Carbohydrate Polymers 92 (2013) 40–47

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Evaluation of the effect of hydroxypropyl-β-cyclodextrin on topical administration of milk thistle extract

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ARTICLE INFO

Article history:
Received 23 May 2012
Received in revised form 7 September 2012
Accepted 8 September 2012
Available online 16 September 2012

Keywords:
Hydroxypropyl-β-cyclodextrin
Skin delivery
Milk thistle dry extract
Silymarine
Antioxidant activity

ABSTRACT

Two water in oil emulsions composed by eudermic ingredients as glycerin, cocoa butter, almond oil and a variety of lipids, were enriched respectively with milk thistle dry extract (MT) or with a binary complex composed by MT and hydroxypropyl-β-cyclodextrin (HP) (1:4 w/w) correspondent to 1% (w/w) in sylimarine in order to obtain two different emulsions designed for the skin delivery and determine influence of hydroxypropyl-β-cyclodextrin on the extract delivery and permeation. Uv–vis spectrophotometric analyses demonstrated that phytocomplex formation influences the finding of MT after the complexation process and the in vitro antioxidant activity.

Further in vitro and ex vivo experiments demonstrated that the penetration capability of MT from formulations is strictly influenced by the phytocomplex able to control MT permeation; moreover phytocomplex increases flavonoids stability during the in vitro tests. Additionally, in vivo studies showed that the penetration into the *stratum corneum* of the active ingredients is effectively achieved by the phytocomplex formation, in fact about 80% of MT is absorbed by the skin along 1 h despite the 30% of MT not complexed absorbed during the same period.

1. Introduction

It is well known that the major obstacle for topic delivery is constituted by the *stratum corneum* conformation, which limits the active ingredients transport even through the most superficial layers of the epidermis (Gillet et al., 2011). Ideally, drugs must possess both lipoidal and aqueous solubility: if administered molecules are too hydrophilic, they will be unable to transfer into the *stratum corneum*; on the contrary topic administration of completely lypophilic compounds can be affected by low absorption and bioavailability (Josef, Zilberman, & Bianco-Peled, 2010). For these reasons several delivery systems were studied in order to favorite skin penetration of active ingredients from natural origin even for systemic than for topic activity into deeper zones of the skin. Inclusion complexes between cyclodextrins (CD) and several kind of lipophilic drugs designed for topic application are widely studied because of CD are able to increase stability and solubility of several kinds of active ingredients as well as protect them from degradation and oxidation (Monti et al., 2011); moreover, CD are able to affect the availability of topically applied drugs modulating their permeability into and through the skin (Simeoni, Scalia, & Benson, 2004) and they can helpfully control skin delivery of many substances avoiding a too deep cutaneous penetration (Cal & Centkowska, 2008).

Milk thistle have been recently studied by Toklu et al. (2007) that demonstrated that the mixture of flavonoidic molecules present onto the MT extract, named sylimarine, has high antiradical and antioxidant activity on skin cells. Indeed, MT extract is noted as able to decrease cellular peroxidation and protect the skin from photo-induced tumors (Katiyar, Korman, Mukhtar, & Agarwal, 1997; Katiyar, 2007; Nichols & Katiyar, 2010; Singh & Agarwal, 2009). Further studies already showed that topical application of silymarin suppresses intracellular production of hydrogen peroxide and nitric oxide and reduces depletion of catalase activity in UVB-irradiated mouse skin. In addition, silymarin inhibits expression of cyclooxygenase-2 and its prostaglandin metabolites implicated in tumor promotion (Katiyar et al., 1997; Křen & Walterová, 2005).

