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Enhancement of *in vivo* human skin penetration of resveratrol by chitosan-coated lipid microparticles

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Highlights

- Resveratrol as a therapeutic agent for the treatment of several skin diseases
- Resveratrol inefficient percutaneous penetration
- Encapsulation of resveratrol in lipid microparticles for skin delivery
- Uncoated- and chitosan-coated lipid microparticles for resveratrol topical administration
- Evaluation of *in vivo* skin permeation by the tape stripping technique

Abstract

In this study, lipid microparticles (LMs) uncoated or coated with chitosan, and containing the antioxidant polyphenol, resveratrol were developed in order to enhance its *in vivo* skin permeation. The LMs loaded with resveratrol were prepared by melt emulsification and sonication, using tristearin as lipidic material and hydrogenated phosphatidylcholine as the surfactant. Two different methods were examined for the coating of the LMs: chitosan addition during LM preparation or treatment of already formed LMs with a chitosan solution. The latter method achieved a better modulation of the *in vitro* release of resveratrol and hence was used for subsequent studies.

The resveratrol loading and mean diameter of the LMs were $4.1 \pm 0.3\%$ (w/w) and $5.7 \mu\text{m}$ and $3.8 \pm 0.2\%$ (w/w) and $6.1 \mu\text{m}$ for the uncoated and the chitosan-coated LMs, respectively. Chitosan coating changed the LM surface charge, from a negative zeta potential value ($-17.8 \pm 4.8 \text{ mV}$) for the uncoated particles, to a higher positive values ($+64.2 \pm 4.4 \text{ mV}$) for the chitosan-coated ones.

Creams containing resveratrol free, encapsulated in the uncoated or chitosan-coated LMs were applied to the forearm of human volunteers and the penetration of the polyphenol in the stratum corneum was investigated *in vivo* by the tape stripping technique. Uncoated LMs did not produce any significant increase in the fraction of the applied resveratrol dose diffused in the stratum corneum ($32.8 \pm 8.9\%$) compared to the control cream containing the non-encapsulated polyphenol ($26.2 \pm 5.6\%$ of the applied dose). On the other hand, application of the cream containing the chitosan-coated LMs produced a significant enhancement in the *in vivo* permeation of resveratrol to $49.3 \pm 5.9\%$ of the applied dose, the effect being more marked in the upper region of the horny layer. The observed improvement in the human stratum corneum penetration of resveratrol achieved by the LMs coated with chitosan should favour the efficiency of its topical application.

Keywords: Resveratrol; Skin penetration; Lipid microparticles; Chitosan-coating; Tape stripping; In vivo stratum corneum penetration profile

1. Introduction

Resveratrol (3,5,4'-trihydroxystilbene), a naturally occurring polyphenolic compound produced by a variety of plants [1,2], is a potent antioxidant which has been shown to exhibit anti-inflammatory, anti-carcinogenic and anti-microbial properties, together with cardioprotective and neuroprotective activities [1,3,4]. In addition to these array of beneficial pharmacological effects, the topical application of resveratrol has been reported to exert a therapeutic action against several cutaneous pathologies such as oxidative damage (e.g., oxidative DNA damage, lipid peroxidation) mediated by excessive formation of free radicals and other reactive species (non-radical oxygen species), inflammation, microbial infections, skin cancer and damage induced by the solar UV radiation [5-10]. In particular, in vitro and in vivo investigations on animal skin have demonstrated that resveratrol reduced tumorogenesis, inhibited inflammatory leukocyte infiltration as well as the increase in collagen degradation and lipid peroxidation triggered by exposure to the sun UV rays [5,7,9]. Moreover, resveratrol has been shown to possess antiproliferative properties against skin carcinogenesis on human epidermal cell lines [9].

For the treatment of skin disorders, the topical administration of resveratrol provides its direct delivery to the area of interest, circumventing the problem of poor bioavailability due to its rapid metabolism and limited aqueous solubility [9, 11,12]. However, the activity of this polyphenol following skin application is hampered by its inefficient percutaneous penetration and chemical instability [4,11, 13-15]. In order to circumvent the above limitations, incorporation of resveratrol in novel carrier systems including liposomes [4,10,13,16] and lipid nanoparticles [1,15,17] has been reported. The effect of these systems on the skin absorption of resveratrol has been evaluated in vitro on excised animal skin mounted in Franz diffusion cells [1,4,10,13,15,16], these conditions being not representative of the actual application of dermatological products in man. Hence, although the in vitro testing represents a useful model for the prediction of

percutaneous penetration, for realistic and conclusive information, *in vivo* studies in humans are required [18,19].

Aim of this work was to evaluate whether the incorporation of resveratrol in lipid-based microparticles, prepared in the absence or presence of chitosan, could enhance the *in vivo* skin permeation of this polyphenol. Lipid microparticles (LMs), which consist of a solid hydrophobic core stabilized by a layer of surfactant molecules, represent an appropriate carrier for topical administration since their constituents are physiologically compatible and biodegradable. Compared to liposomes and lipid nanoparticles, LMs due to their micron dimensions have the advantage of higher stability and reduced amount of potentially toxic surfactants required for their preparation [20-22]. Moreover, LMs exhibit high loading capacity for lipophilic substances, such as resveratrol, and their solid matrix protects the encapsulated compound against degradation [20,21]. In order to improve the performance of the LMs as topical delivery system for resveratrol, their surface characteristics were modified by coating with chitosan, a biocompatible and non-toxic cationic polysaccharide of natural origin which possess absorption enhancing properties and has been shown to increase skin permeation [23-25].

LMs without or with chitosan coating and loaded with resveratrol were prepared, characterized, introduced in a model cream formulation and their effect on the polyphenol skin penetration was assessed *in vivo* on human volunteers using the non-invasive tape stripping technique.

2. Materials and methods

2.1. Materials

Resveratrol was supplied by Fagron Italia (Bologna, Italy). Glyceryl behenate was a gift from Gattefosse' (Cedex, France). Stearic acid and Tween 20 were purchased from Fluka Chemie (Bucks, Switzerland). Hydrogenated phosphatidylcholine was received as a free sample from Cargill (Hamburg, Germany). Tristearin, low- and medium- molecular weight chitosan, high-performance liquid chromatographic (HPLC)-grade methanol and water were from Sigma-Aldrich (Steinheim, Germany). The excipients for the cream

preparations were obtained from Croda (Snaith, UK), Seppic (Paris, France) and Fagron Italia (Bologna, Italy). Transparent adhesive tapes (Scotch Crystal 600, 19 mm width) were purchased from 3M (Cergy-Pontoise, France). All other reagents and solvents were of analytical grade from Sigma.

