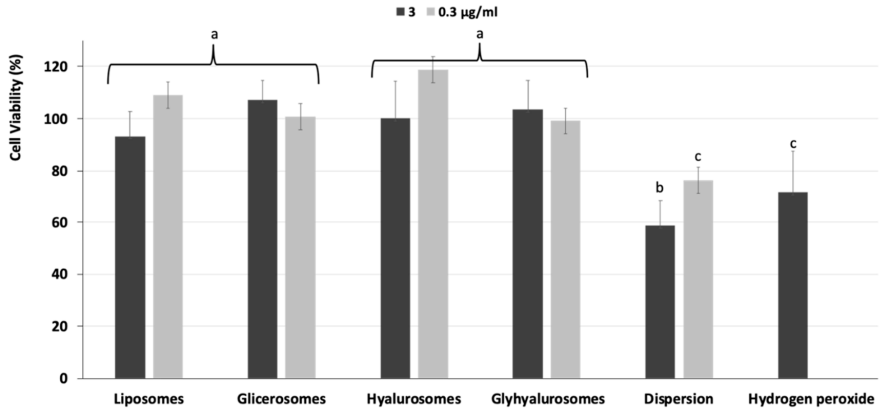

Figure 8. Viability of epithelial cells stressed with hydrogen peroxide and treated with ginger extract in dispersion or loaded in liposomes, glycerosomes, hyalurosomes and glyhyalurosomes. Data are reported as mean values ± standard deviation of cell viability expressed as the percentage of the negative control (100% viability). Each symbol (a, b, c) indicate the same value.

4. Discussion

In the present study, aiming at manufacturing green and natural nanoformulations suitable for the treatment of rhinitis and rhinosinusitis, the *Z. officinalis* extract was loaded in hyalurosomes modified with glycerol [44,45]. Each component was selected based on its beneficial properties on nasal cavity. Indeed, phospholipids, aggregated as lamellar vesicles, strength the barrier function of nasal mucosa, which play an important role in the patho-mechanism of the allergic rhinitis [13]. In a previous study, 60 patients with chronic rhinosinusitis were treated with liposomal nasal spray and 30 patients with steroid-based therapy. The treatment with liposomal nasal spray resulted in a similar reduction of symptoms and a significant improvement of the quality of life, confirming their suitability as valuable alternative intended for nasal administration [14]. Hyaluronan exerts hydrating and lubricating activities, plays a role in controlling inflammatory airway processes and mucociliary clearance, and it is also involved in tissue healing and remodeling [46]. Indeed, its nasal solution was able to significantly reduce symptoms of chronic rhinosinusitis in human patients [44]. In pharmaceutical applications, glycerol has an hydrating and protective effect on skin and *mucosae*, promotes the wound-healing processes and has an antibacterial effect [45–50]. These properties are essential in topical formulations, such as dermal or nasal preparations, to enhance their therapeutic effect. Moreover, glycerol is water-miscible and stable in aqueous phase, thanks to the formation of intramolecular hydrogen bonds [51,52]. Its hygroscopic characteristics, given by three hydrophilic hydroxyl groups, improves its smoothness and humectant capacity [45]. Finally, the extract of *Z. officinalis* has been used as a plant-derived therapeutic date back to more than 2000 years ago. *Z. officinalis* based remedy preparations can be read in Greek, Roman, Arabic and Buddhist medical literature [53,54]. Its anti-inflammatory action was already known to this populations, especially to counteract viral infections and colds [11]. Nowadays many authors are confirming the anti-inflammatory, antioxidant, anti-bacterial and analgesic efficacy of *Z. officials* and it is still widely used as medical plant in therapeutic preparations [55,56]. Recent research studies confirmed its anti-inflammatory and antioxidant effects especially due to several bioactive components such as gingerol (5%), shogaol, paradol, isogingerol, isoshogaol, gingeridone, quercetin and catechin [57–60]. The combinations of these components is expected to counteract the mucosal irritation and dryness typically present in rhinitis and rhinosinusitis [61]. In addition, the traditional therapy based on plant-derived bioactives has been improved by its combination with advanced pharmaceutical nanotechnology by means of a totally green and environmentally friendly approach [62]. Thus, only natural occurring components were used: soy phosphatidylcholine, *Z. officinalis* extract, hyaluronan, glycerol and water. Moreover, vesicles were prepared by direct sonication, which is an easy and low dissipative method, which can be scaled up at industrial level [63]. The resulting vesicles were sized around 150 nm and had an oligolamellar and multicompartment structure, which as reported in a previous study, improved the payload delivery inside the biological membrane [64]. Glyhyalurosomes were the only stable formulation as their physicochemical characteristics remained unchanged during the 10 months of storage, while the other ones aggregated and fused already after 3 months.