For these properties MT was chosen in order to obtain a potential anti-inflammatory and antioxidant topic formulation useful as skin protectant from toxic factors as UV rays, smoke, smog able to induce peroxidation processes and consequent skin damage as already described. Properties of MT are moreover added to the nutritive and restorative qualities of several excipients used for emulsions. However penetration capability of a such lypophilic and complex mixture can be not easily achieved (Gillet et al., 2011), and appear necessary to found a good carrier able to permits the reaction of the flavonoidic molecules with the oxidation factors commonly diffused in the skin. Cyclodextrins, thanks to their complexation capability and their penetration enhancement properties seem the

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http://dx.doi.org/10.1016/j.carbpol.2012.09.018

most quoted excipient for the obtainment of a new formulation designed for antioxidant action and aging prevention.

Studies of active ingredients penetration through the skin are regulated by the indications given in US Pharmacopeia Forum (Vol. 3, 2009) and by the OECD Guideline For The Testing Of Chemicals (2004). However, large possibilities of experimentation are let to the formulator; in fact, since now no one official acceptor fluid for in vitro tests is indicated and several in vitro tests are conducted using different kinds of cells cultures deriving from the skin (Baert, Vansteelandt, & De Spiegeleer, 2011; Gabbanini, Matera, Beltramini, Minghetti, & Valgimigli, 2010; Reichling, Landvatter, Wagner, Kostka, & Schaefer, 2006) and from various types of synthetic membranes (Baert et al., 2011; Frum, Eccleston, & Meidan, 2007; Mitri, Shegokar, Gohla, Anselmi, & Müller, 2011). Despite the different acceptor mediums selected for tests, the ex vivo studies are usually performed using the upper part of the pig skin ear, comprising the *stratum corneum* and the epidermis (Caon, Oliveira Costa, Leal de Oliveira, Micke, & Oliveira Simoes, 2010; Kanikkannan et al., 2001; Moser, Kriwet, Yogeshvar, & Guy, 2001) due to its elevate similarity with the structure and composition of human skin (Simon & Maibach, 2000).

The reliability of the pig skin as permeation membrane simulating the viable skin is commonly verified by the tape stripping test, conducted in health volunteers, indicating the crossing of the *stratum corneum* barrier by the cream applied (Klang et al., 2011; Sgorbini et al., 2010).

Starting from these bases, aim of our work is to evaluate the capability of the extract obtained from the milk thistle's seeds to penetrate through the skin after its vehiculation into proper formulations appositely designed for skin delivery to perform the antioxidant and antiinflammatory activity. In particular it is our goal to verify the effect of a particular kind of CD on the transport of dry MT extract through the *stratum corneum* by exploiting the penetration enhancement activity of CD. Hydroxypropyl-β-cyclodextrin is chosen due to its good water solubility and low toxicity: indeed, it is listed in both The European and The United States Pharmacopeia and it is cited in the FDA's list of inactive pharmaceutical ingredients (Loftsson & Duchene, 2007).

As complex preparation process could strongly influence the effective antioxidant activity of the MT extract, the efficacy of the phytocomplex as antioxidant is also evaluated. Moreover, the pure MT dry extract and the phytocomplex are then vehiculated into two different o/w emulsions designed for topical administration in order to obtain four final formulations. Influence of both complexation and vehiculation into the o/w emulsion on the skin penetration capability were studied. In fact as previously cited, composition of formulations in terms of kind of components and hydrophilicity/lypophilicity ratio could influence the permeation through the skin as well as to maintain healthy skin properties. In order to better understand the effective behaviour of MT during skin administration both ex vivo and in vivo experiments are performed.

2. Experimental

2.1. Materials

Emulsion was obtained using: isopropilmiristate (IPM) and vegetal glycerin both purchased from Prodotti Cruciani CRUAL s.r.l., Rome (Italy); cetyl alcohol produced by Azienda Chimica e Farmaceutica s.p.a, Piacenza (Italy); myristic acid and palmitic acid both obtained by ACROS, Geel (Belgium), Avenopac® obtained by Sinerga, Pero (Italy); hydroxypropylmethylcellulose purchased from The Dow Chemical Company (USA); benzoic acid obtained from Pro Analysi (Germany). Inclusion complexes were obtained starting from: hydroxypropyl-β-cyclodextrin obtained by Waker Chemie (Mw: 1400 g/mol, average degree of substitution per anhydro glucose unit: 0.65) (Germany), Dry extract of milk thistle seeds was purchased from Phoenix s.r.l (Italy); ethanol obtained by Prolabo CE, moreover pure Sylimarine and 2,2-diphenyl-1-picrylhydrazyl (DPPH• radical) were obtained by Sigma–Aldrich (Germany). Other reagents used are all of pure grade.

