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"FROM WASTE TO HEATH BY THE CONVERSION OF GRAPE POMACE IN BIOMEDICAL, COSMECEUTICAL AND NUTRACEUTICAL PRODUCTS"

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"To all those who believed in me,
This thesis is for you"

Resilience, the ability to overcome challenges and bounce back from any situation, is a lesson this thesis has instilled deeply in me. Despite the challenges faced, the journey has been immensely enriching, both personally and professionally, leaving me with only fond memories.

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Abstract

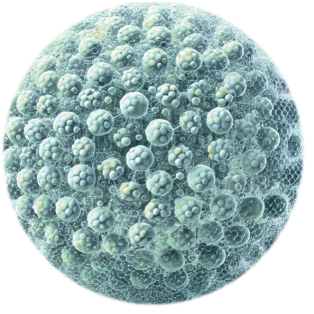
Grapevines, renowned for their high phenolic content, are attracting significant interest due to their potential health benefits, including antioxidant, anti-inflammatory, and anti-cancer properties. This research delves deeply into the primary chemical constituents of grapes and their byproducts, identifying the most effective polyphenol extraction methods. Special emphasis is placed on the development and production of innovative grape-based formulations that enhance human health through various delivery methods such as cutaneous, mucosal, oral, pulmonary, and nasal. The transition from laboratory research to industrial production is also explored, highlighting the challenges and limitations of scaling up these novel health-promoting products. This comprehensive review aims at providing valuable insights into the potential of grape-derived compounds and their applications, paving the way for future innovations in the field of health and nutrition.

Riassunto

Le viti, rinomate per il loro elevato contenuto fenolico, stanno attirando un notevole interesse per i loro potenziali benefici per la salute, inclusi effetti antiossidanti, antinfiammatori e anticancerogeni. Questa ricerca esplora in profondità i principali costituenti chimici dell'uva e dei suoi sottoprodotti, identificando i metodi più efficaci di estrazione dei polifenoli. Un'enfasi particolare viene posta sullo sviluppo e sulla produzione di formulazioni innovative a base di uva, che promuovono la salute umana attraverso vari metodi di somministrazione, come cutaneo, mucosale, orale, polmonare e nasale. Viene inoltre analizzato il passaggio dalla ricerca di laboratorio alla produzione industriale, evidenziando le sfide e le limitazioni dell'upscaling di questi prodotti innovativi per la promozione della salute. Questa revisione completa mira a fornire approfondimenti preziosi sul potenziale dei composti derivati dall'uva e sulle loro applicazioni, aprendo la strada a future innovazioni nel campo della salute e della nutrizione.

INTRODUCTION

Gold Nanoparticles



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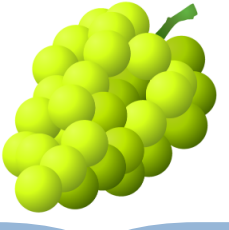
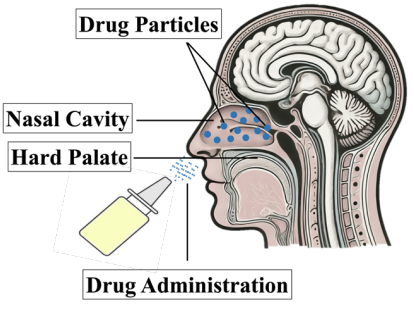


Nasal Administration



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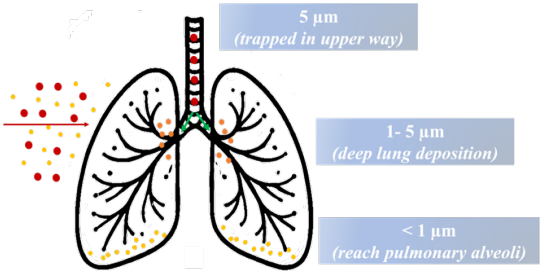
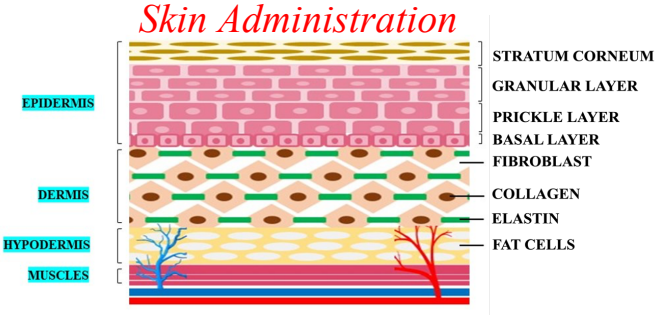


Skin Administration



Review

Exploring the efficacy and industrial potential of polyphenol products from grapes and their by-products

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Abstract

Grapevines, renowned for their high phenolic content, are attracting significant interest due to their potential health benefits, including antioxidant, anti-inflammatory, and anti-cancer properties. This research delves deeply into the primary chemical constituents of grapes and their byproducts, identifying the most effective polyphenol extraction methods. Special emphasis is placed on the development and production of innovative grape-based formulations that enhance human health through various delivery methods such as cutaneous, mucosal, oral, pulmonary, and nasal. The transition from laboratory research to industrial production is also explored, highlighting the challenges and limitations of scaling up these novel health-promoting products. This comprehensive review aims at providing valuable insights into the potential of grape-derived compounds and their applications, paving the way for future innovations in the field of health and nutrition.

Keywords:

Grapes; Polyphenols; Extraction procedures; Nanocarriers; Scaling-up

1. Introduction

Due to its flavour, beneficial content, and non-climate character, grape is extensively cultivated worldwide and is one of the first domesticated fruit crops, grown for thousands of years in the whole Mediterranean basin, where it has immense cultural, economic and ecological importance (1–5). Nowadays, grapes are cultivated in many regions thank to their ability to adapt to a variety of habitats and climates, and to provide higher income to farmers than any other crop, mainly because of their exceptional derivative product, the wine (6). Indeed, the main part of collected grape (~58 million tons), is used to produce wine, resulting in around 18-20% of pomace, 3-5% of stalks and 8-10% of lees, corresponding to approximately 15 million tons per year of by-products produced (7,8). Alternatively, this crop fruit can be directly consumed or used in a variety of products such as juice, jam or raisins or even in healthy products due to the extensive benefits provided by the contained active molecules (9). The complex phytochemistry of the berry is due to a wide variety of compounds, the most abundant are water and mineral salts, followed by sugars, polymers, fibers, fatty acids, organic acids, vitamins, terpenes and polyphenols (10), which may be responsible for the well-known biological activities, such as antioxidant (11,12), gut-microbiota-regulating (13), cardioprotective (14,15), antidiabetic (16) and anticancer (17,18). All these beneficial properties are largely due to polyphenols, considered one of the most interesting class of compounds contained in grape, which have diverse phenolic structures, and are divided into two macro-classes, phenolic acids (i.e., hydroxybenzoic and hydroxycinnamic acids) and flavonoids, which, in turn, are classified as flavones, flavanones, flavanols, isoflavones (19,20). During the winemaking process, as the primary application of grape, a small portion of these valuable molecules contained in the berry, passes in wine, while most of them remain in the by-products (21). The most abundant by-product is pomace, mainly composed of skin, seeds and, in some cases, stalks, that, due to its high organic content needs to be properly treated during harvest, to avoid economic and environmental problems (22,23). Lees are another interesting but problematic by-product formed by the heterogeneous masses deposited after the fermentation activity of must, which make them especially rich in proteins (24). The high amount of both pomace and lees produced every year and especially their interesting content in bioactive molecules stimulated researchers to find new opportunities for the sustainable valorization of these by-products, proposing several transformation processes and applications in various fields/industries (food, nutraceutic, cosmeceutic, among others) (25). Due to the high content of organic matter, several studies have focused on the transformation of grape and especially its byproducts into mature and biostabilized soils, or soil improvers by composting or vermicomposting processes (26,27). As well, others have reported the methods used to generate methane from grape pomace by anaerobic digestion due to its high content in cellulose, hemicellulose, and lignin percentages, in accordance with the worldwide challenge to shift to sustainable green energy sources (28–30). Despite of various strategies adopted, the majority of the performed studies have been focused on the extraction of the phytochemicals, especially phenolic compounds, from grape pomace and the evaluation of their biological effects, such as antioxidant, antiaging, anti-inflammatory, anti-microbial and anti-neurodegenerative (31–35), to be used in pharmaceutical, cosmetic, or dietary products (36,37).

Differently, lees content and applications have been less investigated even if, in the last years they have received increasing attention for their application in the food sector, since they are a good source of functional and bioactive compounds (38).

To the best of our knowledge, few studies have reported the comparison between the composition of the grape berry and its pomace and lees, and on the actual formulation of the extracted polyphenols in sustainable and scalable products, especially those intended for the treatment of human disorders. Accordingly, in this review, the main phytochemicals composition of both grape and the derived biomasses are reported, along with the most effective extraction methods used to separate polyphenols. Special focus has been devoted to the studies reporting the development and manufacturing of conventional and/or innovative products as a function of the proposed administration routes, along with their efficacy and scalability at industrial level.

2. Health-promoting molecules contained in grape and its byproducts (pomace, lees, skin and seed)

2.1. Phytochemical composition of grape

The chemical components of the grape berries were not completely reported in previous works as most of the studies have been performed in a classic targeted approach, frequently evaluating the specific class of compounds, such as organic acids (malic and tartaric), phenolics, volatile compounds or polyamines that are commonly considered key elements during the production of wine (39). In the last few years, more comprehensive studies have been reported, but they are usually limited to the metabolome analysis of specific grape berries of a single cultivar. Additionally, Lee and Rennaker underlined that the quantification of many components (more than 45 compounds) of *Pinot noir* grape strongly depends on the sample preparation and the extraction method used (40). Beyond water, the second class of main components of grape berries are sugars such as glucose, D-fructose, and sucrose, at around 15-20% (41). Other primary metabolites of grape are proteins, lipids, fibers, followed by macronutrients (i.e., potassium, nitrogen, calcium, phosphorus, magnesium, sulfur), and secondary metabolites (Table 1) (42).

Despite the incomplete understanding of the exact molecular composition of grapes, it is well-recognized that grape is a rich source of bioactive compounds, particularly polyphenols, which contribute to a variety of biological activities and health benefits. The polyphenol content varies significantly depending on the part of the grape and the habitat where it grows. Grape seeds exhibit the highest total phenolic content (TPC), followed by the skins and pulp. Remarkably, the total phenolic content in grape seeds is approximately 130 times greater than that in pulp, measured in terms of gallic acid equivalents per gram of dry weight (43). Advanced chromatographic-based analysis techniques have identified a total of 78 phenolic compounds in grape pulp (44). Flavonoids and non-flavonoids constitute the main categories of polyphenols contained in grapes. Among the flavonoids, flavan-3-ols (flavanols), flavonols, and anthocyanins are predominant. Flavan-3-ols, which are the richest flavonoids, are mainly stored in the seeds, with proanthocyanidins being the principal class (45). These compounds include proanthocyanidin monomers, oligomers, and more complex polymers, with (+)-catechin, (-)-epicatechin, procyanidin B1, and procyanidin B2 being the most representative molecules (46). Flavonols, such as quercetin, myricetin, and kaempferol, are significantly accumulated in grape

skins and are also present in the seeds of some grape varieties (47). Anthocyanins, which are water-soluble pigments, are predominantly found in the skins of coloured grape varieties like red or purple grapes, while are completely absent or present just in traces in white grape varieties.

Moreover, phenolic acids and stilbenes are the most representative non-flavonoid polyphenols contained in grapes. Resveratrol, particularly in its trans-form, is the most important stilbene and is primarily found in the grape skin, where it acts as a phytoalexin, providing defence against pathogens and environmental stressors such as fungal infections, injury, and UV irradiation (48). Grapes also contain a variety of phenolic acids, both in free and conjugated forms. The most abundant are hydroxybenzoic acids (including 4-hydroxybenzoic, gallic, protocatechuic, vanillic, and syringic acids) and hydroxycinnamic acids (such as p-coumaric, caffeic, ferulic, and sinapic acids). Additionally, grapes contain melatonin, well known for its role in regulating sleep and circadian rhythms. Alongside resveratrol and anthocyanins, melatonin is nowadays one of the most studied natural compounds.

2.2. Phytochemical composition of grape by-products

After winemaking, the majority of the active molecules remains in the by-products, especially in the pomace (Table 1) (49). Clearly, water and alcohol soluble molecules such as sugars, some oligosaccharides, some polyphenols, and mineral salts partially pass to the wine, giving it not only the flavour, but even its beneficial properties, well known worldwide (50). However, most of the fibers, proteins, lipids, and polyphenols still remain in the pomace and their final content depends on several factors such as grape varieties, soil characteristics, geographic position and winemaking process (51–53). Pomace derived from white wine are usually richer in sugars and polyphenols than those derived from red wine, which in turn are richer in fibers due to the maceration process, during which the water-soluble components pass-on to the wine (54). Indeed, during the white wine making process, the maceration is avoided, thus reducing the amount of active components passing from the grape to the must. On the contrary, the long maceration, which take place during the vinification of red grape, improves the diffusion of more components from grape to must, decreasing the metabolite content of the pomace and increasing that of the lees (55). Lees contains the largest amount of proteins, the lowest of tannins and anthocyanins, and an intermediate value of lipids (Table 1). Grape pomace, in contrast, is valued for its richness in fibers, phenols, and sugars, while grape seeds and skin have a substantial content of macronutrients. From the functional, beneficial, nutritional and therapeutic point of view, the most promising molecules contained in grape biomasses are polyphenols (those reported for grape, section 2.1.), that also have a high economic value (56). The abundance of health-promoting compounds found in grape pomace, peel, seeds, and lees emphasizes their value as excellent sources of functional components, contributing to reaching on one side a closed-loop system and on the other the consumer demand that has definitely shifted towards more sustainable shopping choices.

Table 1. Summary of the main components found in the grape pomace, skin, lees and seeds as a function of the related grape varieties, and method of detection. References for each data are reported as well.

	Grape Pomace	Grape Skin	Grape Lees	Grape Seeds	Grape Varieties	Methods of Detection	References
Total Sugars	39 to 276 g/kg	1.34 to 23.66 g/kg	4.13±0.63 g/kg	100.1 g /kg to 725.3 g /kg	*Silvaner;Blauer Portugieser; Alicante Bouschet*	LC-MS/MS; Refractometer; Phenol sulfuric acid method	(57–59)
Lipids	149.5±0.6 g/kg	140±3 g/kg	48.5±11.5 g/kg to 92±4 g/kg	121.3± 1.7 g/kg	*Muscat Hamburg ; Vitis labrusca cv. Bordeaux; Alicante Bouschet*	Soxhlet extractor; Folch's method; LC-MS/MS	(59–62)
Proteins	120 to 130 g/kg	67.6±0.75 g/kg	203.2±7.5 g/kg to 95±1 g/kg	131.4± 3.1 g/kg	*Cabernet Sauvignon; Grenache; Syrah*	HPLC; A.O.A.C method	(63,64)
Total Phenols	280.6±30g/kg to 105.2 g/kg	111 to 149 g/kg	40.4 to 72.6 g/kg	204 to 245 g/kg	***Muscat; Karaerik* (turkish); *Pinot Noir***	LC-MS/MS	(65–67)
Anthocyanins	0.04 to 0.24 g/kg	0.04 to 0.24 g/kg	0.05 to 0.07 g/kg	3.98 to 57.34 g/kg	*Pinot noir, Gris Grenache ; Syrah; Carignan*	HPLC	(68–70)
Tannins	2.74 to 3.12 g/kg	0.75 to 0.86 g/kg	0.06 to 0.1 g/kg	157.02 to 455.42 g /kg	*Pinot noir, Gris Grenache ; Syrah; Carignan*	LC-MS/MS; Phloroglucinolysis Method; Size Exclusion chromatography	(68–70)

Fibers	560-660 g/kg to 544±14.6 g/kg	129.1±2.2 g/kg	201±3 g/kg	340±8.3 g/kg	*Cabernet Sauvignon; Grenache; Syrah Muscat; Hamburg / Amarone*	A.O.A.C method	(60,61, 63,64,71)
Macronutrients	27.3 g/kg	469 to 799 g/kg	16.09 to 18.16 g/kg	422 to 606 g/kg	*Galician; Kyoho; Red Globe; Cabernet Sauvignon; Chardonnay*	EDTA single-step extraction inductively coupled plasma emission spectrometer (ICP-AES)	(72–74)

3. Beneficial properties of polyphenols contained in grape and its byproducts

As reported above, grapes and winemaking by-products are known to be a useful source of beneficial polyphenols (Figure 1), which in turn display a variety of positive impacts on human health, including control of oxidative stress and chronic inflammation, delay of aging, neuroprotective function, protection of cardiovascular system, and decrease of the incidence of metabolic syndromes and cancer. The wide range of beneficial properties is primarily attributed to their antioxidant activity, which helps to prevent oxidative stress in the human body. Indeed, by reducing or even preventing cellular damage, antioxidants can help avert the development of more serious pathological conditions (19,20,75,76).

Among the various polyphenols contained in both grape and by-products, resveratrol (trans-3, 4, 5-trihydroxystilbene) is known for its multitargeting therapeutic potential due to its antioxidant, anti-inflammatory and anti-microbial properties (77). Different studies have also confirmed promising antiviral activity of resveratrol, whose efficacy seems to be associated with the inhibition of viral replication, protein synthesis, gene expression and nucleic acid synthesis (78–80).

Flavonoids are other important group of polyphenols present in grapes, where, as plant-specialized metabolites, play a key role in the defence against stress from the environment. Since their peculiar chemical structure, they exert also important biological function on human body providing health benefits, including improvement of cardiovascular health and control of inflammation. Clearly, the total content and type of these flavonoids in grape depends on the selected variety, cultivar, geopedological-climatic conditions of growing, but one of the most abundant and always found in both grape and byproducts is quercetin (81). It is a flavonol, having the basic flavonoid skeleton, (two benzene rings attached to a heterocyclic pyrene), and differing for the position of the five hydroxyl groups in the structure (82). Its biological activities are related to 3 functional groups: a catechol group in the second benzene ring; a 2,3-double bond in

conjugation with a 4-oxo function in the pyrene ring; and oxydril group at positions 3 and 5 in heterocyclic ring (83,84). This special molecule has been largely used as fundamental component in the traditional Chinese medicine, specialized in the treatment of nasal and pulmonary disorders (85). Because of its high anti-radical and antioxidant activities, it exserts multiple beneficial properties, similar to those of resveratrol, such as antioxidant, anti-inflammatory, anti-bacterial, antiviral and neuroprotective (86–90). Other class of polyphenols usually present in both grape and by-products is that of anthocyanins, part of flavonoids that contribute to the colour and are usually most abundant in red varieties (53). These chemicals, like the others mentioned above, have strong antioxidant and anti-inflammatory properties, which are both critical for controlling cytokine release in cells, commonly connected with chronic illnesses and aging (91,92).

Another subgroup of flavonoids usually identified in grape is flavan-3-ols, such as monomeric catechin and oligomeric or polymeric procyanidins. These chemicals in part pass in wine providing significant enhancement of its quality and characteristics, molding the aesthetic appeal, affecting bitterness, and maturity (93,94). They are largely contained in the seeds where act as natural preservatives, while, in the human body exsert significant biological functions, especially antioxidant, antidiabetic, and cardioprotective activities (95–97).

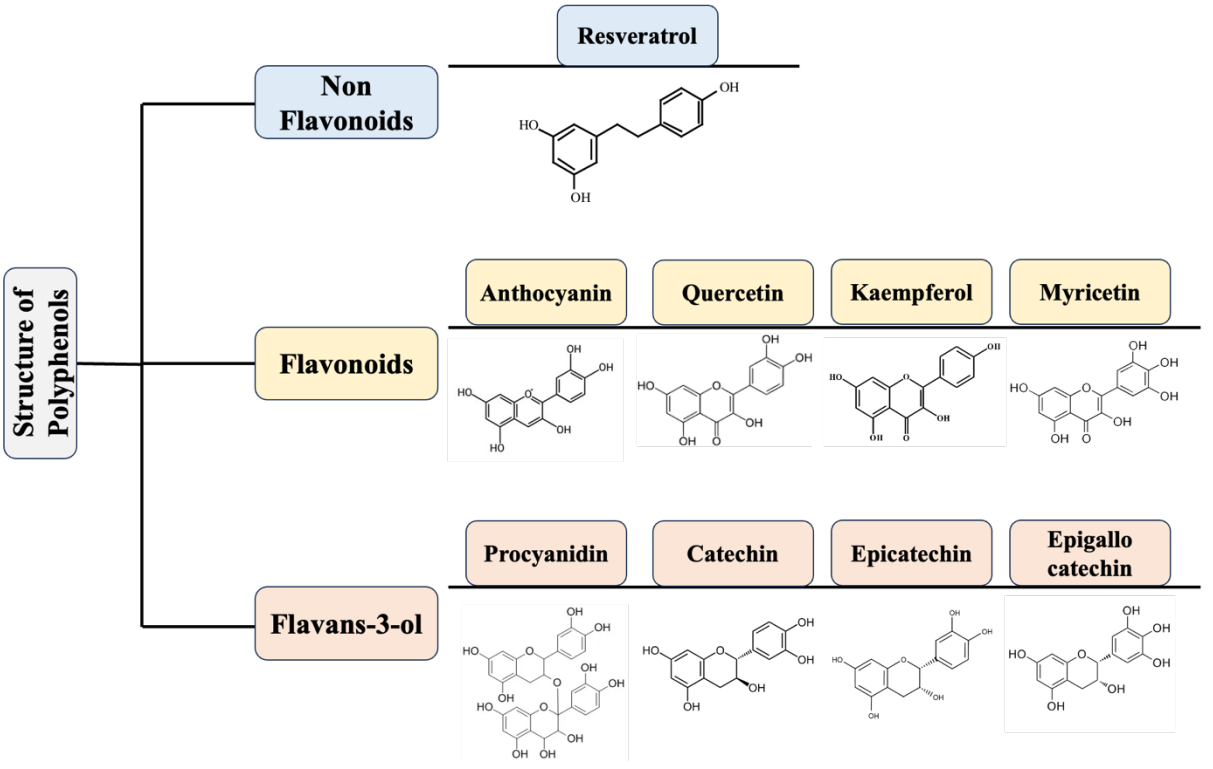

Figure 1. Chemical structure of the main polyphenols found in grape and grape byproducts.