2.2. High-performance liquid chromatography

The HPLC system comprised a Model LabFlow 3000 pump (LabService Analytica, Bologna, Italy), a Model 7125 injection valve with a 20 μ l sample loop (Rheodyne, Cotati, CA, USA) and a Model 975-UV variable wavelength UV-vis detector (Jasco, Tokyo, Japan) set at 306 nm. Data acquisition and processing were performed with a personal computer using Borwin software (JBMS Developpements, Le Fontanil, France). Sample injections were performed with Model 80365 syringe (10 μ l; Hamilton, Bonaduz, Switzerland). Separations were achieved on a 5- μ m Zorbax SB-C18 column (150 mm x 4.6 mm i.d.; Agilent Technologies, Waldbronn, Germany) fitted with a guard column (5- μ m particles, 4 mm x 2 mm i.d.; Phenomenex, Torrance, CA, USA) and eluted isocratically, at a flow-rate of 0.8 ml/min, with methanol-water (65:35, v/v) containing 0.4% (v/v) acetic acid. Chromatography was performed at ambient temperature. The identity of resveratrol peak was assigned by co-chromatography with the authentic standard. Quantification was carried out by integration of the peak areas using the external standardization method. Calibration curves were linear over the range 0.2- 40.0 μ g/ml, with correlation coefficients greater than 0.998. The precision of the method, evaluated by repeated analyses (n=6) of the same sample solution containing resveratrol at levels of 0.2 and 40.0 μ g/ml, was demonstrated by relative standard deviation values lower than 5.0%.

2.3. Microparticle preparation

LMs were prepared by adding pre-heated (75-85 $^{\circ}$ C) deionized water (40 ml) containing the surfactant (0.7-1.4 %, w/v) to the molten lipid phase (3.8 g), in which resveratrol (0.25-0.30 g) was dispersed. The hot aqueous phase was poured into the molten lipid (phase-inversion process) to avoid loss of excipient and drug during the preparation process. The mixture was then subjected to high-shear mixing (17500 rpm for 1-2 min) using an Ultra-Turrax T25 mixer (IKA-Werk, Staufen, Germany) at 75-85 $^{\circ}$ C. For the optimized preparations, the samples were also sonicated at 20 kHz for 5 min

(power output, 10 W), using an ultrasonic probe (Model VCX130; Sonix, Newtown, CT, USA). The resulting oil-in-water emulsion was rapidly cooled at room temperature under magnetic stirring and the formed suspension was subjected to centrifugation (6000 rpm for 15 min) and lyophilization to obtain water-free microparticles.

For the preparation of the chitosan-coated LMs a 1% (w/v) chitosan solution (40 ml) in aqueous acetic acid (pH 4.2) including the surfactant was used as the water phase of the emulsion formulation. Alternatively, the chitosan solution was added to the LM suspension formed during the cooling phase of the emulsion.

Unloaded particles were also prepared with the same procedures, by omitting resveratrol.

2.4. Microparticle characterization

2.4.1. Particle morphology

The morphological features of the LMs were observed by optical microscopy (B-500 TPL microscope, Optika Microscopes, Bergamo, Italy) and variable-pressure (ca. 90 Pa) scanning electron microscopy (VP-SEM; Zeiss EVO40XVP, Arese, Milan, Italy).

2.4.2. Particle size analysis

Preliminary estimation of particle dimensions was performed by computerized image analysis (MicrometricsTM camera 122CU and software vision 2.02) on a minimum of 100 particles using the B-500 TPL optical microscope (Optika Microscopes, Bergamo, Italy). The particle size (expressed as mean diameter), polydispersity index (PDI) and surface charge were determined by dynamic light scattering using a Zetasizer (Model 6.12; Malvern Instruments, Worcs, UK) equipped with a 4 mW He-Ne laser (633 nm) and a DTS software (Version 5.0). The particles were dispersed in Milli-Q water (18.2 MO, Millipore) at a concentration of ca. 5 mg/ml. Analyses were performed in triplicate.

2.4.3. Resveratrol loading of LMs

The amount of resveratrol entrapped in the LMs was determined by dissolving the microparticles (10 mg) in ethanol (5 ml) under heating (80 °C for 5 min) and sonication (10 min), in sealed glass vials. The obtained sample was diluted to volume (20 ml) with

methanol, filtered (0.45 μm membrane filters) and assayed by HPLC. The encapsulation efficiency was calculated as the percentage ratio between the quantity of resveratrol entrapped in the microparticles and the amount of polyphenol initially added to the melted lipid phase. The results were the average of at least three determinations.

2.4.4. *In vitro* dissolution

Resveratrol dissolution and release from the LMs were assessed by adding resveratrol (ca. 0.8 mg) or LMs, containing an equivalent amount of the polyphenol, to 100 ml of phosphate buffer (0.05 M, pH 7.4), containing Tween 20 (0.5%, w/w) as solubilizer to ensure sink conditions [14]. The samples were kept under mechanical stirring at 50 rpm and 32 °C. At appropriate time intervals, 1-ml aliquots of the medium were withdrawn and replaced with an equal volume of fresh fluid. The test samples were filtered (0.45 μm) and assayed for resveratrol by HPLC, as outlined above. The polyphenol release (%) was calculated from the total resveratrol content of each LM preparation. This was determined by extraction of the particles, after the release experiment, using the method described in Section 2.4.3. A minimum of 5 replicates were performed for each formulation.

2.5. *Cream formulations*

Creams (oil-in-water emulsions) containing non-encapsulated resveratrol (0.5%, w/w) in conjunction with blank LMs or an equivalent amount of the polyphenol incorporated in LMs were prepared. The emulsion excipients were: ceteryl isononanoate (7.5%), Phenonip[®] (0.8%; phenoxyethanol and parabens), benzyl alcohol (0.5%) and Montanov[™] 82 (5.0%; ceteryl alcohol and coco-glucoside) or Polawax[®] (4.0%; emulsifying wax based on ceteryl alcohol and ethoxylated fatty acid esters of sorbitan) for the internal phase and glycerin (3.0%), EDTA (0.1%), ethanol (2.5%) and deionized water (qs 100%) for the external phase. The creams were prepared according to the common procedure used in compounding practice. Briefly, the oil- and aqueous-soluble components were separately heated at about 70 °C and the aqueous phase was added to the oil phase while mixing with an Ultra-Turrax at 7000 rpm for 2 min. Resveratrol (solubilized in ethanol) and the LMs (14.5 g per 100 g of cream, dispersed in water) were added in the cooling phase of the cream preparation at about 35°C, under gentle stirring.