2.2. Preparation of dry extract from milk thistle seeds

Dry extract of milk thistle seeds was purified following the method described by Meghreji Moin, Patel, Dave, Badmanaban, and Patel (2010) suitably modified. About 10 g of extract were weighted, dispersed into 200 mL of acetone and kept under magnetic agitation for 15 min in order to achieve extraction. Mixture was filtered using a paper filter and solution obtained was then evaporated under vacuum at 40 °C until complete evaporation of the solvent. The extraction process was repeated three times.

2.3. HP-MT dry extract inclusion complex formation and evaluation

Phytocomplex formation was achieved by dissolution of respectively 0.5 g of extract into 20 mL of ethanol and 1 g of HP into 100 mL of distilled water. Solutions were then mixed together and magnetically stirred for 72 h at room temperature to facilitate complexation. At the end of the process ethanol was evaporated under vacuum at 40 °C. The remaining mixture was congealed at −80 °C and lyophilized using lyophilization apparatus Lio 5P (Cinquepascal srl Milan, Italy); the process was carried out at −54 °C under vacuum (0.909 bar) for 8 h. Inclusion complex obtained was then analyzed in order to evaluate the effective sylimarine content.

Nuclear magnetic resonance (^{1}H NMR) spectra of MT or phytocomplex or hydroxypropyl-β-cyclodextrin (HP) were determined in DMSO-d6, and were recorded in a Brucker 400 MHz spectrophotometer.

2.4. Emulsion preparation

The first step of the work was the preparation of two different unloaded emulsions (coded A and B) and further tests of stability, spreadability and sensorial feeling, necessary for their characterization, were subsequently carried out. The choice to select two different formulation was done in order to verify how their composition and their physical characteristics are able to influence the administration of the active ingredients studied. Table 1 reports each component of both emulsions; both phases were obtained by mixing ingredients at 65 °C. Water phase was then added drop-wise to the oily phase in order to obtain an emulsion homogenized by Ultraturrax T25 (IKA, Switzerland) for 5 min at 20,000 rpm.

Emulsions previously obtained were added with an amount of phytocomplex correspondent to 1% (w/w) in sylimarine in order to obtain final formulations coded AC and BC respectively starting from emulsion A and emulsion B. Moreover, formulations coded AE and BE were obtained by the addition of the same amount of MT dry extract to emulsions A and B.

2.5. Stability studies by centrifugation test

Two different tests were carried out in order to evaluate the stability of the emulsions prepared (AE, BE, AC and BC). Centrifuge stress test was performed by samples centrifugation at 3000 rpm for 30 min at 20 °C (Anchisi, Maccioni, Sinico, & Valenti, 2001).

Phase separation was reported as percentage of stability: measurement was done using a graduated centrifuge tube containing 10 mL of emulsion exactly measured, totally not separated phase

Table 1
Excipients amounts used for preparation of both emulsion A (A) and emulsion B (B). All values are expressed in percentage (%).

Emulsion A	Amount (%)	Emulsion B	Amount (%)
Water	86.4	Water	77.7
Glycerin	5	Propylene glycol	3.8
Avenopac®	2	Theobroma cacao seed butter	3.4
Hydroxypropylmethil cellulose	1	Cetearyl alcohol	3.4
Cetyl alcohol	3	Hamamelis virginiana water	2.6
Isopropylmyristate	1	Capric acid	2.4
Palmitic acid	1.5	Prunus amygdalus Dulcis (sweet almond) Oil	2.4
Formic acid	0.05	Palmitic acid	1.8
Benzoic acid	0.05	Hydroxypropyl methylcellulose	0.8
		Carbopol (971NF)	1.6
		Methyl benzoate	0.1

corresponds to 100% stable emulsion; each mL of separated phase correspond to 10% of not stable emulsion.