Overall, the studies based on the use of polyphenols derived from grape berries, pomace, and lees highlight their important biological impacts, confirming their possible use in boosting human health and well-being.

4. Extraction of polyphenols from grape and its by-products

The majority of grapes are either consumed as fresh fruit or utilized in the production of wine, jam, juice, and raisins, with winemaking representing the largest use of grapes in the food industry (98). Grape berries have also been used to recover pectic substances, sugars, proteins, organic acids, carotenoids, chlorophylls, and several polyphenols (99–101). Additionally, grape by-products, such as grape pomace, seeds, peel and lees, derived from the winemaking process, have garnered increasing research interest due to their significant potential applications in the various industrial sectors (food, nutraceutic, cosmetic, textile, among others). Moreover, their exploitation and transformation into wellbeing products can help mitigate environmental issues associated with their disposal, reduce resource waste, and provide significant economic and health benefits. In the case of by-products, since direct use is not feasible, as it is for grapes, they must be properly processed to recover all the valuable molecules they contain. The processes used to extract pure molecules or phytocomplexes from byproducts vary widely, and the choice of a specific method depends on the selected biological matrix and the type of molecules to be extracted (102). The traditional and easier method used to isolate these molecules from the vegetable matrices is the solid-liquid extraction by static or stirring-assisted or ultrasound-assisted maceration, usually performed using water, methanol, ethanol, glycerol or other organic solvents like hexane, chloroform, benzene, acetone.

Clearly, both the extraction method and the solvent used play a crucial role in terms of efficiency and safety. Consequently, research has increasingly focused on the use of food-grade solvents and the selection of scalable, low-energy methods. So that, an accelerated solvent extraction technique was applied by Rajha et al., to increase the recovery of phenolic molecules from cv. *Cabernet sauvignon* grape pomace. The maximum yields were achieved using wet (16.2 g of gallic acid equivalent/100 g of extract) and dry (7.28 g of gallic acid equivalent/100 g of extract) grape pomace performing the process at 140 °C and using a mixture of ethanol and water (70:30) (103). Rajha et al., also improved the solid-liquid extraction (48 hours at 66.6 °C) of polyphenols from cv. *Syrah* grape pomace adding β-cyclodextrins (37.7 mg/mL) to aqueous extractive solvent, that allowed the extraction of 5.8 mg of gallic acid equivalent/g of dry extract corresponding to 3146 µM trolox equivalent per milliliter of extract (104). According to green chemistry and food-grade practices (105–107), Perra et al., have used a mixture of water and ethanol to extract polyphenols from grape pomace and recovered about 76.5 g of extract for every kilogram of dried pomace (108).

Another method proposed to maximize the extraction performances is the combination of maceration with ultrasounds, involving the generation of high energy capable of easily and quickly disrupting cell membranes and walls (109). It is a simple method that could be used with several solvents, ensuring an improvement in the yield of the extraction and already applied to recover phenols from grape pomace (110). Goula et al., extracted 9.57 mg of gallic acid equivalents/g of dry *Agiorgitiko* grape pomace using a water and ethanol mixture and additionally applying ultrasounds for only 10 minutes (111). The resulted extraction yield increases as a function of the temperature used (up to 35 °C), solvent-biomass ratio, amplitude, and pulse duration. Similarly, De Menezes et al., investigated the extraction of grape cv. *Cabernet sauvignon* and *Ives* seeds oil and discovered that ultrasonic technology allowed to obtain the maximum oil content (7% to 18% of extract) and the highest content of saturated, monounsaturated, and

polyunsaturated fatty acids (124, 187 and 698 mg per gram of oil) (112). Microwave-assisted extraction is another advanced method based on the implementation of extraction by microwaves and its efficiency is linked to the conversion of electromagnetic energy to thermal energy through increased agitation of water molecules and charged ions exposed to the microwaves. The damage caused to the tissue and cell membrane led to an increase in the contact region between the liquid and solid phases (113). Drosou et al., performed the extraction of polyphenols by simple maceration or ultrasound- or microwave-assisted maceration in pure water, water and ethanol (1:1) or only ethanol on cv. *Agiorgitico* red grape pomace and confirmed that the microwave treatment was more efficient in comparison with the others (35).

Physical, electrical, and biochemical impacts of high-voltage electrical discharges were applied by Rajha et al., to improve the extraction of polyphenols from *Grenache blanc* vine shoots. Without any fragmentation and using low particle size (a_0=1885.19 cm^{-1}), the recovery of polyphenols was increased using higher energy output, having an improvement of polyphenol content in the extract by 20 mg/L (114).

Another method to extract polyphenols, that has attracted the attention of food industries, involves the use of a pulsed electric field capable of increasing the permeability of cells, inhibiting microorganism growth and improving the recovery of intracellular components and water from natural sources (115). This method has been used by various authors that have demonstrated the enhanced recovery of polyphenols from low humidity cv. *Dunkelfelder* red grape pomace, coupling the densification with a pretreatment with pulsed electric field, which can be also used at industrial level, since the solvent volume and extraction duration can be minimized ensuring in any case a high recovery (12.9%) (115). Moreover, El Darra et al., tested the impact of pulsed electric field, at moderate or elevated intensities, before and during the alcohol fermentation of red grape cv. *Cabernet sauvignon* (E = 0.8 kV/cm & 5 kV/cm). The application of pulsed electric field during cold maceration (at 0, 2, and 4 days) improved the content of anthocyanins up to 426.3 mg/L of extract (116).

An additional technique suitable for the recovery of active components can be the pressurized extraction, which has been used by Perra et al., testing different blends of water and ethanol to maximize the recovery of phenols from cv. *Cannonau* grape pomace, and obtained an extract extremely rich in polyphenols (~2809 μg /g of extract), macerating at high pressure with a mixture of water (45%) and ethanol (55%) for 22 minutes at 130 °C (117).

The surfactant-based extraction method is another interesting, simple, green and low dissipative method, which offers a novel and efficient solution for solid-liquid extraction of polyphenols from various matrices (118). Aqueous surfactant solutions are gaining recognition as one of the most promising green extraction methods, largely because water is used as the principal solvent. In a previous study, Brij S20 and poloxamer 407 exhibited a high potential for polyphenols extraction, with the extraction efficiency in the range of water: ethanol (1:1) and higher than water and ethanol, individually. Similarly, de Araújo Padilha found that the non-ionic surfactant Triton X-114, used for recovering polyphenols from camu-camu depulping residue via cloud point extraction, proved to be highly effective. The optimal system, comprising 7 wt% Triton X-114 and 80 wt% extract, achieved a maximum extraction efficiency of 95.71% at 30 °C, without the need for pH adjustment (119).

Supercritical Fluid Extraction is another intriguing process that is remarkably effective, environmentally friendly, and adaptable technique for recovering bioactive compounds. The

primary aspect is the utilization of a supercritical fluid, often carbon dioxide, as the solvent. In a recent study, Pazir et al. were capable of retrieving 36% of the anthocyanins found in the *Merlot* red grape within the first 80 minutes of extraction, which is much quicker compared to that of the standard approach (120).

The newest technology is based on the use of the patented Ired-Irad® infrared device, which allows to heat the raw material using infra-red radiations (121). This is an environmentally friendly method, which requires a reduced exposure to radiation, is easy to handle, has low processing fees and addresses high amounts of active compounds (122). Cai et al., used Ired-Irad® to improve the extraction of active molecules from grape seeds obtaining higher amounts of catechin (47.88 mg/g of extract), epicatechin (40.04 mg/g of extract) and procyanidin B2 (12.10 mg/g of extract) than those obtained using microwaves and ultrasound methods (123). Current studies highlight the preferential use of grape byproducts as a source of polyphenols, as they are inexpensive and allow to eliminate the need for new resources. However, some of the proposed methods require more systematic studies to identify the most suitable conditions for good reproducibility, fewer steps, and minimal equipment. The goal is to find the most efficient method in terms of energy conservation and environmental impact minimizing damage of valuable compounds (124,125).

5. Formulation and development of grape-based products for enhanced human health and a closed-loop system

Given their phytochemical composition, grapes and their by-products can be used as natural source of active molecules and/or phytocomplexes to produce herbal medicines, food supplements, fortified foods, nutraceuticals, cosmeceuticals among others (126–128). However, it is widely acknowledged that for grape bioactives to exert a biological effect, they must be present in sufficient concentrations in raw or prepared foods and must be both bio accessible and bioavailable. Bioaccessibility refers to the fraction of these compounds released from the food matrix during gastrointestinal digestion, while bioavailability refers to the fraction that reaches systemic circulation in sufficient amounts to elicit a physiological response (129). Bioavailability can be influenced by the processing methods. For instance, phenolic compounds are susceptible to oxidation during various processing techniques, have limited water solubility, and exhibit low bioavailability (130). Studies have shown that consuming 100% grape juice and whole grapes delivers phenolic compounds to consumers in a different manner. Bioaccessibility of anthocyanins in grape juice ranges from 86% to 135%, compared to 14% to 39% in whole grapes. Similarly, bioaccessibility of flavan-3-ols and phenolic acids is higher in grape juice (48–101% and 39–85%, respectively) than in whole grapes (0–3% and 9–67%, respectively). Therefore, consuming grapes or their products can provide beneficial bioaccessibility and bioavailability, initiating a biological response. However, bioactive compounds such as resveratrol and anthocyanins, which are important in managing various disorders, exhibit low bioavailability (129). Given the high bioavailability of grapes and especially of their derived products, particularly juice and wine, many studies have shifted their focus toward utilizing grape by-products as a primary source of active compounds. This approach ensures similar effectiveness while being more environmentally sustainable. In this respect, Luga et Mironeasa, reported that cv. *Feteasc̆ă regală* grape pomace could potentially be added to wheat pasta, providing nutritional advantages due to their antioxidants and fibers contents (131), and Acan et al., examined the chocolate spread enriched

with cv. *Cabernet sauvignon* grape pomace, both demonstrating the high value of grape byproduct as a cheap and healthy source of valuable functional substances (132). Baldan et al., explored the incorporation of cv. *Syrah* grape pomace powder to gluten-free muffins and demonstrated that it would improve their nutritional value (133).

Moreover, considering that 100 kg of processed grapes generate approximately 8-10 kg of lees, depending on the grape variety and the vinification methods applied, they represent the second largest by-product of the winemaking process (134). They are mud-like biomasses generated during fermentation and storage of wine and are composed of dead yeast, yeast residues and insoluble components that precipitate at the bottom of wine vessels and derived directly from grapes or from the occurrence of different chemical or microbiological changes (135).

Wine lees were previously used to stabilize emulsions due to their promising functional and interfacial properties (being hydrophilic or hydrophobic). The interfacial characterization was carried out by means of dilatational and interfacial rheology, whereas the characterization of emulsion microstructure was assessed by droplet size distribution, rheological characterization and backscattering measurements. The results of measurement of droplet size and rheological behaviour of prepared dispersions indicated that high amounts of polyphenols present in grape lees improve the interfacial behaviour of emulsions (oil/water), reducing the interfacial tension up to ~15 mN/m and increased the antioxidant power of formulations (136). Moreover, lees from Japanese grape *Black queen* were used at increasing concentrations (50, 100 and 150 g lees/kg ice cream), by Hwang et al., as a value-added ingredient in ice cream to enhance the rheological and antioxidant properties of final products (137). In particular, the addition of lees decreased the specific pH, melting rate, firmness, lightness and the amount of freezable water in ice creams, while increased the viscosity, yellowness and fat destabilization as a function of the concentration used. The most suitable concentration appeared to be the lowest (50 g lees/kg ice cream), which ensured a good functionality of the product without any disadvantages in both overrun and particle size.

As noted above, despite the promising results, the bioavailability of molecules in both grapes and by-products is significantly influenced by various external and physiological conditions. To address these challenges, incorporating these molecules into suitable dosage forms, whether conventional or innovative, could be a promising strategy for various industries, including food, nutraceutical, cosmeceutical, and pharmaceutical sectors. Dosage forms or micro- and nano-encapsulation techniques can regulate the release of these molecules during digestion, enhance their ability to cross the intestinal barrier, prevent oxidation, increase stability, and overall improve the quality and efficacy of the final products (138), making them suitable for nutrition, medicine, cosmetics, and agriculture (139,140). Various forms of nano-systems are being used to deliver both hydrophilic and hydrophobic compounds. They are commonly known as polymer and lipid-based nanocarriers, including polymeric micro- and nanoparticles, phospholipid vesicles, nanoemulsions, which are all capable of improving the stability of both natural compounds and phytocomplexes (141–144).

Overall, the potential of grape nutrients to revolutionize various industries, along with their promising impacts and health benefits, is emphasized. These nutrients offer numerous advantages that facilitate their implementation. The following sections will focus on the utilization of these grape nutrients, especially those derived from grape by-products, to formulate effective delivery systems (conventional or innovative), suitable for various administration routes.

5.1. Skin and mucosa administration

The demand for cosmetics and cosmeceuticals with natural ingredients is stronger than ever, presenting a significant challenge to the global economy and society. By recovering bioactive compounds from grape by-products, high-value ingredients for cosmetic formulations can be obtained. Given their composition and the sustainability considerations, several studies have been focused on the extraction of pure molecules or phytocomplexes, and their uses for the treatment of skin and mucosa disorders. Gels and creams, particularly oleogels and hydrogels, are among the most commonly used treatments for topical administration. When enriched with natural antioxidants, especially grape polyphenols, they can effectively slow the progression of oxidative stress-related damage, thereby helping to preserve human wellbeing (145). Salem et al., incorporated grape cv. *Marselan* seeds extract into a commercial cream, which was highly stable on storage, and had a strong antioxidant activity (146). In another study, Salem et al., added white grape cv. *Obeidi* seeds powder into cosmetic scrubs. In vivo tests demonstrated enhanced stability of the scrubs and detected no irritation during application (147). Ferreira et al., explored the potential use of extracts and oils derived from grape waste as antioxidants in cosmetic products as substitutes for synthetic alternatives. The phenolic compounds in the extracts have increased antioxidant and antibacterial activity, effectively inhibiting the growth of gram-positive pathogens such as *Staphylococcus aureus* and *Staphylococcus epidermidis*. The evaluation of durability and lipids oxidation revealed that incorporating grape by-products yielded comparable results to artificially produced antioxidants. Thus, they could be included into cosmetic products (148), as mentioned previously by Salem et al. (146).

However, innovative dosage forms are more suitable than conventional ones, as they effectively address physical, biochemical, and pharmacological requirements while also enhancing stability. These advancements are particularly important for the preservation of crop polyphenols. There is currently a wide variety of new active molecules available that further demonstrate impressive results for topical delivery, especially if they are incorporated into innovative nano-systems (149). Given that, Manca et al. used ultrasound assisted extraction method to obtain an extract from *Cannonau* grape pomace, rich in phenols, and loaded it into phospholipid vesicles obtained by direct sonication, thus avoiding the use of organic solvents, to be specifically used for skin delivery. Formulations were highly biocompatible, promoted keratinocytes and fibroblasts growth, and improved the beneficial effect of the active molecules contained in the extract, as only when it was incorporated in phospholipid vesicles, was capable of effectively protecting these cells against damage caused by oxidative stress (150). Similarly, Perra et al., recovered a valuable phytocomplex rich in catechin, quercetin and gallic acid from cv. *Carignan* grape pomace, by macerating them with a hydro-ethanolic mixture, and loaded it in conventional liposomes and phospholipid vesicles enriched with glycerol or with a commercial mixture of polyglucosides, as natural surfactants. The loading of the extract in vesicles improved its capability of protecting fibroblasts from oxidative damages caused by hydrogen peroxide confirming the importance of formulation, especially if nano, for skin delivery (151). Castangia et al. compared conventional liposomes and vesicles enriched with propylene glycol, both loaded with grape pomace extract and stabilized with grape-silver nanoparticles. This formulation proved to be effective in counteracting infections caused by several pathogens, including *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Additionally, these systems protected fibroblasts and

keratinocytes against oxidative stress, indicating their potential as suitable formulations for the topical treatment of skin lesions (152). Supporting these findings, Vitonyte et al. showed that the combination (0.5% w/v each), of resveratrol and gallic acid, as the main representative molecules contained in grape pomace, co-loaded into phospholipid vesicles effectively protected fibroblasts and keratinocytes from oxidative stress and enhanced antimicrobial activity (153). Simonetti et al. formulated poly(lactic-co-glycolic) acid nanoparticles, small in size (50-150 nm), to improve the antifungal activity of grape pomace extract against Candida albicans, demonstrating their ability of effectively reducing biofilm formation and maturation (154). Furthermore, Felippi et al. demonstrated that the administrations of grape seed oil containing lipid nanoparticles to the skin, reduced the wrinkle intensity in a better extent than other antioxidants (155). In another work, grape seed oil was used as one of the oil phase components of nanoemulsions, specifically tailored for the topical delivery of resveratrol, and Tween 80 was selected as surfactant. The formulation was capable of fostering the stability of resveratrol under UV-light exposure, retaining 88% of the active molecules in comparison with the 50% retained using dimethylsulphoxide (DMSO) (156).

The general findings emphasize that ad hoc formulating extracts or chemicals derived from grape by-products for skin and mucosal applications significantly enhances their beneficial effects. This approach adds value, simplifies application, and improves efficacy, making these formulations effective cosmeceutical ingredients and/or additives.

5.2. Oral administration

The unique combination of grape polyphenols, including flavonoids, anthocyanins, proanthocyanidins, and stilbenes, makes grapes and especially its byproducts, a promising source of active molecules for the development of novel nutraceutical products. In recent years, a wide range of food additives and nutritional products derived from grapes have been introduced to the global market. Most of them are obtained from the processing of pomace during wine or grape juice production. These include various grape skin and seed extracts, grape skin powder, dry seed powder (available in capsules or bulk), pomace powder, and anthocyanin colorants (157). However, variable results in terms of bioavailability have been detected upon oral administration of both grape and derived products. This variability can be attributed to several factors including sources of molecules and/or phytocomplexes, specific chemical properties, type of food matrix, dosage, individual variations and analytical methodologies (158,159). As reported for skin and mucosa formulations, phytochemicals isolated from waste streams are susceptible to chemical degradation when exposed to environmental stresses, as well as during passage through the human gut (160,161). Recent advancements have led to the development of enriched foods, dietary supplements and additives based on grape and its byproducts, that may improve their nutritional profile (162,163). The high amount of dietary fibers contained in grape pomace further improve its value, as the interest in them is increasing due to the relatively low energy content and the notable influence on gut health (164). Additionally, dietary fibers from grapes are notable for their high antioxidant potential, distinguishing them from common fibers. These fibers show promise in the prevention and treatment of diseases such as gut dysbiosis, ulcerative colitis, and hyperglycemia (165,166). Hogan et al., assessed in vitro and in vivo antioxidant and anti-postprandial hyperglycemic effect of an extract obtained, via ethanol-water

extraction, from red (*Cabernet Franc*) and white (*Chardonnay*) grape pomace, and orally administered after its transformation into powder. It was found that pomace extract (400 mg/kg mouse body weight) effectively reduced postprandial hyperglycemia in streptozocin-induced diabetic mice by 35% (167). Kandylis et al., added grape pomace powder into dietary foods (i.e., yogurt, cheese, milk) to enrich the products with antioxidant polyphenols, usually absent in dairy products (168). Iuga et al., reviewed the impacts of chemical components from grape byproducts on the nutritional, functional, rheological, textural, physical, and sensory attributes of baked goods and pasta and stated that their incorporation (i.e., pomace, seeds, peels) enhances these characteristics and increases the content of fibers, minerals, and phenols (169). Theagarajan et al*.,* used red grape cv. *Muscat* pomace powder to prepare functional cookies, which had increased antioxidant potential, and improved preservability with an extended shelf life (170). Other than conventional formulations, nanotechnological systems have attracted the interest of the scientific community thanks to their advanced performances. Indeed, nano-delivery systems offer the possibility to be specifically engineered with various compositions, shapes, sizes, and charges, allowing their functionality to be tailored for specific applications. Perra et al., obtained an extract from pomace of red grape cv. *Cannonau* and loaded it into innovative phospholipid vesicles improved with gelatin and Nutriose, designed to provide intestinal protection. Vesicles ensured effective protection of intestinal cells (Caco-2) against oxidative stress and simultaneously promoted the biofilm formation by probiotic bacteria (171). Parekh et al., loaded the extract of *Nasco* grape pomace in nutriosomes, special phospholipid vesicles formulated with a dextrin and tailored for oral administration of natural antioxidants. Their neuroprotective effect upon oral administration, was explored in an animal model in which Parkinson disease was induced using 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and compared with that provided by the extract in solution. The extract of *Nasco* pomace, only when loaded in nutriosomes effectively prevented the reduction of tyrosine hydroxylase (TH) and dopamine transporter (DAT), along with the reduction in the number of TH-positive cells. The data support the potentially beneficial effects of Nasco pomace extract loaded in nutriosomes as an adjuvant therapy of Parkinson's disease (172). In another study, nutriosomes containing cv. *Cannonau* grape pomace extract have been tested for the protection of the intestinal tract. Formulations further confirmed their high biocompatibility and effectiveness against oxidative stress shielding Caco-2 cells. In vivo findings emphasized that the vesicular formulations did not impact the composition of mouse microbiota (173). Castangia et al., used cv. *Carignano* grape pomace powder in gelatin-liposomes and nutriosomes formulations to enrich yogurts and make them suitable against gastro-intestinal disorders. The obtained vesicles protected Caco-2 cells against oxidative stress, suggesting their suitability as a new approach for the development of health-conscious enriched food (174). Manconi et al. successfully incorporated grape pomace extract (4% or 5% w/v) into small-sized liposomes based on sodium alginate (SA) or arabic gum (AG), achieving good encapsulation efficiency and enhancing its potential in the nutraceutical industry (175).

Overall, the results highlight that formulating extracts or chemical compounds derived from grapes and their byproducts into oral dosage forms, either conventional or innovative, significantly enhances their beneficial effects. Using nanoformulation technology is an emerging strategy to enhance or reinvent traditional ingredients, adding value, facilitating their use, and improving both their nutritional efficacy and consumer acceptance.