2.6. *In vitro* release studies

These studies were performed in Franz-type glass diffusion cells with a cross sectional surface area of 2.5 cm² and a receptor compartment volume of 20.4 ml. Cellulose acetate membranes (pore diameter, 0.20 µm) were inserted between the donor and receptor compartment. The receptor chamber was filled with phosphate buffer (0.05 M, pH 7.4) containing Tween 20 (0.5%, w/w) as solubilizer, to ensure sink conditions. The fluid was maintained at 32 °C and stirred with a magnetic bar throughout the experiment. Portion (20 mg) of the cream formulations containing resveratrol free or encapsulated in the LMs were spread on the membrane surface in the donor chamber. At appropriate time interval, 0.1-ml aliquots of the receptor phase were withdrawn and replaced by an equal volume of fresh fluid. Samples from the receptor phase were assayed for resveratrol by HPLC. Drug release (%) was calculated from the amount of resveratrol deposited on the membrane, which was calculated by adding to the total quantity of drug diffused in the receptor phase, the fraction retained on the cellulose membrane after the experiment. This fraction was measured by heating and extraction of the residual material on the membrane with ethanol under sonication, as described in Section 2.4.3., followed by HPLC assay. Data were determined from the average of at least six experiments.

2.7. *In vivo* skin penetration studies

The *in vivo* human skin penetration assay was carried out by the tape stripping technique. Six volunteers of both sexes, aged 24-28 years and free of dermatological disorders gave signed informed consent for the experimentation. The study protocol was approved by the local Ethics Committee (Comitato Etico della Provincia di Ferrara) and complied with the Declaration of Helsinki guidelines. The subjects were allowed to acclimatize (30 min), before application of the test formulations on the internal region of both their forearms, which were previously wiped with ethanol and water and dried. The test creams (creams containing resveratrol-loaded LMs) and the control formulations (creams containing resveratrol in conjunction with blank LMs) were applied at a dose of 4 mg/cm² and randomly allocated to a delineated area (2 x 5 cm), respectively in the lower and upper part of each forearm of the volunteers. The creams were homogeneously distributed using rubber gloves. After an application time of 60 min [26], which was found to produce a significant accumulation of resveratrol in the stratum corneum, the remaining preparation was removed from the treated area by a cotton swab and then the

stratum corneum was sequentially stripped, from the same area, 15 times with Scotch adhesive tapes, in accordance with published studies [18, 26-28]. In addition, this number of tape strips allowed the collection of the majority of drug in the stratum corneum and the removal of deeper and more intact stratum corneum layers. The tapes were applied to the skin with a constant pressure by a 500 g stainless steel roller (30 mm diameter). The first stripped tape was added to the cotton swab for the assay of the unabsorbed resveratrol [18,26,27]. The successive 14 tape strips were collected separately in 4 groups (group 1: strips 2-4; group 2: strips 5-7; group 3: strips 8-11; group 4: strips 12-15). The obtained samples were extracted with ethanol (9 ml) under heating (75 °C for 5 min) and sonication (5 min) and re-extracted with methanol (9 ml) under sonication (5 min). The combined fractions were adjusted to volume (20 ml), filtered (0.45 µm membrane filters) and analysed for resveratrol by HPLC. The results were expressed as penetrated percentage of the applied dose.

Validation of the tape stripping assay was carried out by spiking adhesive tapes of untreated stratum corneum with 5 and 40 mg of the tested creams. The samples were processed as outlined above and the percentage recoveries calculated by comparing the peak areas of resveratrol extracted from the tape samples with those obtained by direct HPLC analysis of equivalent amounts of the polyphenol dissolved in methanol. The precision of the method was calculated by extraction and HPLC assay of individual tapes (n=6) spiked with 5 mg of the same cream formulation.

2.8. Statistics

Statistical analysis of data was performed by analysis of variance (ANOVA) and differences between groups were identified using Tukey's post-test. P values < 0.05 were considered to be significant. Statistical analysis was carried out using GraphPad InStat software (Graphpad, San Diego, CA).

3. Results

3.1. Lipid microparticle preparation and characterization

For the preparation of LMs loaded with resveratrol, the melt emulsification technique was employed since it circumvents the use of organic solvents [20]. Several lipids (tristearin, stearic acid, glyceryl behenate) were evaluated for the preparation of uncoated LMs in conjunction with hydrogenated phosphatidylcholine (0.7%), as biocompatible and non-irritating for human skin surfactant [29]. Particles with satisfactory morphological characteristics (spherical shape and absence of irregular fragments) were obtained only by the LMs prepared with tristearin, as shown by SEM analysis (Fig. 1) and therefore they were selected for further studies. The release of resveratrol from the LMs was significantly lower (ANOVA and Tukey's post-test) than the dissolution of the pure drug (Fig. 2), which suggested that the polyphenol was entrapped in the particle lipid matrix. The amount of resveratrol incorporated into the LMs was $5.6 \pm 0.5\%$ (w/w), which corresponded to an encapsulation efficiency of 76.8%. Preliminary analysis of the obtained LMs by optical microscopy indicated a particle size between ca. 5 and 38 μm , with the majority of the population in the 15-20 μm range, in accordance with SEM (Fig. 1). Published studies have shown that reduction of the microparticle dimensions to few microns facilitates their diffusion through the superficial layers of the stratum corneum and the hair follicles [30-32]. Consequently, decreasing the size of the foregoing LMs based on tristearin and hydrogenated phosphatidylcholine, should improve their performance for topical delivery. To this aim, a sonication step was combined with high-speed mixing, using a higher surfactant concentration (1.4%), in accordance with a previous study on the incorporation of the flavonoid quercetin in lipid particles [33]. This procedure resulted in a reduction of the particle dimensions, as demonstrated by dynamic light scattering which gave a mean diameter and PDI of 5.7 μm and 0.39, respectively. The resveratrol loading of the latter particle formulation was $4.1 \pm 0.3\%$ (w/w), which corresponded to an encapsulation efficiency of 67.2%. Release studies were also performed on the microparticles produced by high-speed mixing and sonication. The obtained curve was not significantly different (ANOVA) from that measured for the larger LMs (Fig. 2).