2.6. Rheological tests

Reological parameters are indicators of the spreadability of the creams and the good diffusion of the product through the skin. Measurements were performed by modifying the method already cited by Realdon, Perin, Morpurgo, and Ragazzi (2002): emulsions were submitted subsequently to increasing shear rates from 10 to 100 mPa s^{-1} and decreasing shear rates from 100 to 10 mPa s^{-1} at temperature of 20 ± 2 °C; variation of speed was regulated every 10 min and measurements were done using a Brookfied viscometer Alpha series L (Fungilab, SA).

2.7. Flavonoids content determination

2.7.1. Evaluation of flavonoids content into the purified dry extract (MT)

Quantity of total flavonoids contained into the MT dry extract was evaluated following the method described by Dewanto, Xianzhong, Kafui, Adom, and Hai (2000) in which 20 mg of dry extract were suspended into 10 mL of methanol and stirred for about 10 min. A sample of 200 μL was withdrawn and added to 150 μL of 5% $NaNO_2$ water solution, mixture obtained was vortexed and suddenly let repose for 5 min, then 10% of $AlCl_3 \cdot 6H_2O$ water solution was added and, after further 5 min, mixture was centrifuged at 12,000 rpm for 5 min and analyzed by UV–Vis spectrophotometry at 450 nm wavelength. Flavonoids concentration was determined by data extrapolation from previously prepared calibration curve ($r^2 = 0.999$) constructed using several concentrations of sylimarine comprised between 0.05 and 1 mg/mL.

2.7.2. Evaluation of flavonoids content into the inclusion phytocomplex

Inclusion complex was submitted to flavonoids amount determination. This test was carried out in order to verify the stability of flavonoidic molecules after complexation process and evaluate the effective quantity on flavonoids entrapped onto the HP cavity. 20 mg of powder were suspended in 10 mL of methanol and the test was carried out as previously described. Quantity of flavonoids was expressed as mg of sylimarine contained in 1 g of phytocomplex. Analyses were done in triplicate in order to calculate the mean quantity of flavonoids ± standard deviation (SD).

2.7.3. Evaluation of flavonoids quantity contained onto the formulations (AE, BE, AC and BC)

The obtained final formulations were then submitted to new analyses in order to establish the effective amount of flavonoid contents. Tests were carried out by the extraction of 1 g of emulsion with 100 mL of methanol. Suspension was vortexed for 1 min and then magnetically stirred for further 10 min; a sample of 200 μL was then treated with $NaNO_2$ and $AlCl_3$ as already described and analyzed by UV–Vis spectrophotometry at 450 nm wavelength. Amount of flavonoids was expressed as mg of sylimarine contained in 1 g of formulation. Analyses were performed in triplicate and data obtained were expressed as mean value ± SD.

2.8. Evaluation of antioxidant activity of flavonoids contained in AE, BE, AC and BC

This test was carried out by modification of the test already described by Von Gadow, Joubert, and Hansmann (1997) using DPPH 2,2′-difenil-1-picrilidrazil (DPPH•) antioxidant molecule. An exactly weighted amount of MT extract, phytocomplex and emulsions AC and BC containing 20 mg of sylimarine, were submitted to test. 50 mL of diluted DPPH• methanolic solution were obtained starting from 30 mg of free radical DPPH• and used for antioxidant activity tests. Samples of extract or phytocomplex or emulsions were prepared following procedure described for flavonoids quantity determination. After that 500 μL of each sample were added to 500 μL of DPPH• solution and incubated in dark ambient at room temperature for 60 min (t_{60}) in order to guarantee the reaction. After incubation, suspension was centrifuged at 14,000 rpm for 5 min and analyzed by UV–Vis analysis at 517 nm. Values obtained were compared with absorbance values obtained by the DPPH• analysis before incubation (t_o); result was expressed as radical inhibition percentage.