To quantify the deposition of the generated droplets in the target regions represents a critical step especially for the development of nasal products, as in that case it mainly depends of size and velocity of droplets and plume angle along with nasal anatomy and inter-subject variability related to age, gender, and ethnicity among human individuals [65,66]. The size distribution of sprayed droplets, generated from the different formulations, was between ~60 and ~180 μm, suggesting their feasibility and ability to reach the anterior nasal cavity after spray, in accordance with the FDA guidelines [67], and exerting a local effect on the nasal mucosae. All the vesicle dispersions, especially glycerosomes and glyhyalurosomes were atomized in fine droplets homogenously distributed in a full cone plume, with an angle ranging from 20 to 31°. The angle of plume generated by glycerosomes and glyhyalurosomes was narrower than that of liposomes and hyalurosomes, probably due to the presence of glycerol, which led to the formation

of a more viscous system. A narrow plume with an angle < 30° is not the most effective for the deposition of the formulation in the anterior part of the nose and can favour the deposition in the turbinate region as a function of the droplet size and velocity [65,68–70]. Indeed, droplets with high-speed are deposited in the anterior part of the nose [70]. Thus, despite the small plume angle, the size and velocity of droplets generated by glycerosomes and glyhyalurosomes can ensure a good deposition in the anterior part of the nose because of their high kinetic energy that may result in a considerable inertial deposition on the nasal surfaces close to the spray inlet [41]. Deposition efficiencies of approximately 90% could be achieved modifying the degrees, such as using 30° administration angles. After the drop deposition in the nasal mucosa, *Z. officinalis* extract loaded hyalurosomes and glyhyalurosomes can effectively exert its effects strengthening the barrier function and hydrating, lubricating and moisturizing the mucosa. This effect is very important because nasal mucus is the first line defence barrier against a variety of inhaled pathogens. With around 12,000 L of air inhaled daily, the airways may enter in contact with 25 million particles per hour that need to be filtered and/or transported away [1–3]. The barrier protection exerted by vesicles has been further strengthened by the antioxidant protection of the extract. Indeed, the loading of the extract into the vesicles maximize its efficacy, as vesicles were capable of counteracting the damages induced by oxidative stressed with hydrogen peroxide, avoiding the death of cells and maintaining their viability ~100% even after injury. Thus, glycerosomes and hyalurosomes are the most suitable formulations for nasal delivery mainly because of compactness of the generated cone (cone angle between ~20 and 24°), droplets size (comprised between ~70 and 180 μm), and a high-speed (~13 m/s). Between these, glyhyalurosomes, which further contain hyaluronic acid, seem to be the most promising formulation for the treatment of local diseases such as nasal allergic conditions and nasal congestions thanks to their high stability during the whole storage period and to their capability to protect the nasal cavity.

5. Conclusions

The use of *Z. officinalis* extract passes through millennia and nowadays its efficacy continues to be validated by modern scientific research. The incorporation of *Z. officinalis* extract in hyalurosomes enriched with glycerol, allows to obtain a formulation based on natural components, prepared by green method, stable on storage, effective as antioxidant, which can be sprayed in the anterior part of the nasal cavity. The obtained glyhyalurosomes, being the most promising formulation should be further evaluated for the manufacture of natural and green nasal spray for the prevention and treatment of rhinitis and rhinosinusitis.