For the preparation of chitosan-coated LMs containing resveratrol, low- and medium-molecular weight chitosan was utilized, employing the procedure based on high-shear mixing and sonication. Polymer coating of the particles was performed simply by using a chitosan solution (1.0-2.0%) in aqueous acetic acid (pH 4.2) as the water phase of the hot emulsion during the production of the microparticles or by adding the chitosan solution

to the LM suspension formed on cooling of the emulsion. The concentration of chitosan was found to affect the solid state of the particles. Specifically, at chitosan levels higher than 1.0%, soft aggregates were obtained after lyophilization and therefore the concentration of the polysaccharide solution was set at 1.0%. Comparison of the release profiles of the particles obtained by addition of the chitosan solution at the two different stages of the production process, as outlined above, indicated (data not shown) that a better modulation of resveratrol release was achieved by mixing the polysaccharide solution with the LM suspension. Therefore, the latter procedure was utilized for the production of the coated microparticles. Coating of LMs with chitosan caused a greater decrease of resveratrol release (Fig. 3), as compared to the uncoated particles (Fig. 2), the slower release rate being attained with the higher molecular weight form of the polysaccharide (medium molecular weight chitosan). Therefore, the latter particle formulation was selected for subsequent studies.

The SEM micrograph of the particles coated with the medium molecular weight chitosan and obtained by the optimized procedure revealed a spherical shape with a relatively smooth surface and a size $< 12 \mu\text{m}$ (Fig. 4). Their mean diameter, as measured by dynamic light scattering, was $6.1 \mu\text{m}$, in accordance with SEM analysis, and the PDI was 0.14. The resveratrol content was $3.8 \pm 0.2\%$ (w/w) which corresponded to an encapsulation efficiency of 62.3%.

Zeta potential measurements were also performed on the uncoated- and chitosan coated-LMs loaded with resveratrol. The uncoated LMs exhibited a negative zeta potential value of $-17.8 \pm 4.8 \text{ mV}$, whereas for the chitosan-coated LMs the zeta potential was positive ($64.2 \pm 4.4 \text{ mV}$). The change in surface charge for the chitosan-coated LMs indicated the efficient adsorption of the cationic polysaccharide on the surface of the particles. Moreover, the absolute zeta potential value of the LMs increased significantly (more than three times) upon coating of the particles with chitosan.

3.2. Cream formulations

As a model topical vehicle for free and microencapsulated resveratrol, a hydrophilic cream (oil-in-water emulsion) was used, since this formulation represents the most common type of dermatological dosage form [34]. In order to minimize possible interactions between the excipients and the active agent, a simplified emulsion formulation was selected (see Section 2.5.). Preliminary experiments for the development

of the cream preparations indicated that when Montanov 82 (coco-glucoside) was used as nonionic emulsifier, phase separation occurred. This problem was ascribed to the presence of chitosan, since the emulsions containing uncoated particles did not exhibit any phase separation. On the other hand, replacing Montanov 82 with the ethoxylated nonionic surfactant Polawax, lead to physically stable cream formulations. Therefore Polawax was selected as the emulsifier. The chemical stability of resveratrol in the creams was evaluated by HPLC assay of the polyphenol after extraction of the sample with ethanol under heating and sonication. No significant decrease (< 5%) in the initial levels of resveratrol was detected over 6 months storage of the preparations at room temperature and in the dark. Homogeneity of the cream formulations was assessed by analyzing samples from the top, middle and bottom of the preparations. The obtained relative standard deviation values for the assay of resveratrol were < 6.4% , within the acceptance criteria for semisolid formulations, such as creams [35].

3.3. *In vitro* diffusion studies

The cream formulations (ca. 20 mg) containing resveratrol free, incorporated in the uncoated or chitosan-coated LMs obtained by the optimized procedure (high-shear mixing and sonication), were applied to the surface of the cellulose acetate membrane mounted in the donor compartment of the Franz cells and the amount of polyphenol diffused in the receiver fluid was measured by HPLC. As illustrated in Fig. 5, the obtained curves indicated that resveratrol release from the emulsions and diffusion across the synthetic membrane was lower for the formulations containing the polyphenol loaded in the LMs. Compared to the cream with free resveratrol, significance was attained after 3 h (ANOVA and Tukey's post test). However, no significant differences (ANOVA and Tukey's post test) were observed among the creams containing uncoated or chitosan-coated LMs.

3.4. *In vivo* skin penetration studies

The *in vivo* permeation of resveratrol into human skin was evaluated by the tape stripping technique which is based on the successive removal of the stratum corneum layers by repeated application of appropriate adhesive tapes [18,36]. After topical application of the tested cream formulations, the amount of resveratrol fixed to the individual tape strips was quantified to provide the *in vivo* penetration profile of the

polyphenol in human stratum corneum. The average recoveries of resveratrol from the adhesive tape were satisfactory ($> 87.4\%$). Moreover, the precision of the method determined by replicate assays of spiked tapes was shown by a relative standard deviation of 6.7% .

The creams containing resveratrol free, incorporated in the uncoated- or chitosan coated-LMs were applied to the volar forearm of the human volunteers and the polyphenol levels in the collected tapes was measured by HPLC after solvent extraction. The overall resveratrol recoveries obtained as sum of the polyphenol unabsorbed and permeated into the horny layers removed by the tape strips was $> 82.6\%$, which is acceptable considering that the stratum corneum was stripped only 15 times [27]. The majority of the topically administered resveratrol was not absorbed (Table 1) from the examined formulations with the exception of the cream containing the polyphenol loaded in the chitosan-coated LMs. For the latter preparation (Table 1), the fraction of polyphenol penetrated (49.3%) was higher than the unabsorbed one (38.5%). The percentage of the applied resveratrol dose permeated into the stratum corneum from the cream containing the uncoated LM-entrapped polyphenol (32.8%) was greater compared to the control preparation (26.2%), but the difference was not significant (ANOVA and Tukey's post test) (Table 1). On the other hand, the cream containing resveratrol incorporated in the chitosan-coated LMs achieved a marked and statistically significant ($p < 0.01$, ANOVA and Tukey's post test) increase in the fraction of the applied polyphenol dose penetrated into the stratum corneum (49.3%), as compared to the control formulation (29.0%) based on free resveratrol in conjunction with unloaded chitosan-coated LMs (Table 1). Moreover, the chitosan-coated resveratrol-loaded LMs provided a significantly ($p < 0.01$, ANOVA and Tukey's post test) higher accumulation of the polyphenol in the horny layer than the other tested formulations containing the uncoated LMs (Table 1).