5.3. Pulmonary and Nasal administration

As previously mentioned, the high instability and low oral bioavailability of grape derived active molecules, have often limited the translation of their in vitro activities to in vivo effects. Although oral administration of these bioactives, particularly when delivered using appropriate dosage forms, may slow down, prevent, or even inhibit various local or systemic diseases, they have frequently failed to protect mice from chemically induced diseases. Therefore, alternative administration routes must be considered to enhance the beneficial properties of these molecules both in vitro and in vivo. In this regard, a drug delivery method that allows direct administration through inhalation using a specialized device may represent an effective strategy for meeting the needs of most patients. The unique physiological structures of the nose and lungs can reduce metabolism after drug absorption and enable direct entry into the bloodstream, thereby bypassing the first-pass effect that normally take place in the liver (176,177). Recent research found grape seeds polyphenols to be effective at inhibiting the proliferation of respiratory syncytial infections in human airway epithelial cells at concentrations of 5-10 µg/mL of extract (178). Mahmoud Y., explored the impact of grape seeds extract on lung parenchyma histopathology and irritation in an ovalbumin induced mouse asthmatic model. The findings imply the use of the extract as a secure and reliable anti-inflammatory additive to reduce the severity of asthma (179). Zhou et al., evaluated the influence of grape extract on inflammatory diseases and restructuring in a chronic asthmatic model. The extract inhibited inflammation of the airways and modification in a mouse model of long-lasting asthma, suggesting that it might be used to treat asthma (180).

However, to achieve optimal effects, the use of micro- and nano-delivery systems is often necessary especially to successfully overcome mucosal barriers. Hence, phenolics administration requires the development of a complete protective system capable of preserving the structural stability of the polyphenols before application or delivery, thereby increasing their water absorption and bioavailability.

Chang et al., demonstrated that in NCI-H292 cells (human airway epithelial cells), dietary polyphenols (e.g., red wine extract) reduce MUC5AC gene expression, where high expression contributes to chronic rhinosinusitis, as well as their influence on beat frequency of human nasal mucosa. They found that gingerol, quercetin and epigallocatechin gallate might be used as anti-hypersecretory drugs due to their effectiveness in decreasing mucus production of respiratory epithelial cells while preserving regular nasal ciliary activity (181).

Trapani et al., discovered that the integration of grape seed proanthocyanidins with solid lipid nanoparticles and dopamine, not only improves the mucoadhesive performance, but also forms hydrogen bonding connections with mucin macromolecules, efficaciously inhibiting dopamine autoxidation. These discoveries make them intriguing prospects for harmless nose-to-brain transfer, promising breakthroughs for targeted medication administration, and neurological therapy (182). In a pharmacological context, Masiero et al., highlighted the incorporation of plant-based oils in the development of lipid nanoparticles as an emerging tool for addressing a variety of diseases. For example, nano emulsion using palm oil as lipid phase exhibited significant promise towards pulmonary administration in the treatment of lung cancer (183). Trapani et al., encapsulated grape seeds extract into lipid nanoparticles tailored for nasal administration. Outcomes indicated improved nasal mucosa permeation and cell uptake (184). Despite the

limited number of studies conducted on this route of administration, inhalation represents a promising strategy, leveraging the unique physiological structures of the nose and lungs to reduce metabolism after drug absorption and enable direct entry into the bloodstream, thereby bypassing the first-pass effect in the liver. Consequently, pulmonary and nasal administration emerges as a valid alternative to the more conventional oral route.

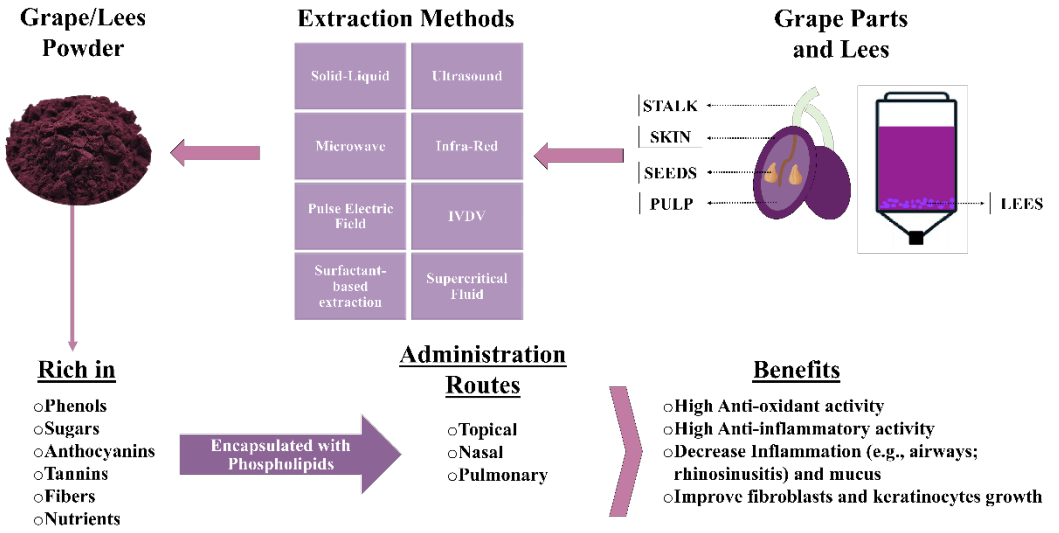

Figure 2. Visual representation summarizing grape, and lees exploitation; their pathway for administration and beneficial effects provided.

6. Scaling Up from Laboratory to Industry: Suitability, Sustainability and Limitations

The utilization of grape pomace in various applications has yielded promising results. Winery by-products are rich sources of phytochemicals, which may act as antioxidants and health-promoting agents in the cosmetic and food industries, as well as in food supplements and pharmaceuticals. To ensure the safety of these phytochemicals in pharmaceutical and cosmetic products, companies must thoroughly assess potential toxicity and be aware of any adverse side effects before incorporating them into formulations. While animal model tests have shown grape seed extract to be safe, it can exhibit pro-oxidant effects depending on dosage, duration of administration, and other dietary components (185,186), continued research is necessary to provide robust evidence of the efficacy and safety of phenolic herbal remedies as alternative or complementary therapies for certain diseases.

From an environmental perspective, consumers, food sectors, and stakeholders are increasingly shifting towards sustainable wine production practices, emphasizing green technologies. The quantity and specific profile of bioactive compounds in winemaking residues vary significantly depending on the type of residue (187). Research should focus on the structural elucidation of novel bioactive compounds derived from vinification residues, such as grape stems and lees. Identifying specific polyphenols in different winery wastes has led to the development of more extensive valorisation options, underscoring the need for further research in this area.

Implementing efficient procedures for integrating these products using time-saving technologies can lead to successful phytochemicals recovery and innovative products, ultimately reducing the environmental impact of winery by-products. Bioactive compound extraction involves complex mechanisms and can be achieved through various techniques. Green technologies, which replace conventional methods, can increase extraction yields, reduce processing time, and minimize environmental damage caused by toxic solvents. While novel extraction processes are often complex and costly, they hold significant potential. Comparative studies of conventional solvent extraction (SLE) and emerging technologies such as supercritical fluid extraction (SFE) and accelerated solvent extraction (ASE) have shown that the latter can achieve similar or better recoveries. However, pilot-scale studies are necessary to determine the optimal technique based on cost-effectiveness for producing commercially viable extracts. Additionally, modelling these extraction processes can enhance understanding and optimization of extraction conditions, facilitating scale-up.

Despite advancements, considerable progress is still needed before all winery residues are effectively recovered, making winemaking a more sustainable activity that reduces costs and environmental impact. A holistic approach to the wine waste chain is essential, focusing on optimizing extraction methods for pilot-scale applications. The future of sustainable separation for agro-industrial waste recovery is promising, as it increasingly integrates various sectors towards more efficient and circular systems.

Moreover, the correct exploitation of these by-products using nanomaterials is rapidly expanding among various industrial sectors, establishing them as next-generation resources with numerous potential applications (188,189). Nanocarriers have proven to be effective for delivering a variety of poorly bioavailable molecules. Furthermore, they possess unique characteristics such as low toxicity, cost-effective scale-up manufacturing, adequate biocompatibility, and high drug incorporation efficiency (190). However, transitioning these nanocarriers from laboratory to industrial scale still presents several challenges.

Ensuring control over the structural features, dimensions, composition, benefits, and physiological properties of nanocarriers from laboratory testing to industrial production is crucial. Additionally, factors such as cost, toxicity, and environmental impact must be considered (190). The progress in nanocarriers relies on technological breakthroughs that enable sustainable methods compliant with Good Manufacturing Practices (GMP) quality requirements. This ensures the reliability of approaches and final products by maintaining adequate guidelines throughout every process (191). The expansion of nanotechnology in healthcare includes ensuring the final systems along with each component are recognized as safe (GRAS), non-toxic, and capable of in vivo biological degradation (192). Prior the selection of solvents and components for generating small particles, considerations regarding financial implications and product acceptability by specialists and patients must be addressed. Beneficial features of nanocarriers may be impacted when scaling up from laboratory methods. For instance, experiments on emulsion-based approaches found that increasing stirring duration and rotor speed reduced particle diameter while maintaining entrapment efficiency (193). Scaling up production requires validation by relevant organizations and must be cost-effective. To ensure product competence and durability while addressing toxicology variables, adjustments to industrial manufacturing techniques are necessary (194). Despite the development of commercial formulations based on nanocarriers, their application in medical/beneficial settings often faces challenges such as cross-

contamination, lack of uniformity, and the complexity of scaling up production (195). To address these issues, innovations like AKVANO® technology, DepoFoam® technology, and lipid multiparticulate systems provide promising solutions. These approaches offer minimal solvent waste, cost-effectiveness, diversity, and adaptability, making them valuable resources for the pharmaceutical, cosmeceutical, and food industries. However, once efficacy is achieved in the laboratory, scientists must focus on large-scale manufacturing. Effective experimental methods may offer solutions to the challenges associated with expanding nanocarrier production (196).

7. Conclusions

This overview highlights that while the complete chemical composition of grapes and their byproducts is not fully understood yet, extensive research has been conducted. The identification and quantification of recovered phenolics after extraction are significantly influenced by the grape cultivar, as well as environmental and geographic growth conditions. Grapes and their byproducts are valuable sources of various molecules with a wide range of beneficial properties. These molecules can be used collectively as phytocomplexes to achieve synergistic effects or as individual compounds, with resveratrol, quercetin, apigenin being the most promising due to their multitarget beneficial properties. Furthermore, nano-formulation significantly enhances their efficacy, proving to be a suitable strategy for improving their pharmacokinetics.

Despite their promising properties, some important limitations have still to be overcome.

First, extensive studies are required to establish robust and scalable methods for stabilizing grape by-products immediately after their recovery. This would ensure the availability of high-quality raw materials year-round, rather than being limited to the short harvest period. Additionally, improving the standardization and certification of phytocomplexes extracted from grape by-products is crucial. Currently, the main barrier to their effective exploitation is the difficulty in obtaining GMP-certified extracts, which hinders their application in final consumer products. Ensuring that these extracts meet strict quality standards will significantly increase the interest of companies in the food, health, and cosmetic sectors, promoting their use in enriching food products or in producing health-promoting additives.

Furthermore, the reproducibility of the extraction and synthesis processes must be optimized to allow for large-scale production. The transition from pilot studies to industrial applications remains a significant hurdle. Without reliable, scalable methods, these innovative nanoformulations cannot reach broader markets.

Lastly, while nanotechnology-based formulations hold immense promise, the lack of extensive clinical trial data means further research is needed to validate their safety and efficacy. Well-designed clinical trials will be essential for gaining regulatory approval and consumer trust, paving the way for these products to be successfully integrated into health and wellness industries.

In conclusion, overcoming these technical, regulatory, and logistical barriers is essential for transforming grape by-products into sustainable, high-value products that can benefit both industry and consumers.

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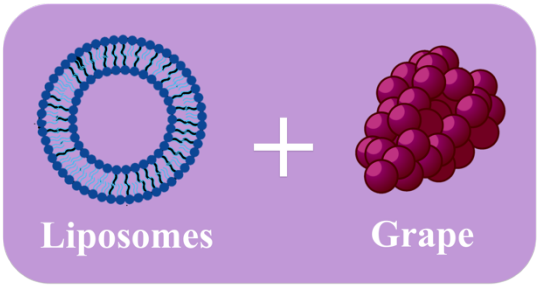
Aim of the Thesis

Encapsulating grape extract polyphenols in lipid nanoparticles (i.e., liposomes, hyalurosomes, glycerosomes) to improve their long-term stability, intake, and effectiveness as therapeutics for various administration route. These nanoparticle-based structures will increase bioactive drug transport by overcoming barriers like inadequate solubility, metabolic processes, and limited penetration into the body. As a result, nanoparticles will have a regulated release and be potentially neurologically beneficial, anti-inflammatory, and antioxidant capabilities for the management of human well-being.

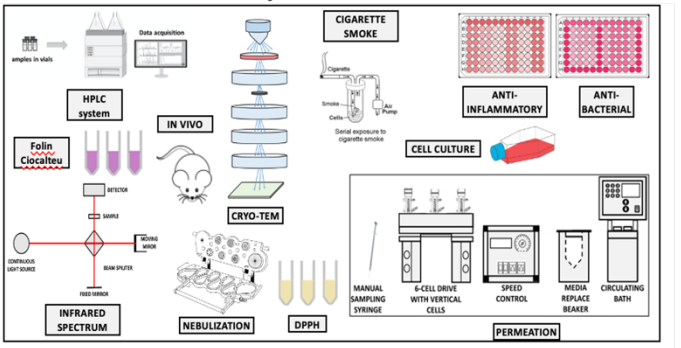
Work 1



Cigarette Smoke Extract

Pulmonary Administration

Analysis Performed



Research Article

"Resveratrol-Enriched Grape Extract Encapsulated in Modified Phospholipid Vesicles: A Promising Approach to Preventing Oxidative Stress Induced by Cigarette Smoke"

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Abstract

In this study, an antioxidant extract was obtained from grape pomace of Lebanese cultivar, Asswad Karech, and was co-loaded with resveratrol in penetration enhancer containing phospholipid vesicles, so called PEVs. Propylene glycol at increasing concentrations (10, 20, 30%) was used as penetration enhancer. The vesicles were prepared by direct sonication, dispersing all components in water solution and sonicating them. Uni and bilamellar vesicles sized ~205 nm, monodispersed (polydispersity index < 0.3) and negatively charged (-55.6 mV) were obtained. Vesicles containing 30% of propylene glycol, when stored at room temperature (25 ºC), maintained these characteristics unchanged during 12 months. When nebulized using the Next Generation Impactor, vesicle dispersions reached the final stages of the impactor that simulate the more profound airways. When incubated with A549 and CuFi-1 cell lines, vesicles loading antioxidants of grape extract and resveratrol were biocompatible and counteract the cell death caused by hydrogen peroxide. When 16HBE bronchial epithelial cells were stressed with cigarette smoke extract, these vesicles, especially those that contain 30% of propylene glycol, competently inhibited the generation of reactive oxygen species (ROS). Thus, grape pomace extract and resveratrol co-loaded in phospholipid vesicles containing 30% of propylene glycol seem to be nebulized in the connective lung tissue for decreasing the harming consequences of cigarette smoke and its related illnesses.

Key words

Grape pomace, sustainability, eco-friendly, phospholipids vesicles, antioxidant, cigarette smoke, oxidative stress, airway, lung delivery.

1. Introduction

The respiratory system is primarily exposed to environmental insults and strongly affected by the air quality. Air pollution can cause frequent respiratory disorders, such as asthma, pneumonia, fungal infections, endobronchial infection, chronic obstructive pulmonary disease and lung cancer, that are often characterized by disruption of the lung endothelial and epithelial barriers [1], [2]. Among the different air contaminants, tobacco smoke is a serious public health threat, as it releases a complex mixture of over 4000 chemicals in form of gases and particulate matter, that are very dangerous first of all for the person smoking who inhales them directly but secondarily also for the people who inhale the contaminated air [3]. It was estimated that only one inspiration of a cigarette contains more than 1015 free radicals and other oxidants, that must be added to indirect ones generated by the stimulation of endogenous inflammatory and immune system [4]. In order to this, cigarette smoke is the main factor associated to the risk to develop chronic obstructive pulmonary disease and lung cancer along with other systemic diseases [5], [6]. It induces significant harm, particularly to the lungs, due to the generated and inhaled hazardous agents such as reactive oxygen species [7]. When these oxidant levels increase

and antioxidant defences are not enough, the balance between oxidants and antioxidants tilts towards an excess of oxidants and oxidative stress arises. This condition can cause local damage in lung tissue and also trigger molecular pathways that lead to inflammation [8]. Moreover, cigarette smoke dangerous chemicals are systemically adsorbed and induces oxidation of human plasma proteins and extensive oxidative degradation also in heart, and liver [9].

Even if the main tool to avoid these damages remains the education to avoid the use of cigarettes, especially in yang people, same approaches have been developed and tested to avoid or lessen the harmful impact of tobacco smoke on the respiratory system and throughout the whole body. It was reported that a diet rich in antioxidants represents a preventative strategy easy to be implemented and cost-effective, reducing the risk of related diseases [10]. However, it was observed that blood concentrations of antioxidants, especially vitamin C and carotenoids, are always lower in smokers than non-smokers even when they are constantly taken by diet, due to inflammatory changes that increase turnover of these micronutrients [4]. However, Panda et al. [9] reported that vitamin C, orally administered, ensured a complete protection against protein damage and lipid peroxidation caused by cigarette smoke in guinea pigs fed with 15 mg per animal per day. Several natural antioxidants have been recently tested and seem to be able to decrease the damage caused by cigarette smoke. A careful literature analysis confirmed that consumption of curcumin attenuates toxicities caused by cigarette smoke, decreasing the risk of developing chronic obstructive pulmonary disease, gastrointestinal toxicity, metabolic diseases, testis injury, and neurotoxicity [11]. Liu et al. [12] disclosed that resveratrol, when orally administered to animals, attenuated oxidative injury in lungs induced by cigarette smoke. To improve the intake and systemic concentration of natural antioxidants, several strategies have been tested. Berberine a natural occurring alkaloid was loaded in laded liquid crystalline nanoparticles and was suggested as a promising alternative for the management of chronic lung disease also caused by cigarette smoke [13]. It was found that curcumin, especially when loaded in lipid-core nanocapsules allowed the maintenance of ion homeostasis and redox balance that usually is broken by the exposure to the cigarette smoke causing excitotoxicity and, consequently, oxidative and nitrosative stress and neurocognitive impairment [14]. In one other study, green tea catechins were loaded in nanoparticles orally administered to animals exposed to cigarette smoke and reduced neuroinflammation, and neuroplasticity [15].

Alternative strategy adopted to improve the effectiveness of polyphenols in counteracting the cigarette smoke toxicity is their local administration to airways. Donato et al. [16] developed a novel cigarette filter with a surface enriched with polyphenols, especially hydroxytyrosol, to neutralize dangerous molecules present in cigarette smoke. Kurus et al. confirmed that local resveratrol exserted a preventive role in the histopathological changes caused by cigarette smoking on the tracheal tissue of rats [17].

Regarding the different polyphenols used to counteract cigarette smoke, resveratrol and curcumin are the most tested phytochemicals, especially by oral administration [11], [12]. Other antioxidants have been tested too, in form of pure chemical or phytocomplex, and results strongly suggested their protective effects, especially if daily administered in diet [18]. These molecules, especially quercetin, catechin and epicatechin, exserted an important antioxidant effect that is usually associate to the anti-inflammatory one. Thus, they directly prevent oxidative damage caused by the oxidative molecules contained in cigarette smoke and, at the same time, reduce inflammation that in turn generates other oxidative species. The double activity provides

protection against systemic diseases and acute lung injury such as chronic obstructive pulmonary disease [19].

Based on these previous findings, in the present study a triple strategy has been adopted to counteract the damaged effects of cigarette smoke: 1) an antioxidant extract rich in resveratrol was obtained from the grape pomace, 2) the extract potential was further improved by co-loading it with resveratrol in phospholipid vesicles, 3) obtained vesicle dispersions were tailored to maximize the lung delivery after aerosolization. The extract was obtained by maceration of grape pomace in water and methanol. Its antioxidant activity and the main polyphenol contained were measured. Phospholipid vesicles were improved with propylene glycol due to its penetration enhancer properties and penetration enhancer containing vesicles (PG-PEVs) were obtained. Three different concentrations (10, 20, 30%) of propylene glycol were used to obtain 10PG-PEVs, 20PG-PEVs, 30PG-PEVs. Their mean diameter, polydispersity index and zeta potential were measured and the morphology observed. Vesicle dispersions were stored for 12 months at room temperature and their physico-chemical characteristics were measured as scheduled time. The aptitude of vesicle dispersions to be nebulized and deposited in the different airways was assayed. The biocompatibility with cells and ability to counteract the cell death caused by hydrogen peroxide and the damage caused by cigarette smoke extract were measured.

2. Materials & Methods

2.1. Materials

Lipoid S75 was obtained from a blend of soybean phospholipids (70% phosphatidylcholine, 9% phosphatidylethanolamine, and 3% lysophosphatidylcholine), triglycerides, and fatty acids (Lipoid, Ludwigshafen, Germany). Sigma-Aldrich supplied the ethanol, propylene glycol, and all other analytical grade products (Milan, Italy). Kaighn modification of Ham F-12 with L-Glutamine, penicillin-streptomycin (PEST), fetal bovine serum (FBS), Dulbecco Phosphate Buffered Saline (DPBS) and trypsin-EDTA (0.5%) without phenol red-free, were acquired from Gibc (Life Technologies, Madrid, Spain). Serum-free Bronchial Epithelial Growth Medium made of BEBM basal medium and SingleQuot additives, was procured from Lonza (Clonetics, Lonza, Walkersville Inc., Walkersville, MD, USA). Dimethyl sulfoxide (DMSO) was obtained from Scharlau (Madrid, Spain). Human Placental Collagen type IV (Sigma Cat. No. C-7521) as well as cell counting kit-8 (CCK-8) were sourced from Sigma Aldrich (Saint Louise, MO, USA).