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Zingiber officinalis & Citral

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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

Eleonora Casula [a], Maria Manconi [a,*], Tania Belen Lopez-Mendez [b,c], Jose Luis Pedraz [b,c,d], Esteban Calvo [e], Antonio Lozano [e], Marco Zaru [f], Ines Castangia [a], Germano Orrù [g], Sara Fais [g], Maria Letizia Manca [a]

[a] Dept. of Scienze della Vita e dell'Ambiente, University of Cagliari, via Ospedale 72, 09124 Cagliari, Italy
[b] NanoBioCel Group, Laboratory of Pharmaceutics, School of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain
[c] Biomedical Research Networking Center in Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), 01006 Vitoria-Gasteiz, Spain
[d] Bioaraba, NanoBioCel Research Group, 01006 Vitoria-Gasteiz, Spain
[e] LIFTEC, CSIC – Universidad de Zaragoza, María de Luna, 10, 50018 Zaragoza, Spain
[f] Icnoderm Srl, Sardegna Ricerche Ed. 5, Pula, 09010 Cagliari, Italy
[g] Molecular Biology Service Lab, Department of Surgical Science, University of Cagliari, Via Ospedale 54, 09124 Cagliari, Italy

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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.

1. 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

* Corresponding author.
E-mail address: manconi@unica.it (M. Manconi).

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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.

2. Materials and methods

2.1. 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).

2.2. Liposome's 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.

2.3. 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 $\lambda = 517$ 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].

2.4. Droplet size distribution

Droplet size distribution was evaluated by 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.

2.5. Spray plume, 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.

2.6. Biocompatibility of vesicles against keratinocytes

Human epithelial cells (HaCaT) were grown as monolayers in 75 cm^2 flasks, incubated with 100% humidity and 5% CO_2 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 7.5×10^3 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).

2.7. Protective ability of vesicles against oxidative damage induced in cells

HaCaT cells (5×10^4 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.

2.8. 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 $1{\cdot}10^7$ 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.

2.9. 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 $p < 0.05$.

3. 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.

3.1. 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 slightly larger than the empty vesicles, indicating a contribution of both bioactives in the bilayer assembling. The zeta potential was highly negative and the low polydispersity index (0.21) indicated a monodispersed sample.

3.2. 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 2).

3.3. 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).

3.4. 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) while the

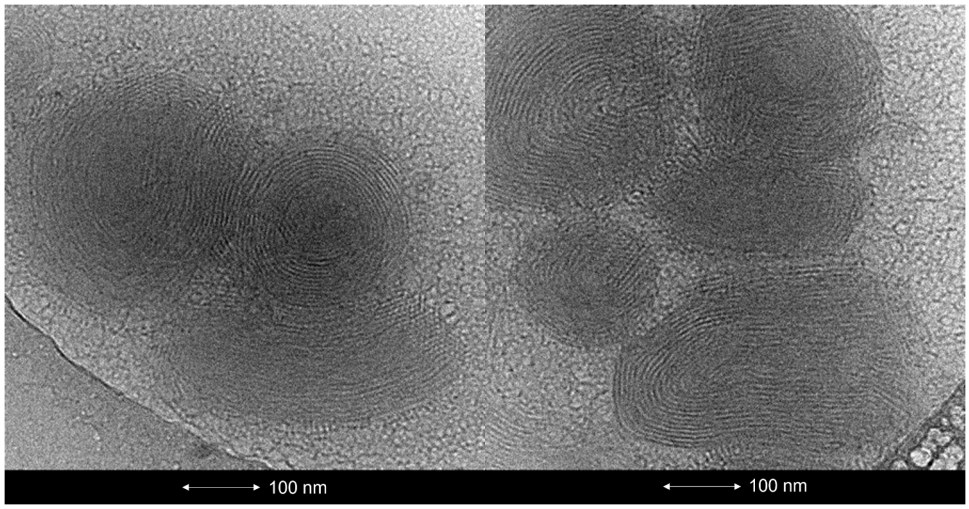

Fig. 1. Representative cryo-TEM images of transfersomes loading citral and *Z. officinalis* extract.

Table 1
Mean diameter (MD), polydispersity index (PI), zeta potential (ZP) of empty and bioactive loaded vesicles and entrapment efficiency of extract loaded vesicles. Data represent the means ± standard deviations of at least six replicates. Each symbol (*, $^{+}$, $^{\#}$, $^{\circ}$) indicates a value statistically different from the others ($p > 0.05$).

	MD (nm)	PI	ZP (mV)	EE (%)
Empty transfersomes	*90 ± 2	0.20	-$^{\circ}$54 ± 5	
Bioactive transfersomes	$^{+}$110 ± 1	0.21	-$^{\#}$44 ± 3	74 ± 10

Table 2
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 ($^{\$}$, $^{\wedge}$, $^{@}$, *, $^{\circ}$, $^{\#}$) indicates the same value.