The levels of resveratrol measured in the adhesive tapes combined in four groups (2-4, 5-7, 8-11, 12-15) are shown in Fig. 6 as a function of strip number (related to depth). For all examined formulations, the main fraction ($64.6-71.6\%$) of the permeated resveratrol was localized in the upper portion of the horny layer (strips 2-4), its concentration decreasing with depth (Fig. 6). However, significant amounts of polyphenol ($9.3-20\%$ of the applied dose) diffused into the deeper and more intact stratum corneum layers (strips 5-15). Differences were observed in the stratum corneum penetration profiles of resveratrol among the studied formulations. In particular, the distribution of resveratrol in

the various stratum corneum layers was not significantly different (ANOVA and Tukey's post test) for the control cream (non-encapsulated resveratrol combined with uncoated blank LMs) and the cream containing resveratrol encapsulated in the uncoated LMs (Fig. 6). Conversely, application of the formulation based on the chitosan-coated LMs loaded with resveratrol yielded a statistically significant (ANOVA and Tukey's post test) increase in the polyphenol amounts measured in each group of tapes, with the exception of the deepest strips 12-15, as compared to the control preparation (non-encapsulated resveratrol in conjunction with coated blank LMs) (Fig.6). Moreover, the cream containing the chitosan-coated LMs induced higher levels of permeated polyphenol than all the other examined formulations, the difference being more marked and statistically significant in the upper portion of the stratum corneum (strips 2-4 and 5-7).

4. Discussion

Despite the therapeutic potential of topical resveratrol against several skin disorders such as oxidative-stress mediated damage, inflammation, microbial infections and skin cancer [6, 9,10], its utilization is hampered by its poor percutaneous penetration [4,10, 11, 37-39]. In fact, in order to elicit its cutaneous activity, resveratrol should permeate into the inner skin layers and in particular in the epidermis, where the majority of resveratrol binding sites that mediated its activity are located [3,4,9,11]. In order to enhance the cutaneous availability of resveratrol, delivery systems such as liposomes and nanoparticles have been designed [1,4,10,13,15,16]. However, the effect of these technologies on the skin uptake of resveratrol has been evaluated only by *in vitro* techniques using excised animal skin [1,4,10,13,15,16]. Although the *in vitro* methodology is commonly used to assess percutaneous penetration, the relevance of the observed effects to realistic conditions is limited due to the structural and functional alteration observed in isolated skin and the differences in cutaneous permeability between animals and humans [18,19]. In order to overcome these drawbacks, in the present study the skin permeation of microencapsulated resveratrol was evaluated *in vivo* on human volunteers. For the *in vivo* percutaneous penetration test, the tape stripping technique was employed since it is a minimally invasive and efficient method to measure the amount of a topically applied substance permeated *in vivo* in subsequent layers of the stratum corneum [36,38]. The obtained horny layer profile reflects the driving concentration that

delivers the drug to the deeper viable skin tissues [18,40] and hence it is related to the fraction that reaches the epidermis, where the resveratrol activity is mainly located [3,4,9,11].

In the present study, LMs uncoated or coated with chitosan were examined as delivery systems to enhance the skin availability of topical resveratrol. Although LMs are less studied than nanoparticles for percutaneous penetration, they were selected for this investigation because of some advantages compared to nanoparticles including simpler production and characterization methods, higher loading and reduced content of potentially toxic surfactants [20,22]. Moreover, the good *in vivo* tolerability and biocompatibility of the LM constituents, many of which are regulatorily accepted [20-22], represent a distinct advantage for the topical application of this carrier. The negative surface charge of the optimized uncoated LMs changed, as expected, to a positive value upon coating with chitosan. In addition, the zeta potential value of the optimized coated LMs was much higher than that of the uncoated LMs and thus the use of chitosan should improve the stability of the particles. The release characteristics of the microparticles were affected by the chitosan coating, the slowest release rate being attained by the particles coated with the higher molecular weight form of the biopolymer. This effect can be traced to the adhesion of chitosan to the LM surface which increases the diffusion path of the incorporated resveratrol through the particle matrix. Furthermore, the reduced dimension of the LMs (mean diameter < 6.1 μm) obtained by the optimized procedure, should facilitate their permeation in the superficial stratum corneum layers [31] and ensure, at the same time, the acceptability of the topical LM formulation, since particles in this size range are not palpable.

At variance with the previously published reports in which a suspension of the resveratrol delivery system in a suitable solvent was used [1,4,10,13,15,16], in the present study the investigated carrier, namely the LMs, was incorporated into a topical formulation (cream) to mimic real conditions of use. The *in vitro* diffusion studies performed on the creams containing free or microencapsulated resveratrol (Fig. 5) demonstrated that the release modulation capacity of the optimized uncoated and chitosan-coated LMs was retained after their incorporation into the cream formulation.

The results obtained from the *in vivo* skin permeation study on human volunteers indicated that the cream incorporating resveratrol loaded in the uncoated LMs did not produce any significant increase in the polyphenol concentration diffused into the stratum

corneum, compared to the control formulation (Table 1). This is in line with reports in the literature indicating that incorporation of different active substances in LMs had no significant effect or decreased their penetration in animal or human skin, both in vitro and in vivo [41-44]. On the other hand, topical application of the cream containing resveratrol encapsulated in the chitosan-coated LMs significantly enhanced the amount of polyphenol permeated in vivo into the stratum corneum, as compared to the control cream and the other tested formulations (Table1). Moreover, the measured stratum corneum penetration profiles (Fig.6) indicated that the formulation containing the chitosan-coated LMs loaded with resveratrol induced the highest polyphenol levels in each examined portion of the stratum corneum, the difference being statistically significant in the upper layers (strips 2-4 and 5-7). The amounts of resveratrol permeated into the stratum corneum from the creams containing the microencapsulated polyphenol were comparable and higher than the levels which have been shown to elicit in vivo a significant antioxidant activity in human horny layer (39). The obtained data also showed that the application of the cream containing free resveratrol in combination with blank LMs coated with chitosan, did not result in an increased stratum corneum uptake of the polyphenol (Table1). This finding suggests that in order to achieve improved horny layer permeation, interaction of chitosan with resveratrol in the LM matrix is required.

The enhanced accumulation of resveratrol in the stratum corneum produced by the chitosan-coated LMs loaded with the polyphenol, increases the concentration gradient that promote the partition/diffusion of resveratrol into the underlying viable epidermis, where it exerts its main activity. In addition, the stratum corneum is more exposed to environmental oxidative damage and therefore higher resveratrol concentrations should favour its protection [15,38].

Comparison with earlier investigations is difficult since the previously described carrier systems for resveratrol skin delivery were based mainly on uncoated liposomes and nanoparticles, which were evaluated in vitro [1,4,10,13,15,16]. In the only report found in the literature on in vivo human skin penetration of resveratrol, a textile material was used as sustained release system with less than 1% of applied resveratrol permeated in the stratum corneum [39]. However, the results presented in this study are consistent with those reported for liposomes by Park et al. [16], showing that the in vitro skin permeation of resveratrol in mouse was increased by application of chitosan-coated liposomes, as compared to uncoated liposomes. In the same line, published studies performed in vitro on animal skin have demonstrated that the topical administration of

liposomes [45], nanoparticles [46,47, 48] or microparticles [49] coated with chitosan, enhanced the percutaneous permeation of the encapsulated drugs.