2.2. Extraction and characterization of extract

The pomace of red grape Asswad Karech (Beqaa Valley, Lebanon) was offered by Château KSARA. It was dried for 48 hours at 35-40 °C in a vented temperature-controlled oven. Aliquots (100 g) of grape pomace were dispersed in 5 L of an ethanol and water (1:1 v/v) mixture and maintained at 50 °C for 2 hours. Solid fraction was separated by centrifugation, ethanol was removed through evaporation, and liquid extractive solution was freezer-dried to obtain the solid extract.

2.3. Determination of Total Phenolic Compounds

The total polyphenol content was determined using the Folin-Ciocalteu technique and gallic acid solutions as calibration [20]. Extract was solubilized in methanol (0.1 g/mL) and 200 µL of this solution was added to 1 mL of Folin solution (Sigma-Aldrich, Darmstadt, Germany) and 800 µL sodium carbonate (Sigma-Aldrich, Darmstadt, Germany). The tubes were heated to 60 °C for 10 minutes and after cooled for 10 minutes. The optical density was measured at 750 nm.

2.4. Antioxidant activities

The ability of the enriched extract to decrease DPPH (2,2-diphenyl-picrylhydrazyl) measured. The DPPH solution (1450 µL, 0.06 mM) was added to 50 µL of grape seed extract solution or Trolox (standard), used as calibration reference (Sigma-Aldrich, St-Quentin Fallavier, France) [21]. The absorbance was measured at 515 nm after 30 min of incubation at room temperature in the dark. A calibration curve was calculated using various Trolox concentrations. The DPPH free radical inhibition was calculated as percentage of absorbance of DPPH less that of sample versus absorbance of pure DPPH [22].

The ability of extract to oxidate copper (II)-neocuproine (2,9- dimethyl-1,10 phenanthroline) was assayed using the Cupric Ion Reducing Antioxidant Capacity (CUPRAC, Bioquochem, Asturias, Spain). 200 µL of reactive solution and 40 µL of tested samples: formulations diluted up to 0.1 g/mL of extract or Trolox as standard were mixed. After 30 minutes of incubation at room temperature, the absorbance at 450 nm was detected with a plate reader. The results were reported as mM Trolox equivalents [23].

The ability of extract to decrease the ferric complex at acidic pH was measured with the ferric reducing antioxidant power assay (FRAP, Bioquochem, Asturias, Spain). 10 µL of formulations, diluted up to 0.1 g/mL of extract or Trolox as standard, were mixed with 220 µL of FRAP reaction mixture. Mixture was maintained for 4 minutes at constant stirring and the absorbance was read with a plate reader at 593 nm. The antioxidant activity was expressed as µM of iron (II) equivalent [23].

2.5. Identification of extract components

A thin stratum of the extract was placed directly into the infrared (IR) beam, and it was scanned by the Fourier-transform infrared (FTIR) spectroscopy.

The components of the extract were separated using a gas chromatograph coupled with a mass spectrophotometer, Shimadzu QP2020 GC-MS fitted with a split-split-less injector, a DB5 MS fused silica column (5% phenyl, 95% polydimethylsiloxane 30 m x 0.25 mm, film thickness 0.25 µm) and a linear temperature program. Temperature was increased of 7 °C /min from 50 °C (beginning temperature) to 280 °C (final temperature) and maintained at 280 °C for 40 minutes for 74 minutes. The injector temperature was 260 °C with a split ratio of 20:1; 1 µL of sample in dichloromethane was injected; the carrier gas was helium; the source and detector temperature was 240 °C; the interface temperature was 250 °C; the ionization energy was 70 eV; and the AMU gain was -492; the AMU offs were -67; the ionization current was 60 µm; and the scan range was 35-500 amu; with a scan speed of 1666. The solvent cut was 3 minutes, and the data were

collected at 4.5 minutes. The mass spectrum of each chemical ingredient was compared to the matching documented spectra (in NIST 2017, and ADAMS-2007 libraries). The detection of compounds was additionally validated through the comparison of their relative retention indices (RRI) versus those reported in literature, including Adam library [24]. The compounds were identified through comparing their retention times (Rt) with the ones of pure authentic samples, assessing their linear indices (LRI) with regard to a series of n-hydrocarbons, as well as computer comparing towards the commercial division [25], [26].

Phenolic molecules were separated and identified using a SciEx UPLC (Exion-UPLC, USA) equipped with a Analyst 1.7 software with an LC-ESI-PDA-MS/MS-4500-QTRAP system (AB Sciex Instrument, located in Framingham, MA, USA). Chromatographic separation was performed at 50 ± 1 °C using a ODS column (100 × 2.1 mm, 5 µm). The initial mobile phase consists of 80% water (containing 1% formic acid, A) and 20% methanol (containing 1% formic acid, B). the separation was performed using a gradient: time 0-1, A 80%, time 12-18, A 0%, B 100%; time 19-22, A 80%, B 20%. The solvent was delivered at a flow rate of 0.35 mL/min and a 5 µL injection volume. The mass spectroscopy analyses were carried out in negative ion mode, with nitrogen gas at a pressure of 60 psi serving as both nebulizing and drying gas. The mass spectra were acquired over an m/z range of 100–900 amu. The calibration curves were prepared using the standard samples of the phenolic compound and phytochemicals and the components of the extract were calculated accordingly.

2.6. Preparation and characterization of vesicles

The extract (20 mg/mL) and resveratrol (5 mg/mL) were mixed with phospholipid S75 (120 mg/mL) and hydrated with 2 mL of water to form liposomes or with a blend of propylene glycol (10, 20, 30%) and water to make PG-PEVs. Dispersions were sonication (40 cycles 5 on / 2 off) with a Soniprep 150 sonicator (MSE Crowley, London, UK). The vesicles were kept at 4 °C after they were prepared.

Using a Zetasizer Ultra, light scattering technology was utilized to measure the average diameter, polydispersity index, and zeta potential of constructed vesicles (Malvern Instruments, Worcestershire, UK). Samples (2 mL) were separated from the non-incorporated extract by dialysis (Spectra/Por® membranes, 3 nm pore size; Spectrum Laboratories Inc., Rancho Dominguez, US) in water at room temperature for 2 hours. This technique was chosen because the principal constituents of the extract are polyphenols with molecular weights lower than the membrane cut-off (Spectrapor, 12-14 kD), allowing them to cross the membrane. Before and after dialysis, the antioxidant activity of formulations was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) colorimetric assay. Samples (20 µL) were dissolved in 1980 mL of DPPH methanolic solution (80 µL/mL) and incubated at room temperature in the dark for 30 minutes. The absorbance was determined at 517 nm against the blank and the antioxidant activity was calculated as percentage of absorbance of DPPH less that of sample versus absorbance of pure DPPH [22]

The vesicle morphology was observed with cryo-TEM, using a Tecnai G2 20 Twin (FEI) at a voltage of 200 KeV in bright-field and low-dose imaging modes. A sample portion (3 µL) was put to glow-discharged 300 mesh Quantifoil TEM grids, and the surplus water was collected with filter paper. To maintain the sample in a frozen solid condition, the prepared sample was plunge

frozen into liquid ethane using an FEI Vitrobot Mark IV (Eindhoven, The Netherlands). The resulting grids were kept at -170 °C and after transferred to microscope at liquid nitrogen temperature (-196 °C). Size and polydispersity distribution of vesicles maintained at 4 °C for 12 months were measured as part of stability research.

The quantification of resveratrol in formulation and permeation samples was performed by the HPLC system (Shimadzu Corporation, Kyoto, Japan) using Hypersil gold C-18 column (250 mm × 4.6 mm, 5 µm) being a stationary phase. Shimadzu Prominence Liquid Chromatography system equipped with LC-20AD quaternary solvent delivery system, auto-sampler having a universal loop injector of capacity (0.1-100 µl), and an SPD-M20A diode array detector monitored the analyte signal between 200 to 350 nm. The mobile phase was provided in an isocratic mode with a flow rate of 1 ml min-1, and the signals were analyzed at 307 nm, with 10 µL as injection volume. All chromatographic conditions were performed at 30 °C. The calibration curve was constructed using known concentrations of standard resveratrol in the range 1.0–100 µg/mL. The retention times for resveratrol were 3.5-3.8 minutes. Resveratrol has been quantified using the linear regression equation of y= 38502x – 14103 (n = 3, r2 = 0.9986).

2.7. Evaluation of *In vitro* permeation of vesicles through pulmonary

Hanson Automated Franz diffusion cell was employed in this analysis; a cell comprised of a controlled sampling, media replacement, single computer workspace, accurate borosilicate glass, and mixer inserts. The system receptor fluid, in this case phosphate buffer, had a pH of 6.8.

2.8. Nebulization Studies of vesicle dispersions

The next-generation impactor (Eur. Ph 7.2, Copley Scientific Ltd., Nottingham, United Kingdom) and the PariSX® air jet nebulizer linked to a ParyBoySX® compressor were used to investigate the *in vitro* deposition of vesicle dispersions. 2 mL of each dispersion was put in the nebulizer for 10 minutes before being aerosolized straight into the throat of the New Generation Impactor. At the end of this process, the dispersion deposited in each step of impactor was recovered with methanol and analysed using a spectrophotometer to quantify the contained extract. The geometric standard deviation (GSD) and mass median aerodynamic diameter (MMAD) measurements were determined without considering the mass deposited in the induction port. The total number of particles with a diameter smaller than the specified size of each phase was expressed as a percentage of retrieved extract versus the cut-off diameter, and the mass median aerodynamic diameter of particles was estimated from the graph using the European Pharmacopeia [27], [28]. Total mass output (TMO), Fine Particle Dose (FPD) was calculated as percentage of extract recovered in the impactor versus the amount initially used in the nebulizer and Fine Particle Fraction (FPF) as the amount of extract contained in droplets smaller than 5 µm versus the proportion of droplets smaller than 5 µm,[29].

2.9. *In vitro* cells experiments

Culture of cells

A549 (ATCC® CCL-185™) and CuFi-1 (ATCC® CRL-4013™) cells were acquired from the American Type Culture Collection (ATCC; Manassas, VA, USA). Human lung epithelial cells (A549) were cultured and maintained in F-12K media enriched with 10% foetal bovine serum, 1% penicillin and streptomycin, and kept at 37 °C with 90% humidity and 5% of carbon dioxide. Cells were cultured to reach 90% of confluence, then, were trypsinized and seeded in separate plates for each experiment. The cystic fibrosis cell line (CuFi-1) was cultured in serum-free BEGM media at 37 °C, 90% humidity, and 5% of carbon dioxide. They were culture in flasks pre-coated for a minimum of 18 hours with a 60 µg/mL solution of Human Placental Collagen type IV, to facilitate their adhesion.
16HBE, an immortal cell line derived from human bronchial epithelial cells, were selected for their shape and functions comparable to normal airway epithelial cell types [30]. Eagle essential medium (MEM)), enhanced with 10% heat-inactivated foetal bovine serum (FBS), 1% of mixture of not essential amino-acids, 2 mM L-glutamine, and 0.5% gentamicin, was used to culture the cells in a humid environment at 5% of carbon dioxide and 37 °C [31].

Cell Viability Assay

Biocompatibility of formulations was assayed using the three different cell lines: A549, CuFi and 16HBE. The A549 were seeded (10 x 103 cells) in well of a 96-well plate for 24 hours and then incubated with resveratrol enriched extract in dispersion or loaded in vesicles at various dilutions (20, 2, 0.2, 0.02 µg/mL) for 48 hours. Cufi cells were seeded in the same condition using plates pre-coated with collagen. Afterwards, 100 µL of cell counting kit-8 (CCK-8) (10% final concentration) was incorporated to each well and plates were incubated for 3 hours; optical density at 570 nm was determined with a microplate reader (Synergy 4 Reader, BioTek Instruments, AHSI S.p.A, Bernareggio, Italy). The vitality of treated cells was calculated as percentage of untreated control cells (100% viability).

Viability of 16HBE was assessed with CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA), a colorimetric technique that allows to detect the metabolically active and therefore vital cells using MTS [3-(4,5-dimethylthiazol-2-yl)-5 (carboxymethoxyphenyl)-2-(4-sulfopheyl) 2H-tetrazolium] [32]. In, Cells (1×104 cells/well) were cultured96-well plates and treated with resveratrol enriched extract in dispersion or loaded in vesicles at various dilutions (20, 2, 0.2 µg/mL). After the treatment, in every well 20 µL of CellTiter 96® Aqueous One Solution reagent was added and the plates were left to incubate for 20 minutes at 37 °C. The absorbance was measured at 490 nm and viability reported as a percentage versus the untreated cells (100% viability) [33].

Ability of vesicles to protect the cells against oxidative stress

The *in vitro* ability of resveratrol enriched extract loaded vesicles to avoid cell damaged induced with hydrogen peroxide was tested. A549 and CuFi were seeded in 96-well plates (10 x

103 cells/well) and incubated for 24 hours. Then, resveratrol enriched extract in dispersion or loaded in vesicles, diluted with DMEM up to 0.2, 0.02 µg/mL of extract, was added and cells were stressed with hydrogen peroxide (1:30,000 dilution). Cells untreated and unstressed were used as positive control and cells untreated and stressed with hydrogen peroxide as negative control. Following 4 hours of incubation, cells were washed with fresh medium, and their viability was determined using the CCK-8 kit, as previously described.

Preparation of Cigarette Smoke Extract (CSE) and Treatment of Cells

Kentucky 3R4F experiment reference cigarettes with no filter (The Tobacco Research Institute, University of Kentucky) were utilized. A peristaltic pump Watson-Marlow 323 E/D (Rotterdam, The Netherlands) was used to remove cigarette smoke. To create the smoke solution (cigarette smoke extract (CSE)), two cigarettes were smoked in 20 mL of PBS mixture every cigarette was smoked for 5 minutes. The solution was then passed through using a 0.22 µm pore filter to eliminate germs and big particles before being utilized within 30 minutes of being prepared. The solution in question was estimated to be 100% cigarette smoke extract, and it was diluted in the medium up to 20% in each well. To establish a standard for this process, we evaluate the Optical Density (O.D.) of smoke solution (i.e., cigarette smoke extract absorbance) at 320 nm. The differences of absorbance of various batches of cigarette smoke extract is very little: 1.37 ± 0.16, as previously documented [34]. A commercially accessible test (Cambrex Corporation, East Rutherford, NJ) was used to identify any presence of contaminated lipopolysaccharide in undiluted cigarette smoke extract solution, and the limit of detection of 0.1 EU/mL was met. In a 12-well plate, cells 16HBE (1.5×105 cells/well) were plated, when they reach 80-90% confluence, were stimulated with: 20% of CSE, PG-PEVs in dispersion or loaded in vesicles, for 24 hours. Every experimental was carried out three times.

Measure of Mitochondrial Superoxide

The evaluation of intracellular ROS, by MitoSOX™ Red mitochondrial superoxide indicator (Molecular Probes Waltham, MA,USA) detects the production of mitochondrial superoxide [35]. 16HBE cells were plated in a 12-well plate, when they reach 80-90% confluence, were stimulated with: 20% of CSE, PG-PEVs in dispersion or loaded in vesicles, for 24 hours. Then, the cells were recovered, washed with PBS, and labelled with 3 □M MitoSOX Red probe for 15 min at 37 °C. Afterwards, cells were washed twice in PBS and analysed by flow cytometry using CytoFLEX (BeckmanCoulter). Results are expressed as a percentage of positive cells.

Measurement of IL-6

The concentrations of IL-6 were measured with enzyme-linked immunosorbent assay (ELISA) (Duo Set R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The data are expressed as pg/mL

2.10. *In Vivo* Preliminary Study

Table 1: Representation of the *in vivo* study

Type of animal	Sex / Age	Number	Period of treatment	Control	Infected
BALB/c mice	Male: 6-9 weeks	6	-	√	-
BALB/c mice	Male: 6-9 weeks	6	-	-	√
BALB/c mice	Male: 6-9 weeks	6	5 days	-	
BALB/c mice	Male: 6-9 weeks	6	10 days	-	

Staphylococcus Aureus was administered into mice through inoculation in a vertical biosafety laminar air flow. To determine its effectiveness, they will get treatment with the 30PG-PEVss formulation (50 μg per day) for 5 and 10 days of treatment. Lungs and trachea will be dissected onto slides to provide the results, which will be assessed with the aid of a qualified pathologist. Additionally, swabs from control, infected, and treated mice are collected, and outcomes are seen using petri dishes probed with the bacteria. Aseptic conditions are used throughout the whole process.

3. Statistical Analysis

Mean values and standard deviation were used to express all results. These statistical analyses were performed with the GraphPad Prism 8 Statistical program, with 0.05 being non-significant. To confirm the substantial difference across results, Two-Way ANOVA test was applied.

3. Experimental Results

3.1 Determination of the optimized experimental conditions of extraction

The method to extract a rich antioxidant phytocomplex from grape pomace was optimized assaying different parameters: the particle size of pomace, the ratio between the solid grided pomace and the solvent, the extraction temperature, the percentage of ethanol added to the water in the extractive solution (Figure 1). The grape pomace was used ungrinded or grinded with increasing sizes of particles (150-250, 250-500, 500-800 mm), it was dispersed in water and left under stirring for 420 minutes at 40 °C. The phenol content of extractive solutions was measured at scheduled time points (Figure 1).

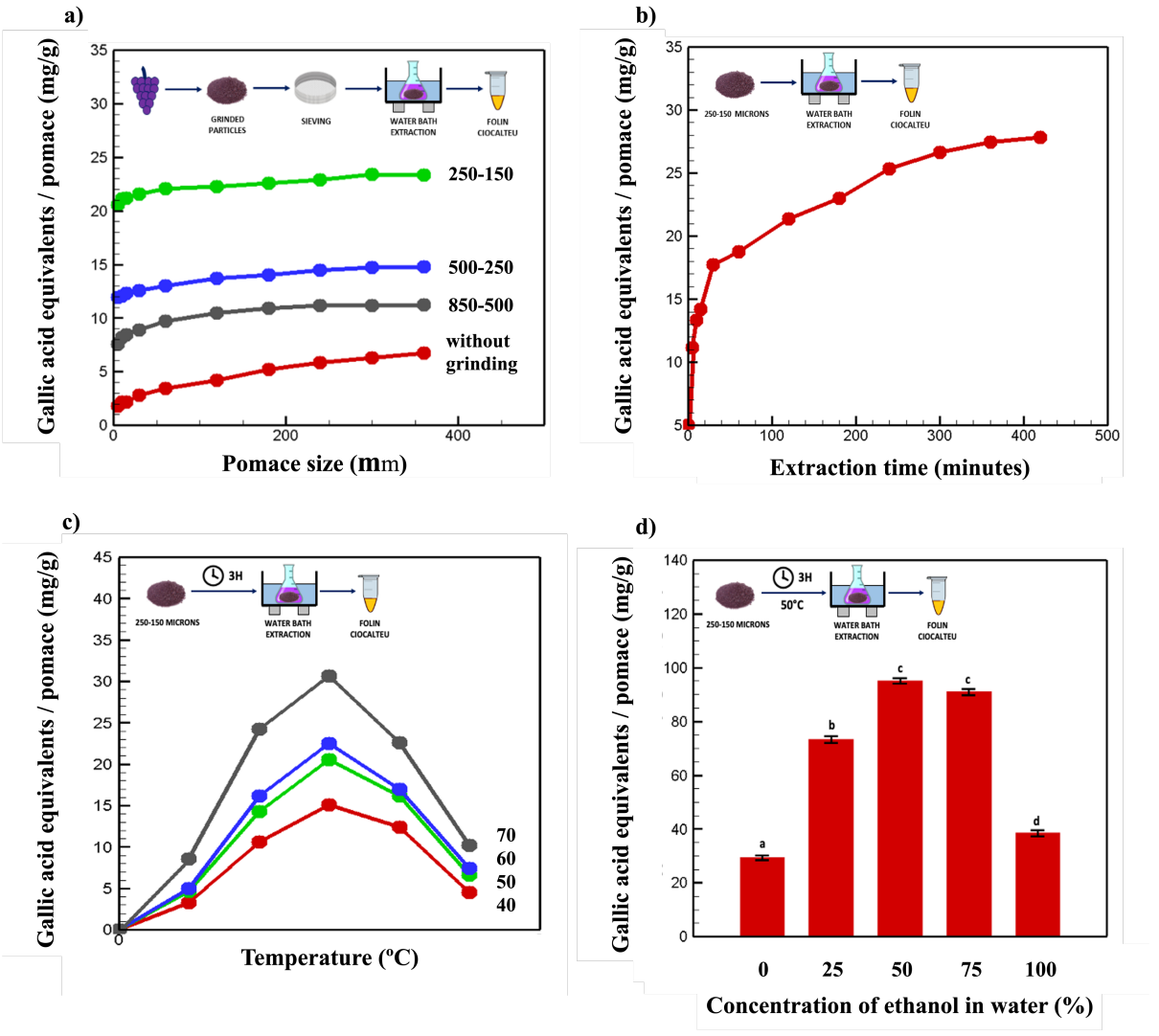

Figure 1. Phenolic content measured with Folin-Ciocalteu reactive in different extracts obtained a) from grape pomace ungrinded or grinding to obtain particles sized 150-250, 250-500, 500-800 mm; b) mixing different ratios of solid extract and solvent (from 1:10 to 1:90 g/mL); c) applying different extraction temperatures (40-70 °C) and mixing different ratios of solid extract and solvent (from 1:10 to 1:90 g/mL); d) using different ethanol percentages in water (0, 25, 50, 75, 100%). Mean values ± standard deviations are reported. Symbols indicate values statistically not different (*p>0.05*).