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

angle of the spray generated by transfersomes was almost the half (~27°) than that of the dispersion (~48°).

3.5. Biocompatibility of vesicles and protective effect against oxidative stress damage

Considering the importance to develop safe formulations, their biocompatibility was measured by using keratinocytes, considered the main cells of epidermis. Cells were incubated with citral and *Z. officinalis* extract loaded in transfersomes or in aqueous dispersion properly diluted with medium (citral 18, 1.8, 0.18 μg/ml and *Z. officinalis* 1, 0.1, 0.01 μg/ml) and their viability was measured at 48 h (Fig. 4A). The viability of cells incubated with the dispersion was ~98% ($p > 0.05$ versus the values obtained with other dilutions) using the highest concentrations of citral (180 μg/ml) and extract (10 μg/ml) and was lower, ~90% ($p < 0.05$ among the three values) when the lower concentration of citral and extract was used. When the cells were incubated with the citral and extract loaded in vesicles, the viability was ~105% ($p < 0.05$ versus other values) by using the higher concentrations (180 and 18 μg/ml of citral and 10 and 1 μg/ml of extract), and was slightly lower (~98%, $p > 0.05$) by using 1.8 or 0.18 μg/ml of citral and 0.1 or 0.01 μg/ml of extract ~98% ($p > 0.05$). Results confirmed the high biocompatibility of prepared transfersomes.

The ability of citral and *Z. officinalis* extract loaded vesicles to protect the cells against both damages and death caused by oxidative stress was evaluated stressing them with hydrogen peroxide (Fig. 4B). The treatment with hydrogen peroxide killed ~30% of cells and the simultaneous treatment with the bioactives in dispersion at the lower concentrations (citral 1.8, 0.18 μg/ml and *Z. officinalis* 0.1, 0.01 μg/ml) did not modify

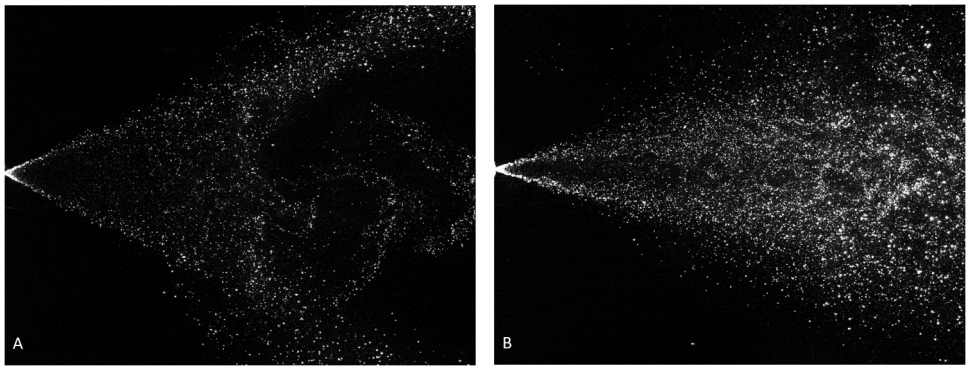

Fig. 2. Representative images of instant visualization of sprays generated by water dispersion (A) and transfersomes (B).