Since microparticles cannot penetrate the horny layer (31), the observed effect of the chitosan-coated LMs, can be ascribed to the skin permeation enhancing properties of this polysaccharide. The mechanisms which have been proposed for the improvement of percutaneous absorption induced by chitosan are based on bioadhesion and reversible opening of the epithelial cell tight junctions [24,25]. More specifically, chitosan can adhere to the skin surface through ionic interactions between its positively charged amine groups and the negative charge in the upper cutaneous layers. Therefore, it can be speculated that chitosan would enhance the contact of the LMs with the stratum corneum and hence the skin delivery of the encapsulated drug. With regard to the cellular tight junctions, their occurrence has been restricted to the epidermal region beneath the stratum corneum [50,51], although in one study their presence in the deepest stratum corneum layers has been suggested [52]. Therefore, the mechanism based on transient opening of tight junctions should not be involved in the increased stratum corneum uptake of resveratrol promoted by the chitosan-coated LMs.

5. Conclusions

In the present study, resveratrol-loaded LMs uncoated and coated with chitosan were developed and evaluated as systems to improve the skin delivery of this polyphenol. In order to provide a more realistic assessment of the degree of percutaneous penetration an *in vivo* methodology, the tape stripping technique, was used. To the best of our knowledge, the effect of particulate carriers on the *in vivo* human skin permeation of resveratrol has not been reported before. The results described in this investigation indicated that the cream containing chitosan-coated LMs significantly enhanced the *in vivo* penetration of resveratrol into human stratum corneum as compared to conventional uncoated LMs. Such increased concentration in the horny layer is of relevance since it should favour the resveratrol diffusion into the viable epidermis which represents its main site of action. Moreover, the incorporation of resveratrol in the particle matrix should improve the stability of this labile polyphenol [15] and prolong its activity due to sustained release.

The data obtained in this study suggest that the use of chitosan-coated LMs as carrier for resveratrol could be an appropriate strategy to enhance the efficiency of its topical administration. However, to confirm the potentiality of the proposed particle system, a

larger number of subjects and longer application times should be investigated.

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Figure legends

Figure 1. SEM micrograph of LMs loaded with resveratrol and produced by high-speed mixing.

Figure 2. Resveratrol dissolution (filled circles) and release profiles from LMs prepared by high-speed mixing (filled squares) or high-speed mixing and sonication (filled triangles). Values are means \pm SD (n=5).

Figure 3. Resveratrol dissolution (filled circles) and release profiles from LMs prepared by high-speed mixing and sonication and coated with low molecular weight chitosan (filled squares) or medium molecular weight chitosan (filled triangles). Values are means \pm SD (n=5).

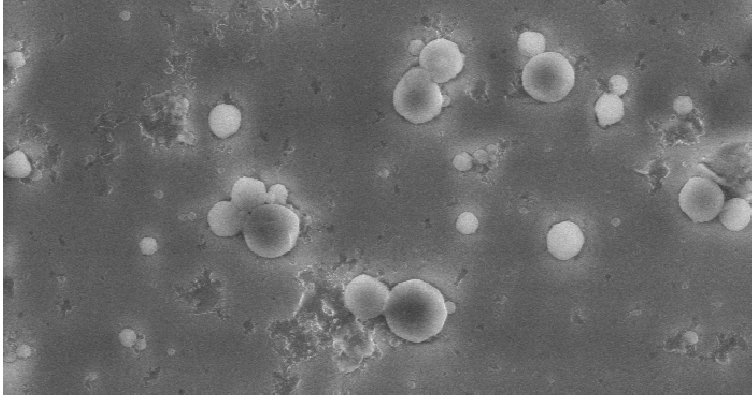
Figure 4. SEM micrograph of chitosan-coated LMs loaded with resveratrol and produced by the optimized procedure.

Figure 5. Franz diffusion cell release profiles of free resveratrol (filled triangles), uncoated resveratrol-loaded LMs (filled squares) or chitosan-coated resveratrol-loaded LMs (filled circles) from their cream formulations. Values are means \pm SD (n=6).

Figure 6. Distribution of resveratrol in human stratum corneum in vivo after application of its cream formulations. The resveratrol amounts (% of applied dose) in the different tape strip groups are shown (mean \pm SD, n=6).

Table 1. In vivo permeation data for resveratrol after application of the different cream preparations: cumulative recovery in strips 2-15 of human stratum corneum (% of the applied dose \pm SD, n=6)

Sample	% of applied dose	
	non-permeated	recovered in strips 2-15
<hr/>		
Cream containing resveratrol		
plus uncoated blank LMs	59.6 ± 9.5	26.2 ± 5.6
Cresveratrol-loaded LMs	54.3 ± 8.0	32.8 ± 8.9
rCream containing resveratrol		
eplus chitosan-coated blank LMs	54.0 ± 4.5	29.0 ± 4.3
Ccoated resveratrol-loaded LMs	38.5 ± 5.3	49.3 ± 5.9
ream containing chitosan-		
am containing uncoated		