As expected, the phenol content was lower when ungrinded pomace was used and it increased as the particle sizes decreased (Figure 1a). Using the particles of 150-250 mm, the phenol content was highest, it was ~19 mg of gallic acid equivalents/g of pomace during the first hour and increased up to ~26 mg of gallic acid equivalents/g of pomace at 120 minutes, without further increase at longer times, reaching a plateau (Figure 1b). Based on these results, pomace with particle size of 150-250 mm and 120 minutes of extraction time were selected as optimized parameters for further tests. The pomace was dispersed in water at different pomace:solvent

ratios (1:10; 1:30; 1:50; 1:70; 1:90). At 1:10 the polyphenol was ~2-8 mg of gallic acid equivalents/g of pomace, at 1:30 increased up to ~32 mg of gallic acid equivalents/g of pomace, at 1:50 ratio up to ~50 mg of gallic acid equivalents/g of pomace, remaining constant when the ethanol further increased, 1:70, and 1:90 ratios (Figure 3b). The ratio 1:50 was selected and used performed to optimize the temperature. The last was seated at 40, 50, 60, 70 °C to 50 °C, and the obtained phenol content was ~38 mg of gallic acid equivalents/g of pomace at 40 °C and enhanced at ~56 mg of gallic acid equivalents/g of pomace at 60 and 70 °C (Figure 3C). The last temperature was the higher tested as it corresponds to that of vinification processes and higher temperatures were not applied to avoid oxidative degradation of phenolic compounds [36], [37]. Classical phenol extraction and concentration are normally carried out at temperatures ranging from 20 to 50 °C because temperatures exceeding 70 °C promote fast polyphenol destruction and temperature higher that 25 °C may improve extraction yield because facilitate the solvent penetration in the cell walls and decrease the viscosity of the solvent, raising the diffusion rates of the molecules in the extractive solution [38]. Therefore, 50 °C that allowed to reach the highest phenol content in the extractive solution was selected as ideal temperature. Finally, ethanol was added in the extractive solution at different ethanol and water ratio: 0; 25; 50; 75; 100%. Using 50% ethanol of ethanol the highest phenol content was found in the extractive solution, ~85 mg of gallic acid equivalents/g of pomace (Figure 3D). This finding is congruent with the outcomes of Caldas et al., (2018), who found that using 50% of ethanol in water as extractive solution to recover phenols from grape, the antioxidant activity was the highest [39]. Thus, the final extract was obtained dispersing 10 mg of extract in 500 mL of ethanol and water (1:1) and lest macerate at 50 °C for 120 minutes. The obtained liquid extract was freeze dried to obtain the powder used in the analysis.

3.2. Characterization of grape pomace extract

a

Analysis	Concentration
Total phenolic content	179 ± 3 gallic acid equivalents / extract (mg/g)
DPPH	343 ± 4 µg/mL of Trolox equivalents
Cuprac	2.7 µg/mL ± 0.1 mM of Trolox equivalents
Frap	2.0 µM ± 1.7 mM of Iron (II) equivalents

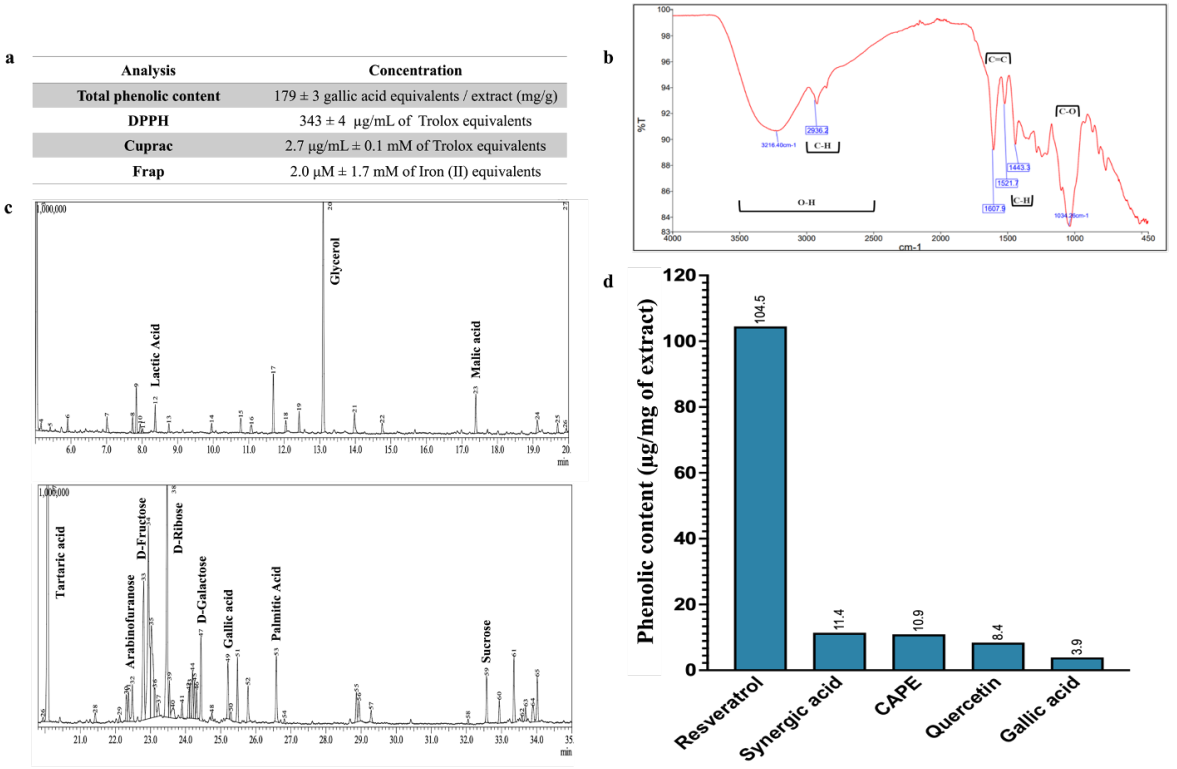

Figure 2. Phenol content and antioxidant activity measured with DPPH, CUPRAC and FRAP (a); Fourier transform infrared spectrum (FTIR) reporting the wavenumbers (cm-1) as a function of the transmittance (%) showing abundant liaison between chemical elements (b); Gas Chromatography Mass Spectrometry (GC-MS) chromatogram (c); Liquid chromatography coupled to mass spectroscopy (LC-MS/MS) chromatogram (d).

In accordance with these results, increased TPC concentrations led to strong antioxidant activity (Figure 2a). The analysis of the FTIR spectrum of sample showed the O-H band from 3550 cm-1 to 2500 cm-1, and the C-H stretching was observed between 2936 cm-1 (asym. str.) and 2875 cm-1 (sym. str.). Moreover, C=C double-bond stretching at 1608 and 1522 cm-1 and strong C-O stretching at 1034 cm-1 was detected. Band corresponding to 1443 cm-1 is C-H asymmetric deformation. The aromatic C-H signals were not visible (Figure 2b). Moving on to the chromatogram, main pics are summarized and shown at the different levels. Highest pics revealed major presence of sugars e.g. fructose, ribose, galactose. In addition to acids e.g. lactic acid, tartaric acid, palmitic acid (Figure 2c). Moreover, the quantification of Asswad Karech powder extract showed the presence of Resveratrol with the highest amount of 104 µg/g, followed by Synergic acid, Caffeic Acid Phenethyl Ester (CAPE), Quercetin and Gallic acid (Figure

2d). HPLC data reveals that the extract powder was stable during storage and there was no degradation product observed in the sample.

3.3. Characterization of the vesicles

Taking into account the advantages obtained using a penetration enhancer in the phospholipid vesicles, they were prepared with increasing concentrations (10, 20, 30%) of propylene glycol, thus obtaining 10PG-PEVs, 20PG-PEVs, 30PG-PEVs PEVs [40]. The optimized extract obtained from pomace of Asswad Karech, a Lebanese autochthonous grape cultivar, was co-loaded with resveratrol (resveratrol-enriched extract) to improve the retention of antioxidant molecules in the target site. The mean diameter, the polydispersity and the zeta potential of prepared vesicles were firstly measured to select their suitability for lung delivery (Table 2).

Table 2. Mean diameter (MD), polydispersity index (PI), zeta potential (ZP), entrapment efficiency (EE) and antioxidant activity (AA) of resveratrol-enriched extract loaded phospholipid vesicles containing 10% of propylene glycol (10PG-PEVs), 20% of propylene glycol (20PG-PEVs), 30% of propylene glycol (30PG-PEVs). Mean values ± standard deviations are reported.

	MD (nm)	PI	ZP (mV)	EE (%)	AA (%)
Liposomes	234±2	0.250±0.01	-56±2	90±1	82±1
10PG-PEVs	225±3	0.245±0.03	-55±1	92±1	84±1
20PG-PEVs	216±4	0.239±0.02	-54±2	93±1	85±1
30PG-PEVs	205±4	0.227±0.01	-56±3	93±1	87±1

Liposomes, prepared without propylene glycol and used as reference, had an average size of ~234 nm. The addition of propylene glycol allowed a reduction of the dimension of vesicles that was inversely related to the used concentration of this polyalcohol. Indeed, 10PG-PEVs were sized ~225 nm, 20PG-PEVs ~216 nm and 30PG-PEVs ~205 nm ($p < 0.05$ among the three values). All vesicle dispersions were monodispersed as the polydispersity index was <0.250 au. Zeta potential was strongly negative (~ −56 mV), regardless of the presence of propylene glycol as previous reported in previous studies [41], [42]. The entrapment efficiency of resveratrol-enriched extract in liposomes was slightly lower (~90%) than that of PEVs, especially that containing 30% of propylene glycol (30PG-PEVs, ~93%), probably due to the higher capability of this solvent to solubilize low hydrophilic molecules than the water. The antioxidant activity was remarkably high, and the composition of the vesicles had no effect on the antioxidant activity of the payloads.

At the cryo-TEM observation, liposomes and PEVs were spherical and oligolamellar (Figure 3).

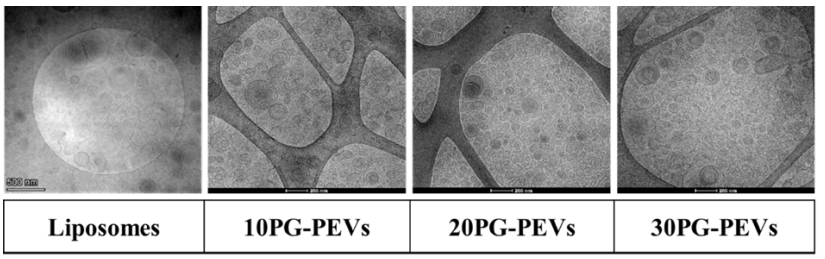

Figure 3. Representative images of resveratrol-enriched extract t loaded phospholipid vesicles containing 10% of propylene glycol (10PG-PEVs), 20% of propylene glycol (20PG-PEVs), 30% of propylene glycol (30PG-PEVs). Scale bar: 200-500 nm.

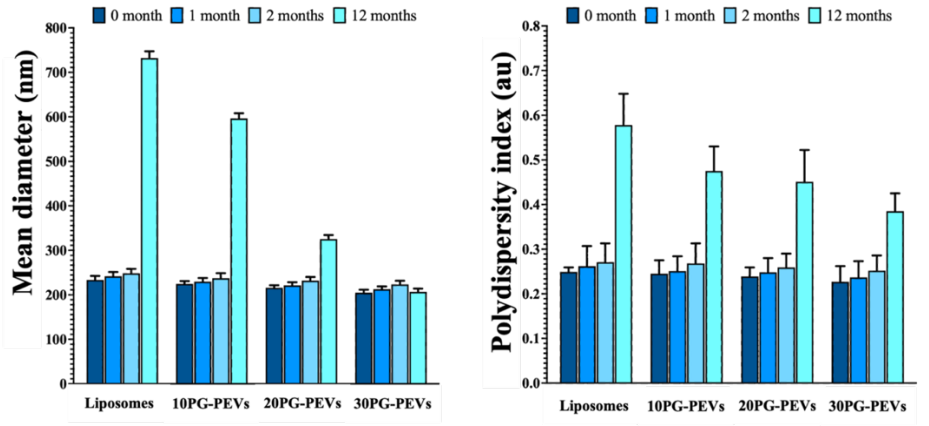

Figure 4. Mean diameter and polydispersity index, of resveratrol-enriched extract loaded vesicles containing 10% of propylene glycol (10PG-PEVs), 20% of propylene glycol (20PG-PEVs), 30% of propylene glycol (30PG-PEVs) and store for 12 months. Mean values ± standard deviations are reported. Symbols represent values statistically not different corresponding to each month of stability (*p>0.05*).

Vesicle dispersions were stored at 25 °C for 12 months and their mean diameter and polydispersity index were measured at 1, 2 and 12 months (Figure 4). At 2 months the parameters of all formulations were stable. At 12 months, liposomes, 10PG-PEVs and 20PG-PEVs underwent a strong increase of their size and polydispersity, especially the first two became larger than 500 nm and the polydispersity index larger than 0.5 au. Only 30PG-PEVs remained stable as their size were 206.6 nm and the polydispersity index 0.365, probably due to the beneficial effect of propylene glycol at the highest concentration.

3.4. *In Vitro* release of resveratrol enriched extract from vesicles

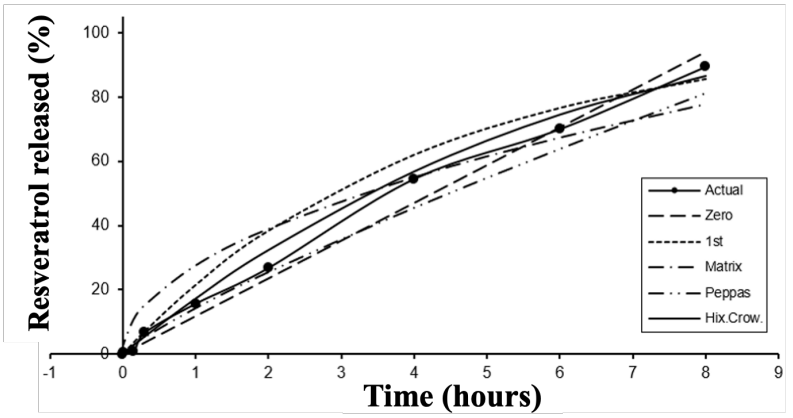

Figure 5. Amount of resveratrol enriched extract released from vesicles.

The amount of from various developed formulations was examined and adapted to the suitable model. Once the proportion of accumulated active pharmacological components, releases, across every created system was measured against a period of time, the software signalled (pass) for the fitting models based on the correlation coefficient (R) and student T-test values (T). The cumulative percentage release of bioactive compounds is reported for 30PG-PEVs since it proved to be to most effective amongst the other formulations. The improved formulation is capable of releasing more than 16% of bioactive compounds necessary for meaningful biological activity *in vivo* within an hour. Within 8 hours, 90% of the bioactive compounds was released cumulatively.

3.5. Nebulization of formulations and evaluation of aerodynamic behaviour

Table 3. Total mass output (TMO), fine particle dose (FPD), fine particle fraction (FPF) and aerodynamic diameter (MMAD) of Asswad Karech loaded into vesicles. Average values ± standard deviations are reported (n = 3).

Samples	TMO (%)	FPD (mg)	FPF (%)	MMAD (µm)
Dispersion	68±2	15±1	56±3	3.9±1.27
Liposomes	70±3	15±1	54±1	3.8±1.3
10PG-PEVs	73±1	17±1	57±1	3.7±1.3
20PG-PEVs	72±2	16±2	56±2	3.8±1.4
30PG-PEVs	76±1	19±2	59±1	3.7±1.2

Extract dispersion, liposomes and PG-PEVs were nebulized with the PariSX® air jet nebulizer connected to the next generation impactor and their *in vitro* deposition parameters were measured to evaluate their ability to deposit in the lungs (Table 3). The water dispersion of

extract as well as the vesicle dispersions were not completely aerosolized, and a small part remained in the inhaler device, especially when the extract was dispersed without vesicles. Specifically, the total mass output of extract dispersion was ~ 68%, that of liposomes and 10-20PG-PEVS was ~72% ($p > 0.05$ among the three values), that of 30PG-PEVs was slightly higher ~ 76% ($p < 0.05$ versus the other values). The fine particle fraction of the last formulation, the most aerosolized, was the highest, ~19 mg and the fine particle dose as well, ~59%. The particle aerodynamic diameter of droplets generated from dispersions was always ≤ 4 µm, with no significant difference among samples. These sizes are appropriate to be deposited in the lungs, as the particles having a mean diameter > 5 µm are considered suitable to be deposited in the higher airways (mouth, trachea, and primary bronchi) via inertial impaction, and those with a mean diameter ranging from 1 to 5 µm in the lungs [43].

3.6. Biocompatibility of formulations with A549 and CuFi cells and ability to protect them from oxidative stress

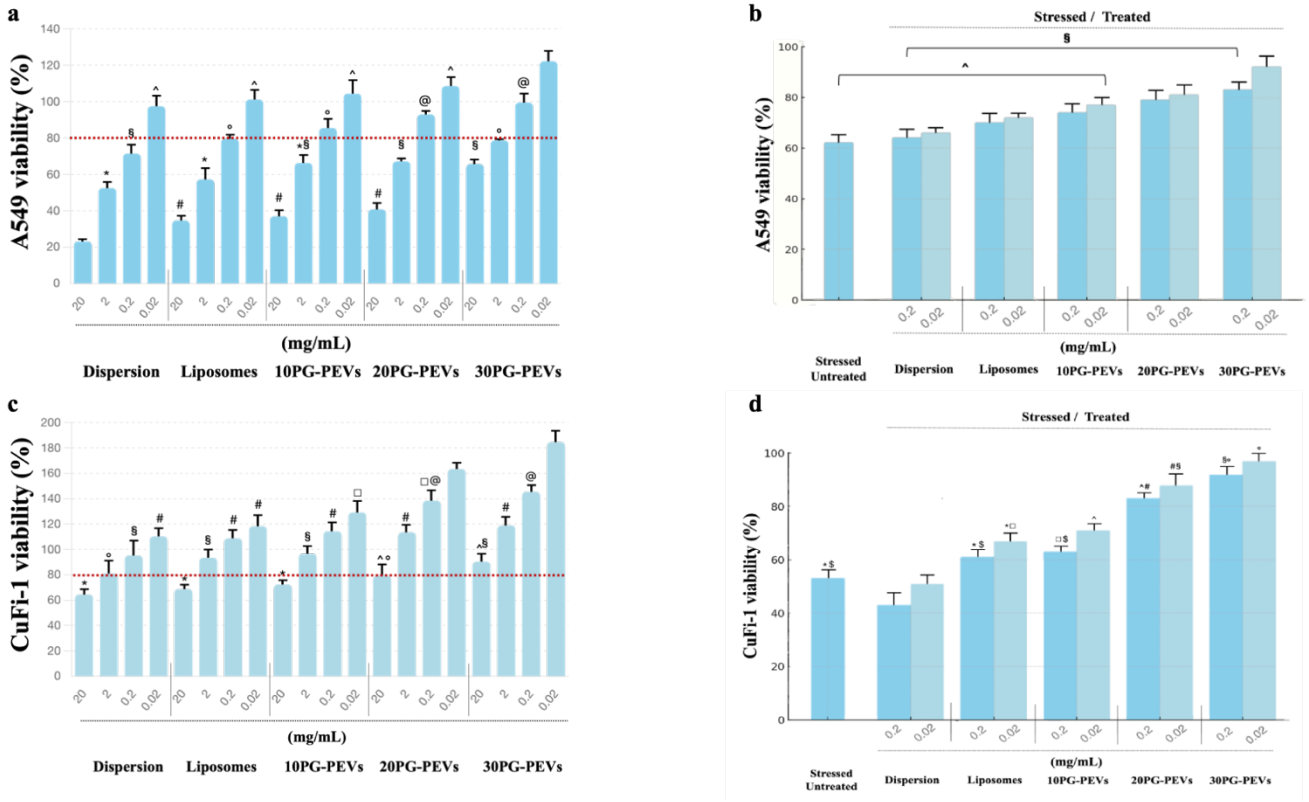

Figure 6. Viability of A549 epithelial cells (a) and CuFi-1 cystic fibrosis cells (c) directly incubated for 48 hours with the extract in dispersion of loaded in vesicles (diluted in the medium up to 20, 2, 0.2, 0.02 mg/mL of extract) and viability of cells (b-d) stressed with hydrogen and treated with the extract in dispersion of loaded in vesicles (diluted in the medium up to 0.2, 0.02 mg/mL of extract). Data (bars) are reported as mean values ± standard deviations of cell viability. Symbols indicate values that are statistically not different (*p>0.05*).

The viability of A549 bronchial epithelial cells incubated with the extract in dispersion or loaded in liposomes and 10-20PG-PEVs was the same and it increased as the extract concentration decrease (Figure 6 A and C). Only using the highest dilution (0.02 mg/mL of extract), the cell viability was ≥ 80%, confirming the biocompatibility of this sample at this dilution. The viability of A549 cells incubated with extract loaded in 30PG-PEVs was ≥ 80%, at the last two dilutions (0.2 and 0.02 mg/mL of extract), confirming the ability of these vesicles to reduce the extract toxicity. The CuFi cells were less sensitive to the extract as their viability, when incubated with the extract in dispersion, was ≥ 80%, yet using the second dilution (2 mg/mL of extract) and the highest (0.2 and 0.02 mg/mL of extract). The viability of CuFi cells incubated with extract loaded liposomes and 10PG-PEVs was comparable and those of cells incubated with 20-30PG-PEVs as higher. Viability was ≥ 80% when cells were incubated with these formulations at the all the dilutions (20, 2, 0.2 and 0.02 mg/mL of extract), and with the highest one reached ≈170%.

The ability of extract loaded vesicles to counteract the cell death caused by hydrogen peroxide was also tested (Figure 6 B and D). A549 cells stressed with hydrogen peroxide and untreated had a viability (~62%), when they were treated with the extract in dispersion, the viability was comparable ~65% ($p > 0.05$ versus viability of stressed and untreated cells). The addition of the extract loaded vesicles slightly decreased the negative effect of the hydrogen peroxide, as the viability was higher (Liposomes ~70-72%; 10PG-PEVs ~74-77%; 20PG-PEVs:~79-81%; 30PG-PEVs at 0.2 mg/mL of extract ~83-92%, $p > 0.05$ among these values) but not statistically different versus that of cells treated with the extract dispersion. Only using 30PG-PEVs at the highest dilution (0.02 mg/mL of extract), the cell viability was higher than that of cells treated with the extract ~ 93%. The protective effect of extract loaded vesicles against CuFi-1 cells was more evident according to the low extract toxicity. The viability of cells stressed with the hydrogen peroxide and untreated was lower, ~53%, than that of A549 stressed and untreated (~62%). When thy were treated with extract in dispersion the cell viability was comparable, ~54% ($p > 0.05$ versus viability of stressed and untreated cells) progressively increased when the extract was loaded in vesicles: liposomes ~61-67%; 10PG-PEVs ~63-713%; 20PG-PEVs ~83-88%; 30PG-PEVs ~92-97%.