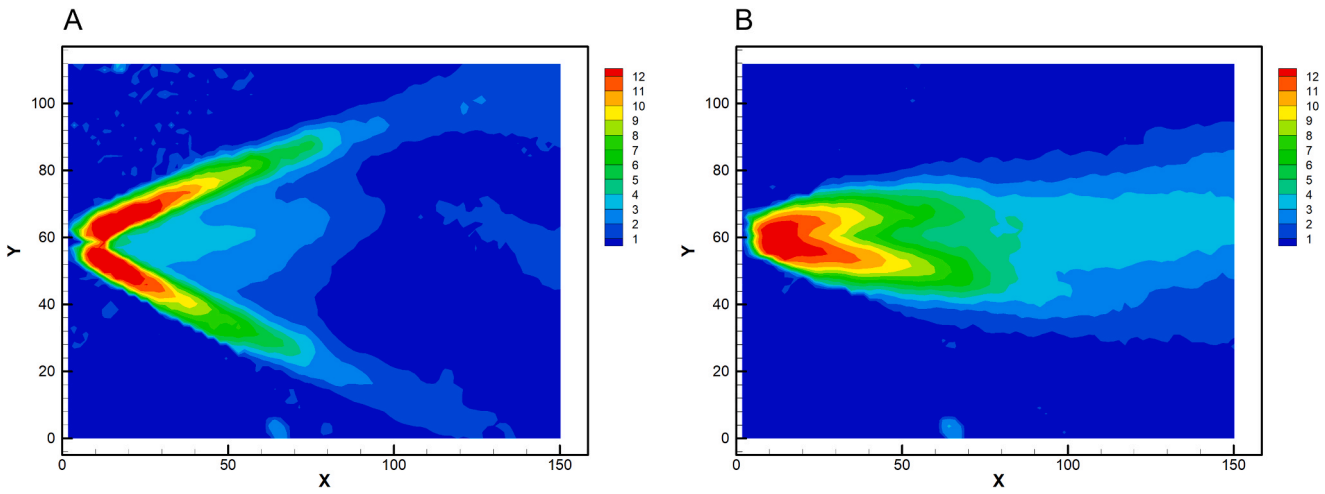

Fig. 3. Images of distribution of average velocity of drops generated by spraying transfersomes (A) and water dispersion (B).

Table 3
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

the viability value (~70%, $p > 0.05$ versus viability of cells stressed with hydrogen peroxide and untreated). The treatment with the dispersion of both citral and extract at the higher concentration improved the cell viability up to ~86%, while the incorporation into vesicles effectively protected the cells (viability ≥100%, $p > 0.05$ among the concentrations) irrespective to the used concentration.

3.6. 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.

4. Discussion

Previous studies disclosed that liposomes dispersions locally sprayed

Table 4
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	30 ± 5	27 ± 3
Citral dispersion	15 ± 1	–	11 ± 1
Extract dispersion	–	–	–
Citral and extract transfersomes	32 ± 1	31 ± 1	32 ± 1

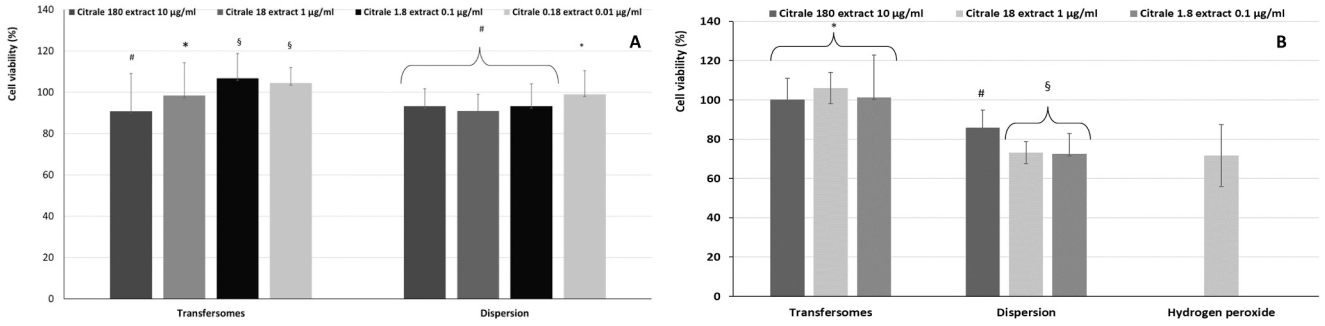

Fig. 4. A) Viability of keratinocytes treated with citral and *Z. officinalis* extract in water dispersion or loaded in transfersomes at different concentrations (citral 18, 1.8, 0.18 µg/ml and *Z. officinalis* 1, 0.1, 0.01 µg/ml). Data represent the means±standard deviations of at least three experimental 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.

on nasal cavity are a valuable alternative to corticoids or antihistaminic drugs with an appreciable potential to reduce and care allergic and not-allergic 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].

5. 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 ensure the delivery in the anterior region of nose exerting at the same time protection of cells against oxidative stress, antibacterial activity against microorganisms typically photogenic for the first tract of the respiratory system and reinforcing the barrier effect. The fruitful association of phospholipid vesicles, citral and *Z. officinalis* extract permit to obtain a synergic and multifactorial effect, which seems to be ideal for the local and natural treatment of rhinitis.

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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