— 15 μm

Fig. 1

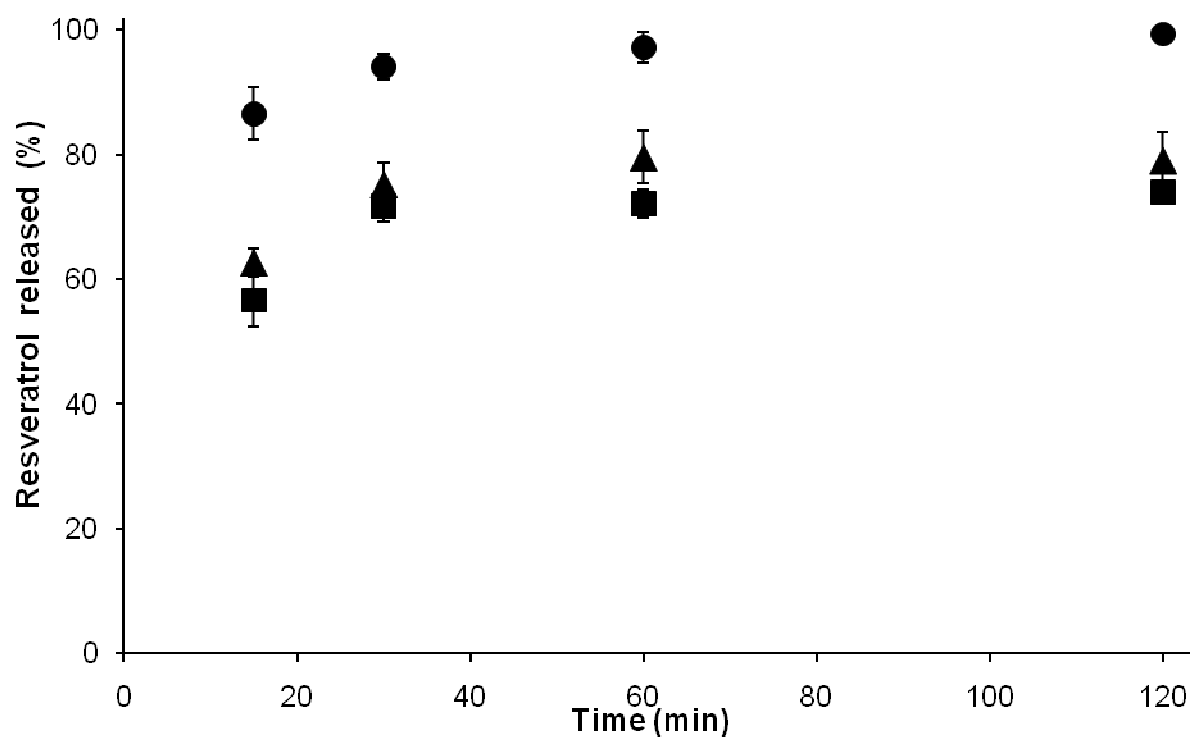


Fig. 2

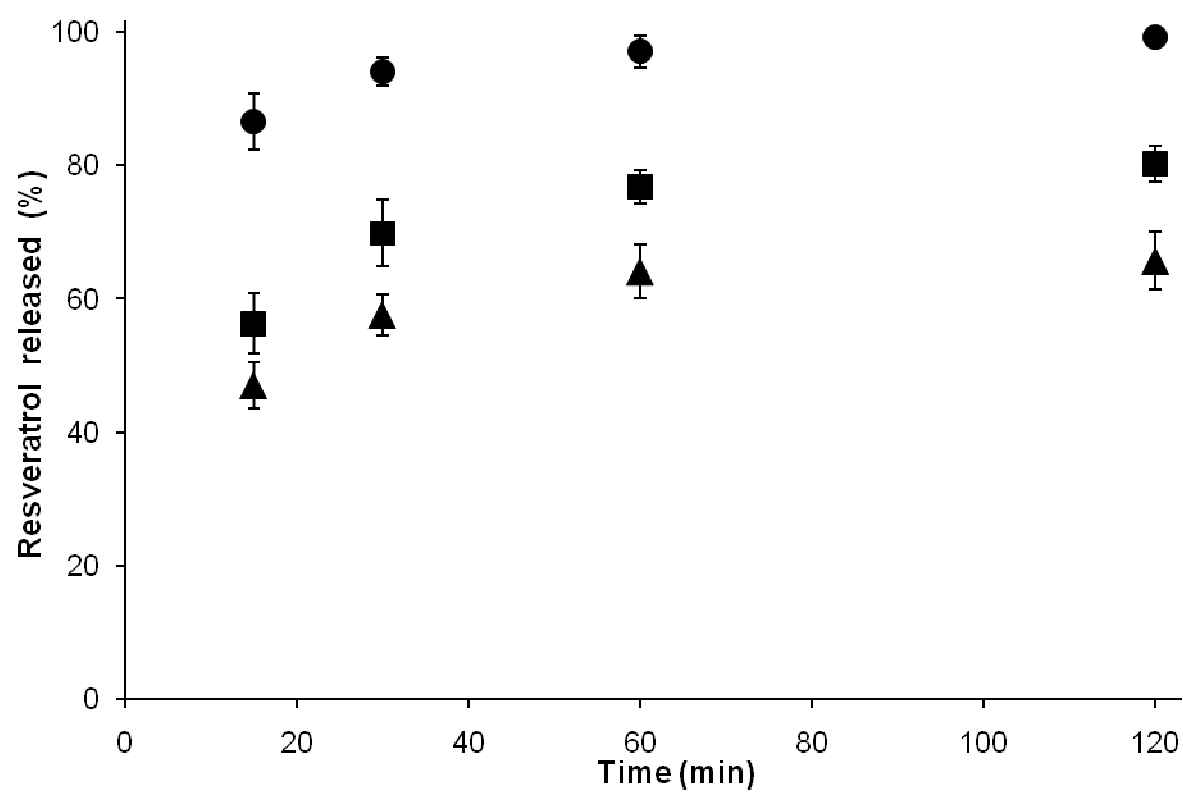
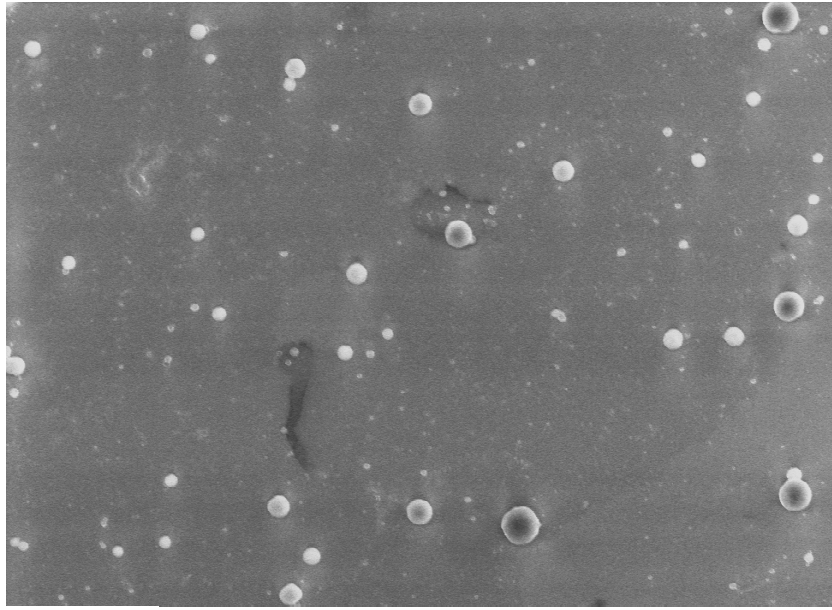