3.7. Biocompatibility of formulations with 16HBS cells and protection against cigarette smoke

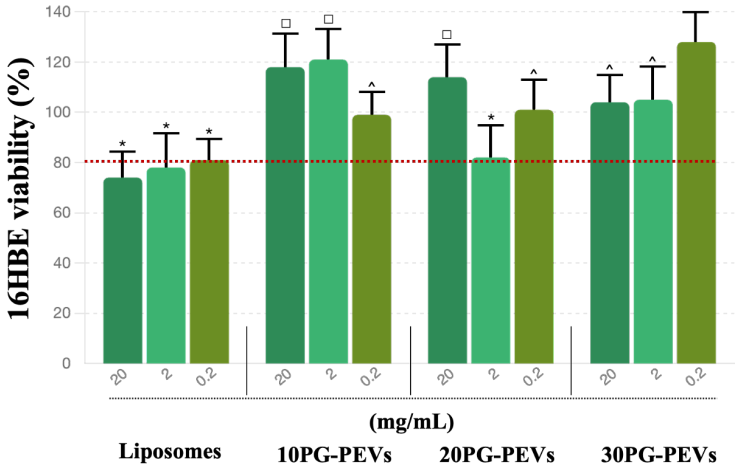

Figure 7. Cell viability of 16HBE incubated for 24 h with extract in dispersion or loaded in vesicles. Mean values ± standard deviations were reported. Symbols indicate values statistically not different (*p>0.05*).

The *in vitro* biocompatibility of extract loaded vesicles was also evaluated using bronchial epithelial cells, 16HBE. Their viability was ≈78% using the extract in dispersion and always ≥ 80% using the extract loaded vesicles, confirming their biocompatibility at the used dilutions (Figure 7). To test the protective effect of formulations, the IL-6 and ROS generated by cells stressed with cigarette smoke extract were measured ((Figure 8). When the cells were not damaged and untreated the basal value of IL-6 was ~270 pg/mL and that of ROS was ~19%. When they were damaged with cigarette smoke extract and untreated with formulations, the IL-6 become ~270 pg/mL and ROS ~34%. The protection of damaged cells with extract loaded in PG-PEVs reduced the production of IL-6, and the values become lower than the basal ones of undamaged cells, 200-150 pg/mL irrespective to the used formulation and dilution. The ROS generated in cells damaged with cigarette smoke extract and treated with the extract loaded 10PG-PEVs was higher (~35-40%) than that of untreated cells, ~34%. The values decreased up to ~35-40%, when the cells were protected with extract loaded 20PG-PEVs and up to ≈20% with extract loaded 30PG-PEVs(irrespective to the used dilution), being statistical equal to health cells, ~19%, $p < 0.01$.

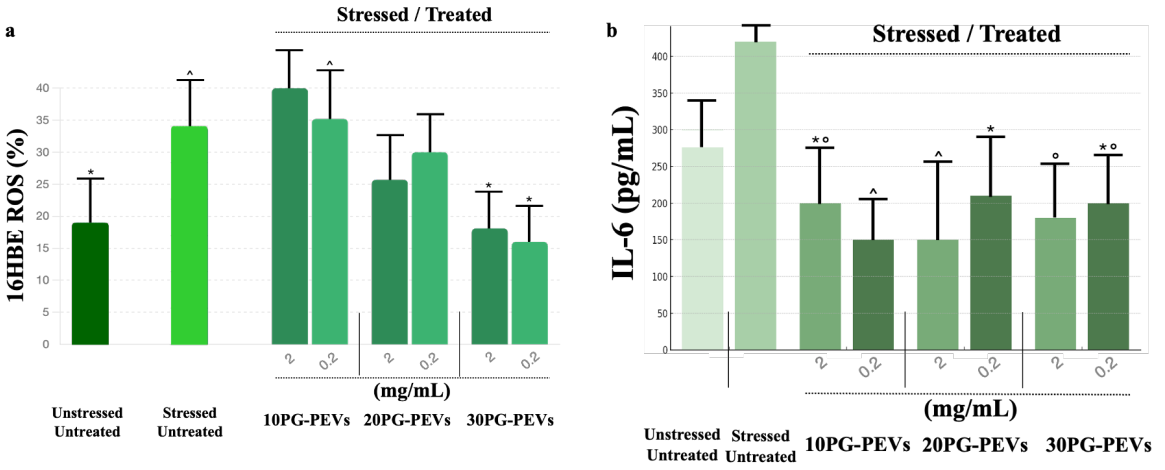

Figure 8. Amount of ROS (a) and cytokine IL-6 (b) generated by 16HBE cells unstressed or stressed with cigarette smoke extract and treated with extract in dispersion or loaded in vesicles. Mean values ± standard deviations are reported. Symbols indicate values statistically not different (*p>0.05*).

3.8. *In vivo* preliminary study

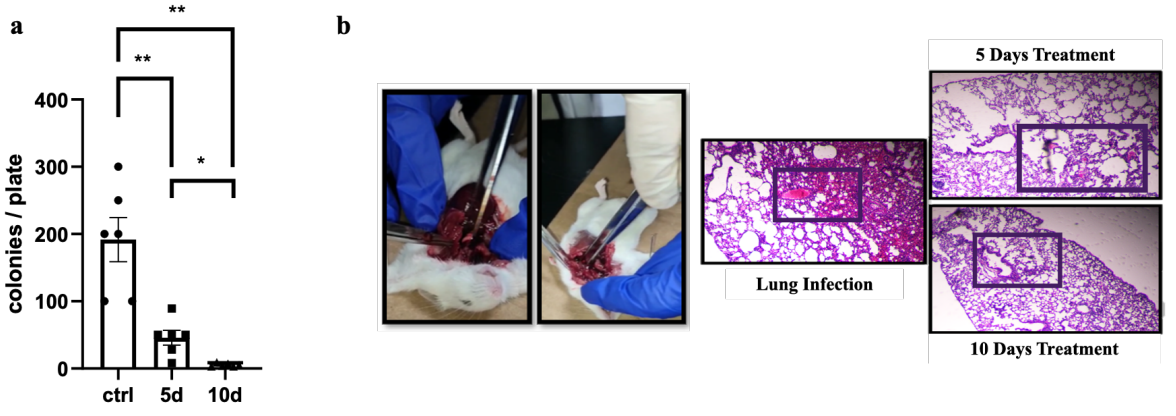

Figure 9. Representation of the *in vivo* preliminary study showing the bacterial colonies' average number differences between infected and treated mice (a), as well as lungs and trachea dissections (b).

The figure presents a comparison of the average bacterial colony counts in infected mice before and after administering two treatment dosages at 5 and 10 days. Primarily, the elevated abundance of bacterial colonies established a positive infection. Though, a significant drop in colony numbers after 5 days of treatment indicates a strong antibacterial effect, which further was intensify by day 10 (Figure 8a).

The left panels in Figure 8b, depict the surgical procedure used to induce or confirm infection in the lungs. The central image shows infected lung tissue with signs of structural damage, indicating successful infection establishment. The right panels, display lung tissue samples after

5 and 10 days of treatment, with outlined areas highlighting improvements in tissue structure. A noticeable reduction in infection markers from 5 to 10 days. These outcomes provision the hypothesis that the treatment, using resveratrol-enriched grape extract loaded in modified phospholipid vesicles, effectively reduces bacterial infection or inflammation over time.

4. Discussion

In this study, an extract antioxidant was obtained from the pomace of Lebanese red grape Asswad Karech. The extract contained several sugars and polyphenols, among which the most abundant was the resveratrol, which is well known to its antioxidant and anti-inflammatory activity and protective effects against ROS and oxidative damage. It is considered to be effective in attenuate lung oxidative injury induce by cigarette smoke, because decreased the activity of necrosis factor-κB in animals and reduced apoptosis of cells [44]. Due its important role, the extract was enriched with an additional amount of resveratrol and both were coloaded in phospholipid vesicles improved with propylene glycol, that is a water soluble polyalcohol able to facilitate the passage of small molecules through the biological membranes [45]. Propylene glycol is commonly used as safe vehicle on electronic cigarette and medical aerosols [46]. Its addition in the vesicles increased the solubility of resveratrol and extract components facilitating their encapsulation in the aqueous phase of vesicles and reducing their intercalation inside the lipid bilayer. According to this the mean diameter of vesicles decreased when the propylene glycol increased (10PG-PEVs were sized ~224 nm, 20PG-PEVs ~215 nm and 30PG-PEVs ~204 nm). As previous confirmed, 30% of propylene glycol in the water phase of vesicles contributed on the stabilization of vesicles that maintained their size unchanged during 12 months of storage and become more elastic [47]. This high stability was a key parameter that supported the application of PG-PEVs as carriers for aerosol inhalation, because during the aerosolization the formulation are sprayed and collected in the device several times, thus are subject to strong impacting force than can break their structure. Moreover, it was reported that the addition of propylene glycol in the vesicles improved the payload permeability and efficacy in different tissues [48]. In this study, their promising properties were applied to improve the deposition of resveratrol and extract antioxidants in the lung epithelium to counteract the damages caused by oxidative stress and cigarette smoke. The delivered antioxidant molecules can locally avoid and rehabilitate these impairments, particularly in individuals who are predisposed to asthmatic or severe lung dysfunction [49], [50]. The vesicle dispersions, especially 30PG-PEVs were aerosolized in higher amount that the corresponding extract in dispersions and their aerodynamic diameter was suitable for the deposition in the lungs thanks to their surface qualities [51], [52].

Biocompatibility with cells is a fundamental prerequisite needed for formulations and usually a viability ≥ 80% is required to consider them “nontoxic” [53]. Resveratrol enriched extract loaded in PG-PEVs at the higher dilutions were biocompatible with A549, CuFi and 16HB, thus suitable to be used as aerosol formulation to protect lung tissue. In particular, the viability of A549, adenocarcinoma human alveolar basal epithelial, cells incubated with 30PG-PEVs (0.02 mg/mL) reached ~122%, and that of CuFi ~184%, implying a boost in proliferation capabilities. Zerboni et al., analysed the combined *in vitro* effects of a standard diesel exhaust particles (DEP), and commercial metal oxides nanoparticles on human lung cells A549 as a model of the respiratory system. The cell viability reached maximum 110% [54]. The improved vitality obtained with

resveratrol enriched extract loaded PG-PEVs might be related to the vesicle ability to interact with cells, boosting the uptake of the antioxidant chemicals [55], [56]. The CuFi viability previously obtained Huguet-Casquero et al., was ~140% using lipid carriers [57]. Thus, resveratrol enriched extract loaded in 30PG-PEVs (0.02 mg/mL) seem to be very safe for lung tissue that they can stably reach when aerosolized. Moreover, they protect the cells from death induced by oxidative stress, that in turn has been found linked to the development of important respiratory pathologies such as pneumonia, chronic obstructive pulmonary disease and cancer genesis pathways [58], [59], [60]. The formulation counteracted the damage caused by hydrogen peroxide and avoid cell death as the viability of A549 and CuFi was ≈95%, when they were stressed with hydrogen peroxide and protected with formulation. Moreover, this formulation also effectively protected the bronchial epithelial cells (16HBE) from damage caused by cigarette smoke extract. These cells were selected since they are the primary lung barrier towards breathed atmospheric and toxic compounds, such as cigarette smoke, in addition to the initial target of inhaled medications [61]. Cells damaged with cigarette smoke and protected with resveratrol enriched extract loaded in 30PG-PEVs (0.2 mg/mL) allowed to control the mitochondrial production of ROS induced by cigarette smoke extract. Differently, in previous study it was found that resveratrol in dispersion or suspension was unable of controlling the ROS production in bronchial epithelial cells [62]. It is possible that propylene glycol targeting the CD44, cell membrane proteoglycan implicated in the attachment of cells to cellular-matrix interactions, as well as lymphocyte stimulation, can increase vesicle adherence to bronchial epithelial cells [63]. Oxidative stress induced airway inflammation by stimulating the release of inflammatory cytokines, such as IL-6 [64][65]. Resveratrol enriched extract loaded in 30PG-PEVs (2, 0.2 mg/mL) inhibited the production of this inflammatory cytokine, on epithelial bronchial cells caused by cigarette smoke exposure.

5. Conclusion

To summarize, the extract obtained from the pomace of Lebanese red grape Asswad Karech is strongly antioxidant polyphenols and the most abundant polyphenol is resveratrol. It can be co-laded with additional resveratrol in propylene glycol containing vesicles, so called PG-PEVs. The resulting vesicles, especially 30PG-PEVs were nanosized and stable, when aerosolised generated droplets suitable reach the lug alveolar tissue and are biocompatible with these cells. Additionally reduced the cell death caused by hydrogen peroxide and the ROS and IL-l production generated by cigarette smoke exposure. The capacity of these vesicles to efficiently carry active chemicals to the respiratory tract highlights their potential as an alternative to traditional medicines. The next step should focus on improving formulation and understanding the mechanisms of cellular absorption and intracellular transport, allowing for the development of more effective and tailored respiratory illness treatments.

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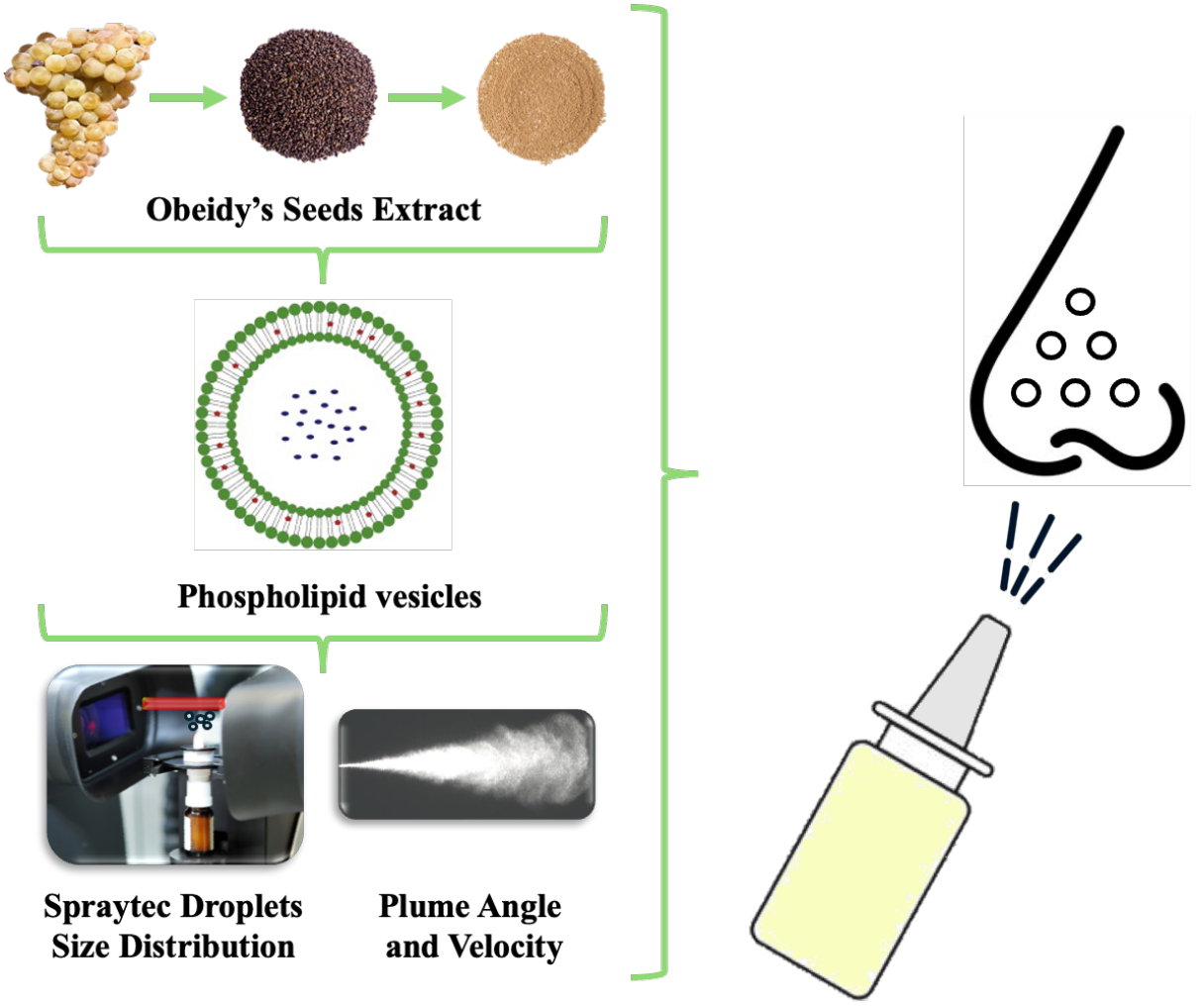
Work 2


Obeidy's Seeds Extract
Phospholipid vesicles
Spraytec Droplets Size Distribution
Plume Angle and Velocity

Research Article

Synergistic effect of antioxidant grape seed extract, thymol, and camphor loaded in carrageenan-enriched liposomes tailored for intranasal therapy

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Abstract: In this study, an extract was obtained from the seeds of ***Obeidi*** grape, a Lebanese autochthonous variety, showing antioxidant and anti-inflammatory properties. It was co-loaded with thymol and camphor in phospholipid vesicles containing propylene glycol and improved with carrageenan to also ensure an antimicrobial effect. The main physico-chemical (size, zeta potential, polydispersity index) and technological characteristics (entrapment efficiency, spray ability and regional nasal deposition) of the obtained vesicles were measured. Vesicles were highly stable as their mean diameter slightly increased (10%) only after 12 months of storage but remaining within the acceptable range. Vesicle dispersions were biocompatible and protected human lung adenocarcinoma epithelial cells (A549) and cystic fibrosis (CuFi-1) cells from damages caused by hydrogen peroxide-induced oxidative stress to a better extent than the extract in dispersion. The formulations used in this study, prevented 90% of protein denaturation and inhibited the bacterial growth of *Staphylococcus aureus* which was further evaluated in a preliminary *in vivo* study.

Keywords: Grape seeds; phospholipid vesicles; oxidative stress; nasal spray; droplet distribution; sustainability; eco-friendly technology

1. Introduction

The nose cavity is the most cephalic part of the respiratory tract, which communicates with the external environment and is responsible for the conditioning of inhaled air through its humidification, warming, and filtration [1]. This functional filtration removes harmful chemicals or microbes, protecting the more sensitive tissues in the lower tracheobronchial and pulmonary airways parenchyma. Consequently, it may also be a prime target for many inhaled toxics that cause acute, subacute or chronic rhinitis limited to the nasal cavity, sinusitis involving the paranasal sinuses, and rhinosinusitis in both sites [2]. The latter comprises a heterogeneous group of diseases affecting 5 to 15% of the population worldwide, significantly affecting the quality of life [3]. In the past, the starting cause was considered related to viral, fungal, or bacterial infection, while, recently, the inflammation model linked to immunological factors is the most accepted hypothesis [4]. Treatments such as antibiotics, anti-histaminic, and steroids are commonly prescribed to eliminate infection, reduce inflammation, and revert the diseased mucosa to normal. As an alternative to systemic formulations, nasal sprays in solutions or dispersions are commonly used to locally deliver drugs and treat acute or chronic congestions or obstructions [5]. Moreover, nasal sprays are often prepared with natural chemicals, whose therapeutic properties have been reevaluated under the light of medical and pharmacological research, pushed by a constantly growing market demand [6]. Francioso ***et al.*** found that the use of resveratrol in association with carboxymethyl-β-glucan solution as aerosol successfully reduced the spread of rhinoviruses in human nasal epithelial tissues [7], [8]. Esposito ***et al.*** demonstrated that administering a nasal spray of propolis containing polyphenols contributed to controlling the symptoms related to bacterial infections in a short time, resulting in a rapid recovery and avoiding the need for acute treatments [9]. Among the different natural

formulations, liposomes have also been used in the treatment of rhinitis due to their moisturizing and hydrating effects, as they are composed of phospholipids that naturally occur in this mucosa, making up 75% of the protective nasal surfactant layer [10]. Their application does not damage the nasal function and allows an improved mucociliary clearance. Recently, it was demonstrated that their positive effects on barrier function can be further improved using hyalurosomes, special phospholipid vesicles containing sodium hyaluronate [11]. Liposomes and hyalurosomes have been tested in nasal sprays with the double objective of improving mucosal health and delivering natural chemicals. Hyalurosomes have been used to formulate different nasal sprays to deliver an extract of *Zingiber officinale* having anti-inflammatory and antioxidant properties. These formulations, especially glycerohyalurosomes, were stable on storage, effective as antioxidants, and sprayable in the anterior part of the nasal cavity, and thus had ideal characteristics for the manufacture of natural and green nasal sprays designed for the prevention and treatment of rhinitis and rhinosinusitis. Beyond this, few studies have been performed to test the efficacy of nasal sprays containing liposomes, hyalurosomes or other phospholipid vesicles loaded with natural extracts, although several of them are valuable candidates due to their antioxidant, anti-inflammatory, and antibacterial activities. Extracts obtained from agri-food by-products deserve special attention as they are a rich and cost-effective source of beneficial molecules that align with the principles of a circular economy to address environmental concerns effectively. Among different by-products, one of the most used is grape pomace [12], [13], which is particularly rich in bioactive components such as polyphenols, anthocyanins, and flavonoids and is broadly available worldwide [14], [15]. Grape pomace consists of skin, stalks, and seeds, having the highest content in polyphenols, sugars, unsaturated lipids - particularly linoleic acid (58-78%) and oleic acid (15-20%) [16] - and saturated lipids such as palmitic acid (7-10%) and stearic acid (4-6%) [17].

According to these promising properties, in this study, an antioxidant and anti-inflammatory extract was obtained from the seeds of ***Obeidi*** grape seeds, which was co-loaded with thymol and camphor in liposomes improved with carrageenan. These natural ingredients are used in green formulations with a focus on sustainability. This methodology not only uses renewable resources, but it also supports eco-friendly technologies that are consistent with sustainable formulation practices [18]. The main physico-chemical (size, zeta potential, polydispersity index) and technological characteristics (entrapment efficiency, spray ability and airway deposition) of the obtained formulations were measured. Their biocompatibility and ability to protect human lung adenocarcinoma epithelial cells (A549) and cystic fibrosis cells (CuFi-1) from damages caused by hydrogen peroxide-induced oxidative stress were evaluated. The antibacterial and anti-inflammatory effects were also evaluated *in vitro* and preliminarily confirmed in an initial *in vivo* study using mice as animal models. These results open the way to several industrial and therapeutical applications using industrial byproducts as valuable resources of natural antioxidants.

2-. Materials & Methods

2.1. Plant Material

Chateau Saint-Thomas, a vineyard in the Lebanon Beqaa Valley, supplied the autochthonous ***Obeidi*** grape variety that was dehydrated in an airflow oven at 50 °C for 48 h. Seeds were separated from the skin, grinded, and passed through the multi-sieve to achieve particles ranging in size from 850 to 425 µm. They were placed in plastic bags and kept at ambient temperature in darkness until further processed.

2.2. Chemicals

Lipoid S75, a blend of soybean phospholipids (70% phosphatidylcholine, 9% phosphatidylethanolamine, and 3% lysophosphatidylcholine), triglycerides, and fatty acids was obtained from Lipoid (Ludwigshafen, Germany). Sigma-Aldrich (Milan, Italy) supplied ethanol, propylene glycol, and all other analytical grade products. Ultrapure water was from a Milli-Q Water System (Milford, MA). Thymol and camphor were also provided from Sigma-Aldrich (Milan, Italy).

2.3. Cell Culture Reagents

A549 (ATCC® CCL-185™) and CuFi-1 (ATCC® CRL-4013™) cells were acquired from the American Type Culture Collection (ATCC; Manassas, VA, USA). Kaighn modification of Ham F-12 with L-Glutamine, penicillin-streptomycin, foetal bovine serum, Dulbecco Phosphate Buffered Saline (DPBS) and trypsin-EDTA (0.5%) without phenol red were purchased from Gibco™ (Life Technologies, Madrid, Spain). Serum-free Bronchial Epithelial Growth Medium (BEGM Bullet Kit; CC-3170) and SingleQuot additives were purchased from Lonza (Clonetics, Lonza, Walkersville Inc.; Walkersville, MD, USA). Dimethyl sulfoxide was bought from Scharlau (Madrid, Spain). Human Placental Collagen Type IV (Sigma Cat. No. C-7521) and Cell Counting Kit-8 were purchased from Sigma-Aldrich (Saint Louis, MO, USA).

2.4. Extraction Procedure

The ***Obeidi*** extract was obtained by dispersing 0.1 g/mL of the grinded seeds in 80% ethanol. The dispersion was left for 2 h at 50 °C in a JSWB-22T digital water bath (JS Research Inc., Gongju-City, Korea). The ethanol content was removed, and the water solution was spray-dried using an automated atomizer (Shanghai Attainpak, China) to obtain powder extract.

2.5. Characterization of Extract

2.5.1. Determination of Total Phenolic Compounds

The total polyphenol content was determined with the Folin-Ciocalteu colorimetric assay [19],using gallic acid as standard. 200 µL of the extracted solution (0.1 g/mL) was put into tubes containing 1 mL Folin-Ciocalteu solution (Sigma-Aldrich, Darmstadt, Germany) and 800 µL sodium carbonate (Sigma-Aldrich, Darmstadt, Germany). The tubes were heated to 60 °C for 10 min before being cooled for another 10 min. The optical density was measured at a wavelength of 750 nm.

2.5.2. Determination of Antioxidant Activity

Diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity

The ability of the phenolic compounds to scavenge DPPH free radicals was used to determine their antioxidant activity. The DPPH solution (1450 µL, 0.06 mM) was added to 50 µL of solution of grape seed extract or Trolox (standard) [20]. The absorbance was measured at 515 nm after 30 min of incubation at room temperature in the dark. A calibration curve was obtained using different Trolox concentrations. The DPPH free radical inhibition expressed as antioxidant activity was calculated according to the equation:

$$\boldsymbol{Antioxidant\ activity}\ (\%) = \frac{(\boldsymbol{absorbance\ of\ DPPH\ solution - absorbance\ of\ sample})}{\boldsymbol{absorbance\ of\ DPPH\ solution}} \times 100$$

Cupric ion reducing antioxidant capacity assay (CUPRAC)

The total antioxidant activity of the extract was also measured using the CUPRAC test kit (Bioquochem, Asturias, Spain) by oxidation of copper (II)-neocuproine (2,9- dimethyl-1,10 phenanthroline). To 200 µL of working solution, 40 µL of the diluted extract (0.1 g/mL) or the Trolox standard were added. After 30 min of incubation at room temperature, the absorbance at 450 nm was read in a plate reader. The results were given in mM Trolox Equivalents (mM TE) [21].

Ferric reducing antioxidant power assay (FRAP)

The FRAP antioxidant capacity kit (Bioquochem, Asturias, Spain) was used to assess the ability of extract components to reduce the ferric complex at an acidic pH. To summarize, 10 µL of the diluted samples (0.1 g/mL) or the standard were mixed with 220 µL of ready-to-use FRAP reaction mixture. After 4 min of mixing with constant stirring, the absorbance was read with a plate reader at 593 nm. The antioxidant activity was measured in µM of iron (II) equivalent (Iron (II) µM) [22].

2.5.3. Acquisition of Infra-Red Spectra

The extract was analysed by Fourier-transform infrared (FTIR) spectroscopy, which shows characteristic signals between 4000-500 cm^{-1}.

2.5.4. Analysis of Extract with Gas Chromatography-Mass Spectroscopy (GC-MS)

A Shimadzu QP2020 GC-MS equipment (Kyoto, Japan) fitted with a split-split-less injector and DB5 MS fused silica column (5% phenyl, 95% polydimethylsiloxane coated 30 m × 0.25 mm capillary column, film thickness 0.25 µm) was used. To separate the components, a linear temperature program was applied at a heating rate of 7 °C /min, starting from 50 °C up to 280 °C, and the system was maintained at 280 °C for a total runtime of 74 min. The injector temperature was 260 °C with a split ratio of 20:1; the injection volume was 1 µL, and the carrier gas was helium. The source and detector temperature of mass spectroscopy was 240 °C; the interface temperature was 250 °C; the ionization energy was 70 eV; the atomic mass unit gain was –492 and offs were –67; and the scan range was 35-500 atomic mass units (amu); with a scan speed of 1666. The solvent cut was 3 min, and the data were collected from 4.5 min. The mass spectrum of each molecule was compared to the matching documented spectra in NIST and ADAMS libraries [23]. The relative retention indices of molecules were additionally validated through the comparison with published data of n-alkanes (C8-C35) from the Adam library [24]. The compounds were identified by comparing their retention times (Rt) with those of standard molecules, assessing their linear indices relative to a series of n-hydrocarbons.

2.5.5. Analysis of Extract with Liquid Chromatography-Mass Spectroscopy (LC-MS)

Extract phenols were separated and identified by ultra performance liquid chromatography (UPLC) with a SciEx equipment (Exion-UPLC, USA), equipped with Analyst 1.7 software and an LC-ESI-MS/MS-4500-QTRAP system (AB SciEx Instruments; Framingham, MA, USA). Chromatographic separation was performed at 50 °C using a ODS column (100 × 2.1 mm, 5 µm). The mobile phase was a mixture of water containing 1% formic acid (solution A) and methanol containing 1% formic acid (solution B). A gradient program was used as follows: 80% A and 20% B at time 0; 80% A and 20% B at time 1 min.; 0% A and 100% B at time 12 min.; 0% A and 100% B at time 18 min.; 80% A and 20% B at time 19 min.; 80% A and 20% B at time 22 min. The solvent

was delivered at a flow rate of 0.35 mL/min and a 5 µL injection volume. Mass spectroscopy analyses were carried out in negative ion mode, with nitrogen gas at a pressure of 60 psi serving as nebulizing and drying gas. The mass spectra were acquired over an m/z range of 100–900 amu. The calibration curves were prepared using the standard samples of the phenols and the components of the extract were calculated accordingly [25]

2.6. Preparation and Characterization of Vesicles

2.6.1. Preparation of Vesicles

To prepare propylene glycol vesicles (PGvesicles), ***Obeidi*** extract (10 mg/mL), phospholipid S75 (90 mg/mL), thymol (10 mg/mL), and camphor (10 mg/mL) were mixed and hydrated with an aqueous solution of propylene glycol (30%). To prepare carrageenan-PGvesicles, carrageenan was dispersed in the aqueous solution at 0.02 mg/mL. Dispersions were sonicated (30 cycles, 5 on and 2 off, probe amplitude 13 µm) with a Soniprep 150 sonicator (MSE; Crowley, London, UK) aiming at achieving eco-friendly formulations, with preventing solvents usage. Empty vesicles without extract were also prepared and used as a control. The vesicles were kept at 4 °C after they were prepared.

2.6.2. Evaluation of Physico-Chemical Properties

The vesicle morphology was observed by cryogenic transmission electron microscopy (cryo-TEM) in a Tecnai G2 20 Twin equipment (FEI Company; Hillsboro, OR, USA), at a voltage of 200 KeV. A sample portion (3 µL) was placed on glow-discharged 300 mesh Quantifoil TEM grids, and excess liquid was removed with filter paper. The grids were plunge-frozen into liquid ethane in a FEI Vitrobot Mark IV (Eindhoven, The Netherlands), transported to a 626 DH Single Tilt Cryo-Holder (Gatan, France), kept beneath –170 °C and finally transferred to the microscope at liquid nitrogen temperature (–196 °C).

The mean diameter and polydispersity index of vesicles were measured with a Zetasizer Ultra (Malvern Instruments; Worcestershire, UK) based on their light scattering. The zeta potential was measured with the same apparatus as a function of their electrophoretic mobility.

The vesicle dispersions (2 mL) were purified from the non-incorporated extract molecules by dialysis (Spectra/Por® membranes, 3 nm pore size, 12-14 kDa., Spectrum Laboratories Inc.; Rancho Dominguez, CA, USA) in water at room temperature for 2 h. The antioxidant activity of dispersions was measured before and after dialysis. Samples (20 µL) were dissolved in 1980 mL of DPPH methanol solution (80 µL DPPH/mL methanol) and incubated at room temperature in the dark for 30 min. The antioxidant activity was calculated as reported in section 2.5.2.

2.6.3. Stability Studies

Vesicle dispersions were stored at 4 °C in the dark for 12 months and their size and polydispersity index were measured at scheduled time points.

2.6.4. Technological Characterization of Droplet Size Distribution

Droplet size distribution was assessed by laser diffraction, placing the samples at 4 and 7 cm starting from the nozzle output and rotating 45° the spray device, according to established protocols [23]. The equipment provided D10, D50, and D90 volume diameter percentiles, corresponding to 10%, 50%, and 90% of the overall volume undersize. To quantify its breadth, the distribution span defined as (D90–D10)/D50 was further calculated.

2.6.5. Regional Deposition of Droplets

The regional deposition of vesicle dispersions was assessed with the realistic nasal replica Alberta Idealised Nasal Inlet (AINI), quantifying the amount of extract recovered in each region (vestibule, olfactory region, turbinate, and nasopharynx) (Copley). To this end, the AINI was connected to the Next Generation Impactor (Eur. Ph 7.2, Copley Scientific Ltd., Nottingham, UK) [26], [27] and a vacuum pump operating at a 7.5 mL/min flow rate, which mimics a steady and slow inhalation through a nostril [28]. Samples were transferred into a commercial device (Nasonex®) and 5 actuations were made positioning the device at 45° and 60° to the nasal replica. A glass collector was held below the inlet to collect any formulation that may have dripped out of the vestibule. After the deposition of the droplets of vesicle dispersion, each region was washed with an appropriate volume of methanol to allow for the disruption of the vesicles and the recovery of the extract. The amount of extract deposited was determined at the maximum adsorption wavelength (290 nm) using a microplate reader (Synergy 4, Synergy ™ Multi-Detection Microplate Reader, Bio-Tek Instruments, AHSI SPA, Bernareggio, Italy). Empty vesicles were used as a reference.

2.7. Determination of Anti-Inflammatory Activity

2.7.1. Inhibition of protein denaturation method

Inhibition of protein denaturation was estimated using the reported procedure with minor modifications. Different solutions for the assay procedure were prepared: test solution, test control, product control, and standard solution. All the required solutions were prepared using a pH 6.3 phosphate buffer. The samples were incubated for 20 min at 37 °C, then the temperature was raised to 50 °C and incubated for a 15-min period. After cooling, the absorbance was determined at 416 nm with a Synergy HTC multimode reader (BioTek, USA). The percent inhibition of protein denaturation was calculated using the given formula. The control represents 100% protein denaturation. Diclofenac potassium was used as a standard drug [29].

$$\text{Percent Inhibition} = 100 - \left(\frac{Abs\ of\ Test\ solution - Abs\ of\ Product\ solution}{Abs\ of\ Test\ control}\ x\ 100\right)$$

2.8. Determination of Bactericidal Activity

2.8.1. Broth dilution method

This procedure was modified from the reported literature [30]. Stock suspensions were prepared by dispersing appropriate aliquots up to 5% in 5 mL dimethyl sulfoxide (DMSO). Progressive serial dilutions of the stock suspensions were made with nutrient broth ranging from 1250 to 9.8 µg/mL *Staphylococcus aureus* bacterial strain. The bacterial suspension was standardized according to the McFarland method as described by the National Committee for Clinical Laboratories Standard [31]. One drop (50 µL) of bacterial suspension was added to the test tubes containing different concentrations of formulations up to 1 to 5×10^6 colony-forming units/mL. Test tubes were incubated at 37 °C for 24 h and their turbidity was measured. Each experiment was done in duplicate. The bacterial suspension diluted with the culture medium without the formulations was used as positive growth control, and the medium without bacterial suspension and formulations as negative growth control. Positive and negative controls contained DMSO at the same concentration as in the test samples.

2.9. In Vitro Studies with Cells

2.9.1. Culture of Cells

Human lung adenocarcinoma epithelial cells (A549) were cultured and sustained in F-12K medium enriched with 10% (v/v) foetal bovine serum, 1% penicillin and streptomycin and kept at 37 °C, 90% humidity and 5% CO_2; the cystic fibrosis (CuFi-1) cell line was cultivated in the same conditions in serum-free BEGM. Before each experiment, culture flasks were pre-coated for at least 18 h with a solution of Type IV human placental collagen (60 µg/mL), then air-dried and washed 2-3 times with DPBS.

2.9.2. Biocompatibility and Protective Effect Against Oxidative Stress of Extract-Loaded Vesicles

Cells (1×10^4) were transferred to 96-well plates for 24 h and then treated with extract, either in dispersion or loaded in vesicles, at various dilutions (10 mg/mL of extract) for 48 h. Afterwards, 100 µL of Cell Counting Kit-8 (10% final concentration) was added to each well and plates were incubated for 3 h. Optical density was measured at 570 nm with a microplate reader (Synergy 4 Reader, BioTek Instruments, AHSI S.p.A, Bernareggio, Italy). Analyses were carried out in triplicate. The viability of cells was reported relative to untreated control cells (100% viability).

To determine the ability of formulations to protect cells from oxidative stress damage, cells were seeded in 96-well plates (1×10^4 cells/well) and incubated for 24 h. The extract, in dispersion or in vesicles, diluted with medium to 10 and 1 □g/mL, was added to cells that were stressed with hydrogen peroxide (1:30,000 dilution). After 4 h of incubation, cells were washed with fresh medium, and their viability was determined using the Cell Counting Kit-8 (10% final concentration).

2.10. In Vivo Preliminary Study

Table 1. Representation of the *in vivo* study.

Type of animal	Sex / Age	Number	Treated	Control	Infected
BALB/c mice	Male: 6-9 weeks	6	-	√	-
BALB/c mice	Male: 6-9 weeks	6	-	-	√
BALB/c mice	Male: 6-9 weeks	6	1 mL inhaled formulation	-	

Staphylococcus aureus was administered to mice via nasal swab under a vertical biosafety laminar airflow, leading to observable behavioural changes, such as nasal redness and agitation, confirming the establishment of infection. To assess the efficacy of carrageenan-PGvesicles, infected mice were treated by inhaling 1 mL of the liquid formulation, after 24 hours of infection. After 5 days of giving the treatment, swabs were collected from control, infected, and treated mice, and bacterial cultures were analysed on Petri dishes under aseptic conditions to evaluate the treatment outcomes.

2.11. Statistical Analysis

All data were reported as average values and standard deviation and were calculated using GraphPad Prism 8 Statistical software. When p was > 0.05, the differences were considered non-significant. Values obtained from studies with cells were analysed using the two-way ANOVA test and the rest with Student's t-test.

3. Results

3.1. Characterization of Grape Extract

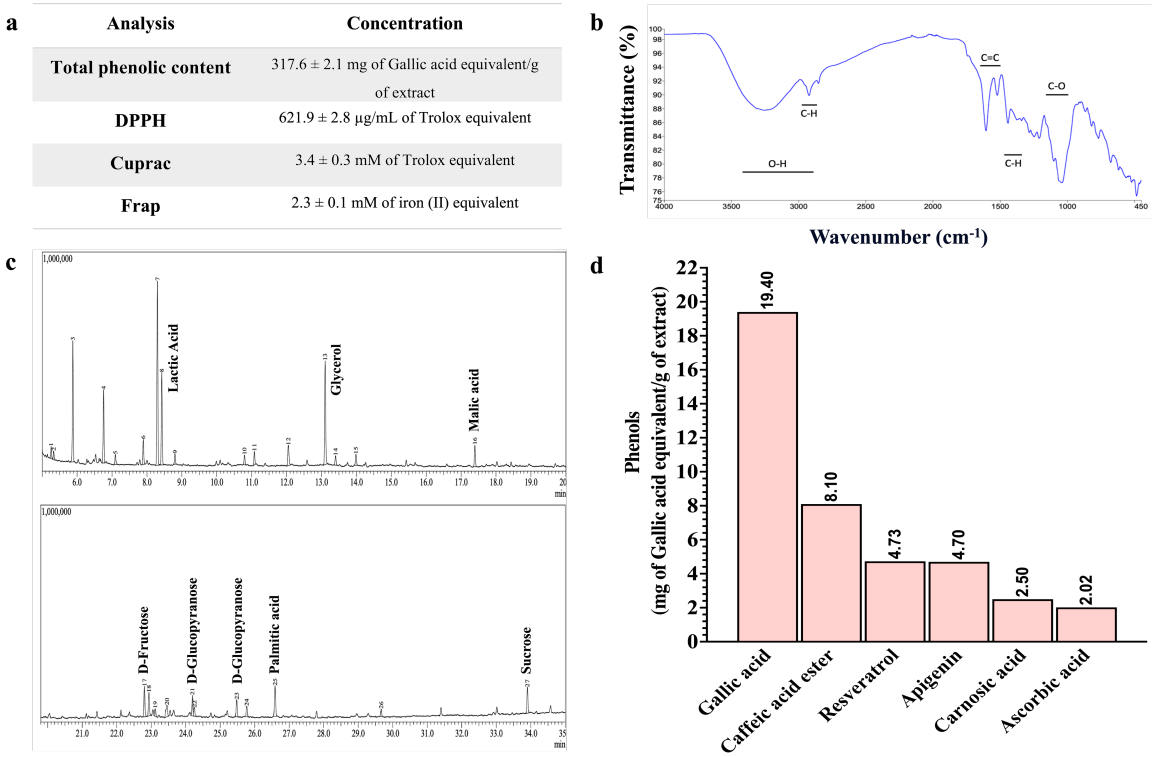

Figure 1. (**a**) Total phenolic content and antioxidant activity measured with DPPH, CUPRAC and FRAP. (**b**) FTIR spectra report wavelengths as a function of the percentage of transmittance. (**c**) GC-MS chromatogram displaying the most abundantly recognized molecules (full list in supplementary material 1). (**d**) Amount of main phenols identified in the extract by LC-MS (see the full list in supplementary material 2).

An estimation of the total antioxidant activity of the extract was performed using different chromogenic redox reagents to obtain its overall potential in different media. The extract contained an amount of phenols equivalent to 317.6 ± 2.1 mg of Trolox in 1 g of extract, indicating a good ability to scavenge free radicals in methanol by its ability to donate an electron or hydrogen atom (Figure 1a) [20]. The equivalents of Trolox measured with CUPRAC reactive (3.4 ± 0.3 mg in 1 g of extract) and those of iron assayed with FRAP (2.3 ± 0.1 mg in 1 g of extract) were lower, probably because they were measured in water solution, in which different polyphenols are less soluble than in methanol [32]. Additionally, these tests only provide a measure of the antioxidant ability to neutralize the radicals by donating electrons, while polyphenols can also transfer a hydrogen atom.

The FTIR spectra of the extract had an O-H band ranging from 3500 cm^{-1} to 2700 cm^{-1}, as well as a C-H band extending between 3050 cm^{-1} (asymmetric stretch) and 2850 cm^{-1} (symmetric stretch). Furthermore, C=C double-bond stretching was present at 1600 and 1510 cm^{-1} and substantial C-O stretching at 1030 cm^{-1}. The 1450 cm^{-1} band is a C-H asymmetric distortion (Figure 1b). Thus, the peaks of the spectra corroborate the presence of different organic molecules with unsaturated links and hydroxylic groups. HPLC data reveals that the drug was stable during storage and no degradation product was observed in the sample.

The main components were separated and identified by LC-MS/MS (Figure 1c). The most concentrated peaks were those of sugars such as fructose and sucrose, alongside with organic acids (lactic acid, malic acid, palmitic acid) and glycerol. No signs of molecule degradation were detected in the chromatogram. The peaks of phenols were less evident, but their amount could be quantified (Figure 1d). Among these, gallic acid was the most abundant (19.40 μg/g), followed by caffeic acid ester (8.10 μg/g), resveratrol (4.73 μg/g), and apigenin (4.70 μg/g).