Fig. 3



— 15 μm

Fig. 4

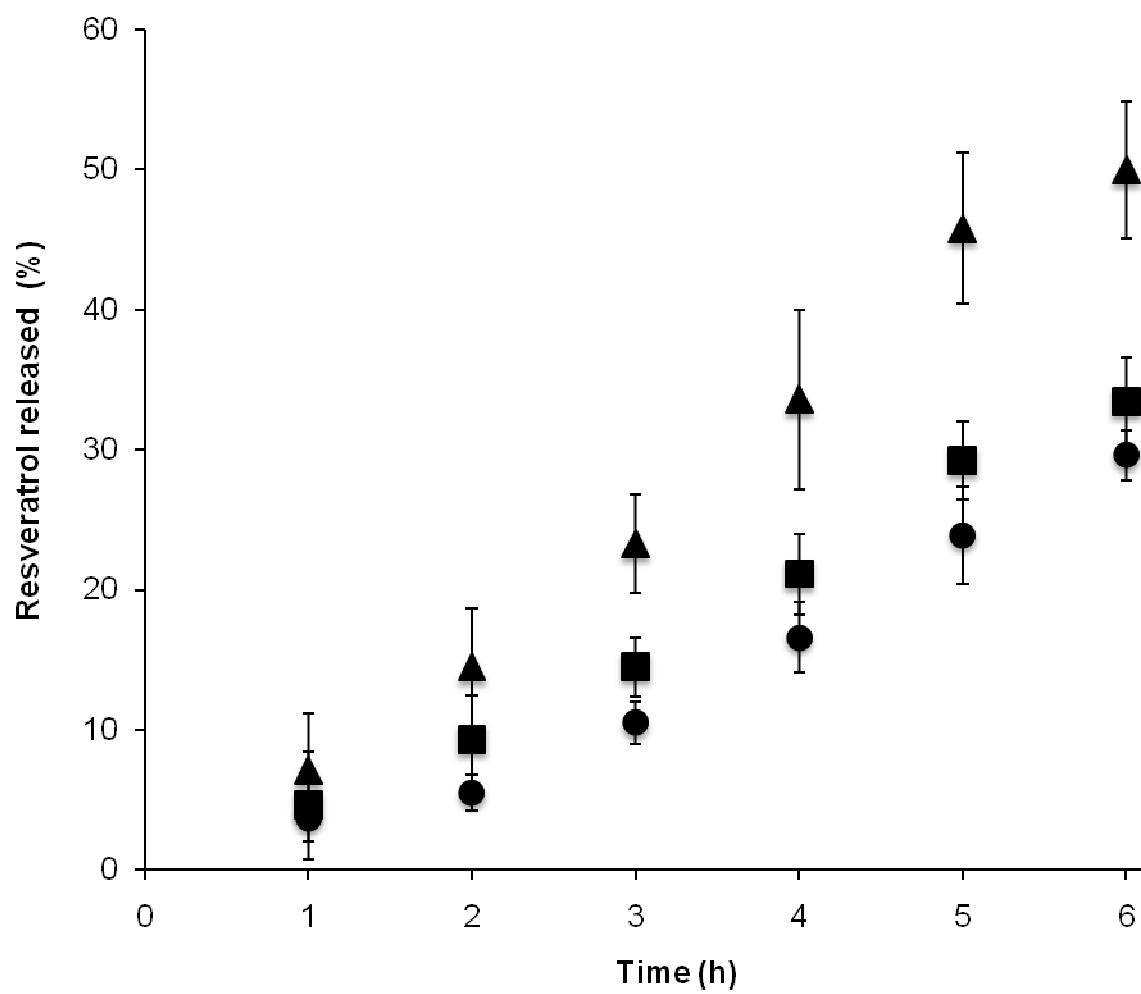


Fig. 5

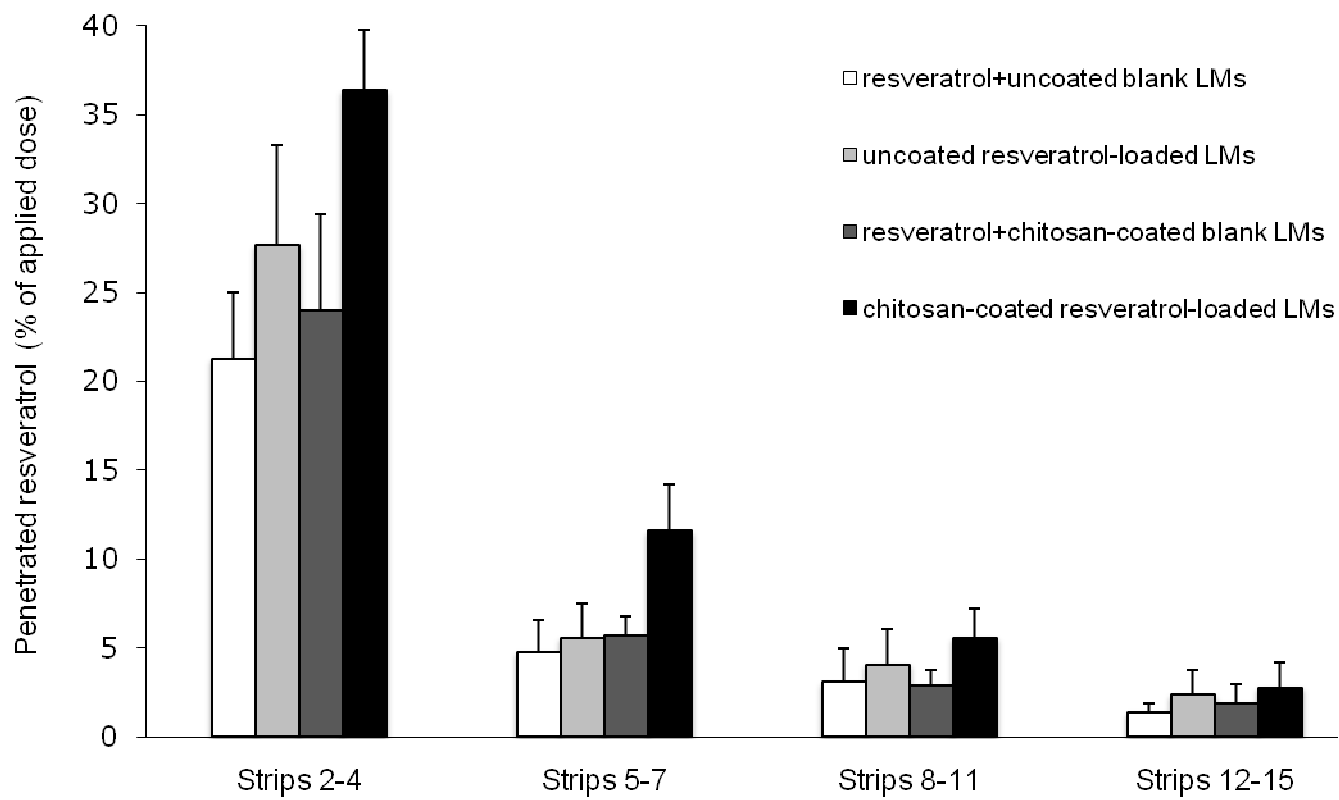


Fig. 6