3.2. Characterization of Vesicles

The extract of the seeds separated from the pomace of ***Obeidi*** was co-loaded with camphor and thymol in PGvesicles and carrageenan-PGvesicles. The dispersions were observed by cryo-TEM to confirm the formation of vesicles and their structure (Figure 2a). Irrespective of the presence of carrageenan, mostly unilamellar spherical structures were present. Still, in some cases, tangled lamellae inside the vesicles were observed, likely because of the presence of multiple active components that can stimulate localized disruptions in the lipid bilayers while maintaining a low polydispersity. Mean diameter, polydispersity, zeta potential and entrapment efficiency of the vesicles were measured (Figure 2b). The average diameter of PGvesicles was ~177 nm and that of carrageenan-PGvesicles was slightly bigger (~193 nm). Both formulations were homogeneously dispersed as the polydispersity index ranged from 0.25 to 0.27. The zeta potential was substantially negative, ~ –62 mV, due to the negative charge of phosphatidylcholine and the presence of propylene glycol [33], [34]. The entrapment efficiency of extract inside the vesicles was ~99%, irrespective of the presence of carrageenan.

Table 2. Mean diameter (MD), polydispersity index (PI), zeta potential (ZP), entrapment efficiency (EE), and antioxidant activity (AA) of extract-loaded PGvesicles and carrageenan-PGvesicles. Mean values ± standard deviations are reported.

	MD (nm)	PI	ZP (mV)	EE (%)	AA (%)
PGvesicles	177±5	0.25±0.03	-63	90±0.9	85±0.7
Carrageenan-PGvesicles	194±6	0.27±0.02	-60	87±1.1	88±0.5

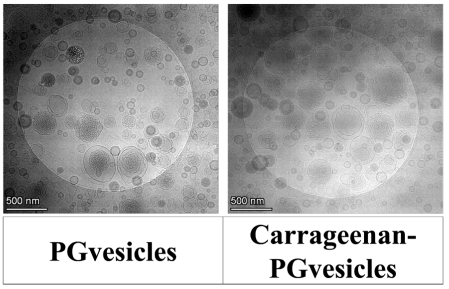

Figure 2. Cryo-TEM images of extract-loaded PGvesicles and carrageenan-PGvesicles.

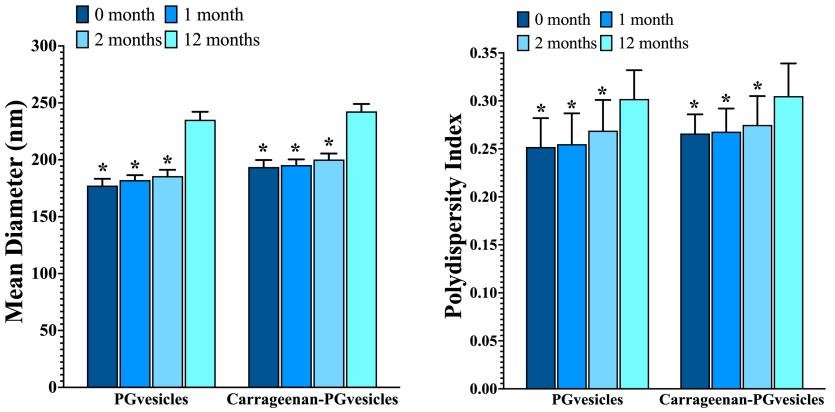

Figure 3. Mean diameter and polydispersity index of extract-loaded PGvesicles and carrageenan-PGvesicles measured for up to 12 months. Mean values ± standard deviations (bars) are reported. Symbols represent values statistically not different corresponding to each month of stability (*p>0.05*).

The antioxidant activity of the dispersions was ~87%, irrespective of the used vesicles. To evaluate the stability of vesicles in dispersion, their mean diameter and polydispersity index were measured at scheduled time points up to 12 months (Figure 3). The mean diameter was stable up to 2 months and slightly increased to ~240 nm at 12 months, similarly, the polydispersity index remained stable at first, and after 12 months reached 0.3.

3.3. Determination of Droplet Size Distribution

To prevent inhalation in the lungs of sprayed formulations, the Food and Drug Administration (FDA) and European Medicines Agency recommend that these must form droplets larger than 10 µm [35]. Indeed, nasal sprays typically form particles larger than 120 µm that are primarily dispersed in the nose anterior region [36]. Thus, the effectiveness of vesicle dispersions to be deposited into the nose was evaluated by measuring the size of droplets generated by the spray (Table 2). In compliance with the recommendations of the FDA, measures were taken at 4 cm and 7 cm from the nozzle exit, having a pump apparatus rotated 45° relative to the laser beam [37]. The size of 50% of droplets (Dv50) generated was lower than 120 µm (70 µm at 4 cm and 65 µm at 7 cm by the PGvesicles and 73 µm at 4 cm and 69 µm at 7 cm from by the carrageenan-PGvesicles). However, for 90% of droplets generated by carrageenan-PGvesicles, the size was 118 µm at 4 cm and 135 µm at 7 cm, thus with appropriate dimensions for deposition in the anterior portion of the nose.

Table 3. Size distribution of droplets generated by extract-loaded PGvesicles and carrageenan-PGvesicles sprayed 4 cm and 7 cm from the laser beam. Dv(10), Dv(50) and Dv(90) indicate, respectively, 10%, 50% and 90% of droplets.

PGvesicles

Distance: 4 cm		Distance: 7 cm	
Dv(10)	50 µm	Dv(10)	55 µm
Dv(50)	70 µm	Dv(50)	65 µm
Dv(90)	96 µm	Dv(90)	106 µm
Span	0.724	Span	0.823

Carrageenan-PGvesicles

Distance: 4 cm		Distance: 7 cm	
Dv(10)	49 µm	Dv(10)	52 µm
Dv(50)	73 µm	Dv(50)	69 µm
Dv(90)	118 µm	Dv(90)	135 µm
Span	0,467	Span	0,421

3.4. Regional Droplet Deposition

The formulations were sprayed on the Next Generation Impactor coupled with the AINI realistic nasal replica, and the amount of extract recovered in each region was measured (Figure 6). The device was positioned at 45° and 60° relative to the nasal replica. Irrespective of the used angle, the extract did not reach the lung mimic region, and the main amount of extract was found in the vestibules and turbinate mimic areas. Using both formulations, the amount deposited was higher at 45° than at 60°. Consequently, the 45° angle would allow the highest extract deposition in the anterior and posterior portions of the nose and especially in the vestibular region.

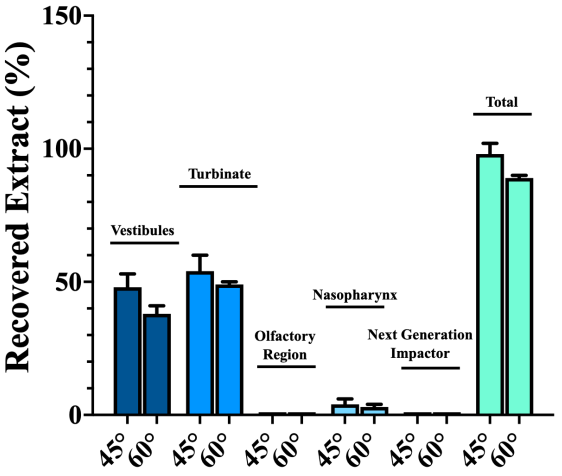

Figure 4. Amount of extract loaded in PGvesicles and carrageenan-PGvesicles and deposited in the different regions of the Next Generation Impactor coupled with the AINI realistic nasal replica (vestibules, turbinate, olfactory region, nasopharynx, lungs). Mean values ± standard deviations are reported.

*3.5. Biocompatibility and Protective Effect Against Oxidative Stress of **Obeidi** Extract-Loaded Vesicles*

The biocompatibility of vesicles was evaluated in an *in vitro* study using two different cell lines that were chosen due to their similarity in morphology and functions to the airway epithelial cells: human lung adenocarcinoma epithelial cells (A549) and cystic fibrosis (CuFi-1) cells (Figure 4). The viability of both increased inversely to the extract concentration, either in dispersion or loaded in vesicles. When A549 were incubated with the extract in dispersion at the higher concentration (10 µg/mL), cell viability was ~42% and increased up to ~100% using the lower concentration (0.01µg/mL). When incubated with extract-loaded PGvesicles at the higher concentration (10 µg/mL) the viability was ~60% and increased up to ~117% using the lower concentration (0.01µg/mL). Using the extract-loaded carrageenan-PGvesicles, cell viability further increased up to 134% at the lower extract concentration (0.1µg/mL), indicating that the extract toxicity decreased using the vesicles. CuFi-1 cells were less sensitive to the extract, since when it was used at the higher concentration (10 µg/mL), cell viability was ~60% and increased up to ~110% using the lower concentration (0.01µg/mL). Similarly, at the same concentration, cell viability was higher when the extract was loaded in PGvesicles (~139%) and further improved in carrageenan-PGvesicles (~160%).

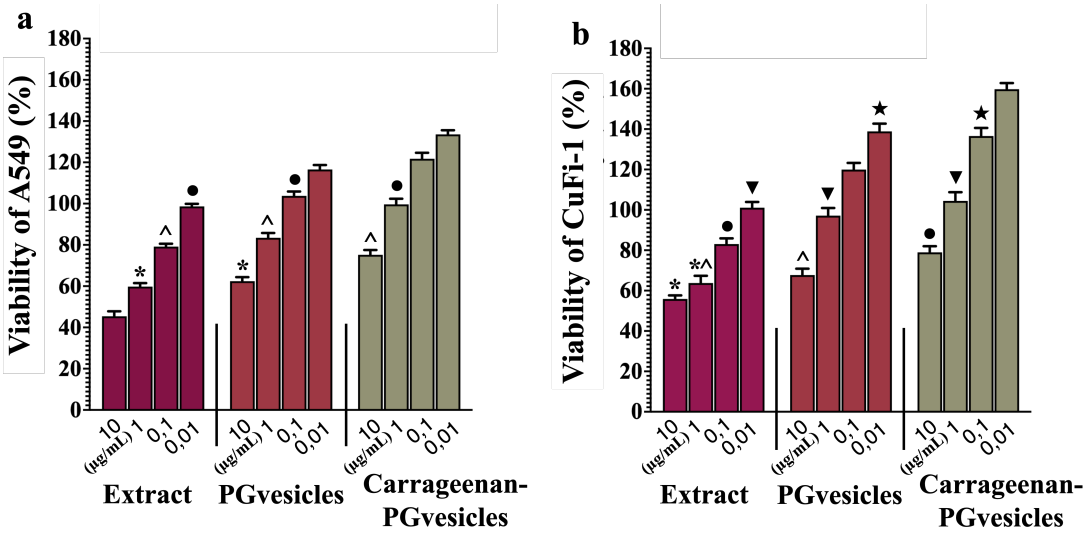

Figure 5. Viability of (**a**) A549 epithelial cells and (**b**) CuFi-1 cystic fibrosis cells incubated for 48 h with extract in aqueous dispersion or loaded in PGvesicles and carrageenan-PGvesicles. Data (bars) are reported as mean values ± standard deviations of cell viability, expressed as the percentage of control (untreated cells, 100%). Symbols indicate values that are statistically not different (*p>0.05*).

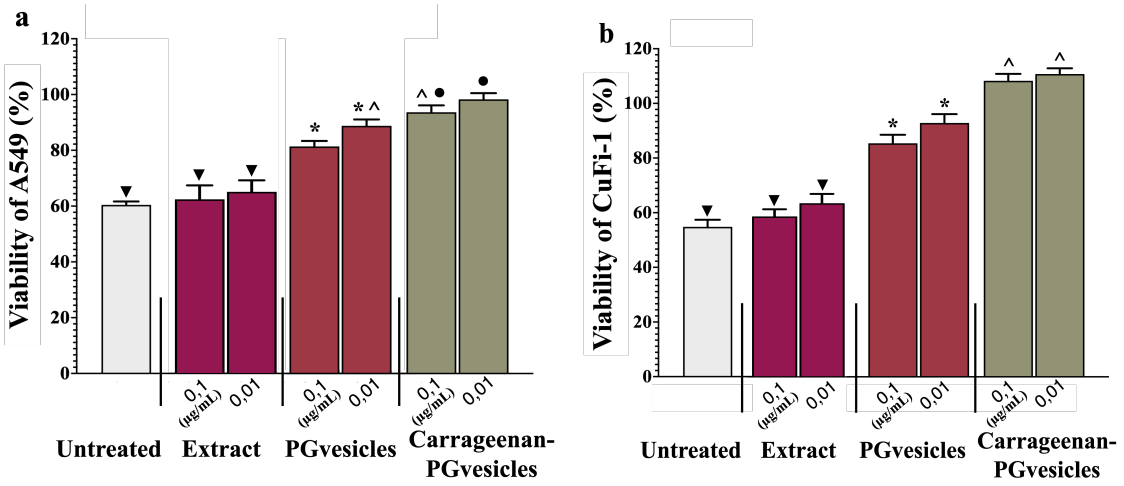

Figure 6. Viability of (**a**) A549 epithelial cells and (**b**) CuFi-1 cystic fibrosis cells stressed with hydrogen peroxide and treated with extract in aqueous dispersion or loaded in PGvesicles and carrageenan-PGvesicles. Data (bars) are reported as mean values ± standard deviations of cell viability, expressed as the percentage of control (untreated cells, 100%). Symbols indicate values that are statistically not different (*p>0.05*).

When cells were stressed with hydrogen peroxide, the viability of A549 was ~60% and that of CuFi-1 was ~57% (Figure 5). The protective treatment with vesicles at the lower and non-toxic concentration (0.01 µg/mL) allowed an increase in their viability as the damages caused in cells by hydrogen peroxide were counteracted. This increase was affected by the cell type, formulation, and concentration. Specifically, the viability of A549 treated with extract-loaded PGvesicles was ~89%, and with carrageenan-PGvesicles ~98%. The viability of CuFi-1 treated with extract-loaded PGvesicles was ~93% and with carrageenan-PGvesicles ~110%. These results indicated that extract, when loaded in PGvesicles and especially in carrageenan-PGvesicles, counteracted the damage caused in cells by hydrogen peroxide.

3.6. Antibacterial Activity and Protein Denaturation Inhibition

The antibacterial activity of ***Obeidi*** seeds extract in aqueous dispersion or loaded in PGvesicles and carrageenan-PGvesicles was also tested. Carrageenan-PGvesicles inhibited bacterial growth with an IC50 of ~937.5 µg/mL. The antibacterial activity was related to the addition of camphor and thymol in the vesicles. The extract encapsulated in liposomes showed enhanced antibacterial efficacy, reducing the minimum inhibitory concentration (MIC) [38]. As for the anti-inflammatory activity, our results demonstrate that using carrageenan-PGvesicles offers better results. The inhibition of the protein denaturation was around 90% using 4 to 8 µg/mL.

3.7. In Vivo Preliminary Study

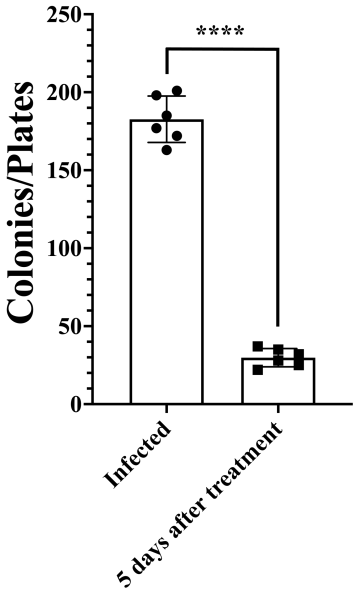

Figure 7. Representation of the *in vivo* preliminary study showing the bacterial colonies' average number differences between infected and treated mice.

The data in the figure illustrates a comparison between the average number of bacterial colonies in infected mice and the results after administrating the treatment. Initially, the bacterial colonies in the infected mice were abundant, indicating the successful establishment of the infection. However, after 5 days of applying the treatment, the considerable decrease in colony numbers points to a robust antibacterial effect of the treatment. This outcome supports the hypothesis that the treatment, involving the use of carrageenan-PGvesicles, significantly curbed bacterial growth.

4. Discussion

Liposomes are composed of phospholipids that also constitute 75% of the natural surfactant layer of the nose mucosa [39]. Thus, these vesicles provide a double advantage in the local nasal administration due to their composition and delivery performances and seem ideal carriers for nasal protection. Indeed, liposomes have been used on the inflamed nasal mucosa to alleviate the symptoms of seasonal allergic rhinitis [40]. Currently, a liposome-based spray is commercially available in Germany and a spray containing hyalurosomes (phospholipid vesicles immobilized with hyaluronate) is present on the Italian market [41]. A nasal spray containing phospholipid vesicles enriched with gelatine or chondroitin sulphate loading *Cardiospermum halicacabum* extract tested *in vitro* could protect the epithelial cells against oxidative damage, thus preventing the inflammatory state [42], adding to that, another study carried out by Al-Samydai ***et al,*** where they added phenolic compounds (gallic acid and quercetin) to their liposome formulation, proved effective acting against oxidative damage [42]. Considering these promising findings, in the present study, phospholipid vesicles were enriched with carrageenan, a natural gelling polymer, which produces a thick barrier in the nasal mucosa. Carrageenan prevents infectious viruses from binding the mucosal cells, thus reducing the release of freshly produced viral particles, and it is

expected to ameliorate the vesicle antimicrobial effect [43]. Building on previous studies, an antioxidant extract was loaded in vesicles to control oxidative conditions, which negatively impact the nasal mucosa. Specifically, the extract was obtained from ***Obeidi*** grape seeds and had a powerful antioxidant action, due to its elevated load of phenolic compounds (e.g., gallic acid, resveratrol), as already reported in *in vitro* studies by Gibis ***et al.*** and Delgado Adámez ***et al.*** [44], [45]. To simultaneously improve the antimicrobial activity of the formulations, camphor and thymol were co-loaded into the vesicles. The versatility of phospholipid vesicles were further improved with propylene glycol, allowing the loading of three different payloads (extract, camphor, and thymol), which exerted complementary effects. The resulting PGvesicles were sized ~177 nm and carrageenan-PGvesicles ~194 nm, both formulations being withing PI in accepted range and negatively charged. These characteristics are consistent with previous results by Manca ***et al.***, where an extract from grape pomace seeds was loaded in vesicles that were monodisperse and negatively charged (–74 to –81 mV). After 12 months of storage their size slightly increased (10%) but the samples remained monodispersed, confirming that only some internal rearrangements occurred [46]. The small vesicle size also confirmed their suitability for nasal delivery, as sizes from 100 to 200 nm are considered optimal for bypassing mucosal limitations and enhancing absorption in the nasal cavity [47], [48]. The spray ability of vesicle dispersions into the respiratory airways was also assessed quantifying the mean diameter of droplets formed [49], [50]. The spraying instrument produced a conical shape, known as a plume, of aerosolized droplets, which based on their size would be mainly deposited in the nasal cavity. Usually, droplets larger than 10 µm can reach the nasal cavity and those larger than 120 µm tend to settle in the anterior region of the nose, creating a regional impact [51], [52]. The dispersions of PGvesicles and carrageen-PGvesicles generated 90% of droplets sized between 118-135 µm, confirming their suitability for nasal spraying and possible deposition in the anterior nasal cavity, where they can exert their action against local allergies and congestion [53]. These positive results were further supported by the amount of extract recovered in each stage of the nasal path using a realistic nasal replica. The main fraction of the extract was recovered in the middle upper section, corresponding to the nasal cavity, while it was not found in the deeper stages, corresponding to deeper airways, according to a previous study by Seifelnasr ***et al.*** [54]. The loading of the extract, camphor and thymol in vesicles increased their biocompatibility versus the dispersed formulation and enhanced their capability to stop the oxidative damage caused by hydrogen peroxide. Similarly, Diaz ***et al.*** found that lipid-encapsulated grape tannins loading grape polyphenols significantly reduced the oxidative damage of cells, reducing the level of intracellular reactive oxygen species by 38% [55], adding to that, Matalqah ***et al.*** talked about the effectiveness of solid lipid nanoparticles in providing a suitable treatment encapsulation, contributing to a controlled drug release and stability [56]. Vesicle formulations increased the payload ability to almost completely avoid (90%) protein denaturation, indicating a possible anti-inflammatory effect. Moreover, vesicle dispersions exerted an antibacterial effect as they inhibited the growth of *Staphylococcus aureus.* This synergistic effect can be likely related especially to camphor and thymol co-loaded in the vesicles, and to carrageenan as well, whose mucoadhesive properties can prolong the residence time of vesicles in the nasal cavity [57]. Regarding the *in vivo* preliminary study, the drastic reduction in bacterial colonies could be attributed to several factors, including the mechanism of action of the treatment, such as disrupting bacterial cell walls, inhibiting bacterial replication, or enhancing the mice's immune response to eliminate the infection. The results provide strong evidence for the efficacy of the treatment.

It is essential to note, however, that while the results are promising, additional studies would be necessary to confirm the consistency of these findings over a longer period or across different bacterial strains. Future experiments should also explore the potential for resistance development, dosage optimization, and potential side effects of the treatment. To sum up, the marked decrease in bacterial colonies after 5 days underscores the potential effectiveness of the treatment in managing infections, providing a appropriate foundation for further investigation into its clinical applicability.

5. Conclusions

The overall results of the present study demonstrate that the extract obtained from ***Obeidi's*** seeds, a Lebanese autochthonous variety is rich in polyphenols and strongly antioxidant. It is possible to co-load this extract with camphor and thymol in propylene glycol-containing phospholipid vesicles, which are stable and small. Vesicles can be further improved by adding carrageenan, which is mucoadhesive, has an antimicrobial effect and can synergistically enhance the formulation efficacy. Vesicle dispersions, especially those enriched with carrageenan, deposited in a mimic nasal cavity without reaching the deeper airways, were biocompatible, protected the cells from oxidative stress, avoided protein denaturation and inhibited the growth of *Staphylococcus aureus.* Thus, these formulations seem convenient for the local treatment of rhinitis preventing the use of systemic therapies. Our study highlights as well the importance of industrial food waste valorisation through the extraction of natural antioxidants compounds and their use as potential therapeutic agents.

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