2.9. In vitro drug release tests

Formulations AC and BC or AE and BE were submitted to in vitro release test in order to evaluate the influence of complexation process and of the emulsion composition on the release rate of the active ingredient. After preliminary solubility studies conducted with several fluids, a mixture of ethanol:water 30:70 was chosen as dissolution medium for formulations. In vitro release tests were carried out by dissolving into 100 mL of medium 50 mg of formulation containing or 3.1 mg of phytocomplex (AC or BC) or 0.5 mg of MT extract (AE or BE); tests were carried out at 32 ± 1 °C along 2 h at 50 rpm. Samples of 1 mL of medium were collected at regular time lags and, in order to establish the amount of flavonoids delivered from emulsions a sample of 200 μL of it were analyzed by UV–vis spectrophotometry at 450 nm wavelength after colorimetric reaction previously described.

2.10. In vitro permeation tests

Evaluation of permeation across synthetic membranes was performed on formulations using three in-line flow-through diffusion cells as already described by Gavini et al. (2011).

Aim of this part of work was to evaluate possible different behaviors occurred on permeation rate of emulsions due to membranes or mediums used. Starting from in vitro tests 3 different kind of

synthetic membrane (regenerate cellulose, nylon and PTFE), having pores diameter of 0.45 μm, were disposed on flow-through diffusion cells in order to separate the donor to the acceptor compartments, acceptor fluid selected was a mixture of ethanol and distilled water 30:70. Tests were carried out at 32 °C for 2 h; a quantity of 50 mg of samples AE and BE, containing 0.5 mg of MT extract or 3.1 mg of phytocomplex (AC or BC) were homogeneously distributed on the upper face of the synthetic membrane constituting the donor portion of the permeation apparatus. At selected times, 1 mL of sample was withdrawn from acceptor fluid and analyzed in order to evaluate the amount of flavonoids permeated in. Samples were replaced with the same amount of fresh acceptor fluid in order to maintain sink conditions. Analyses were conduced as previously described by mixing of a sample of 200 μL with 150 μL of 5% $NaNO_2$ and 10% of $AlCl_3 \cdot 6H_2O$, mixture was analyzed by UV–vis spectrophotometry at 450 nm wavelength.

Tests were done in triplicate in order to express data as mean ± SD.

2.11. Permeation through ear epidermis of pig

Porcine ears obtained from nearest slaughterhouse were treated following indications reported by several authors (Caon et al., 2010; Kanikkannan et al., 2001; Moser et al., 2001): the ears were cleaned with water to eliminate the bloodstains and kindly dried. The hairs on the surface of the pig ear were shaved and the ear was then immersed in water at about 60 °C for 2 min in order to favorite the epidermis separation from the dermis. The outer layer was gently detached from the ear and stored at −20 °C until use. Before the test epidermis was let thaw at room temperature and successively disposed on the in-line flow-through apparatus already described by Gavini et al. (2011). The same amounts of samples used for in vitro permeation tests were homogeneously applied on the entire skin surface by a 30 s long massage in order to simulate the in vivo emulsion application and favorite the passage through the epidermis. Skin layer was then applied to the cells and permeation tests were carried out for 2 h at 32 ± 1 °C. At selected time lags 1 mL of sample was withdrawn and analyzed in order to evaluate the amount of flavonoids permeated through the skin layer. Three different acceptor mediums were used in order to evaluate their influence on permeation behavior: mediums named respectively "artificial human sweat" (AHS) and "simulated body fluid" (SBF) obtained by following literature indications (Baert et al., 2011; Shimamura et al., 2004) were compared with the mixture ethanol:water 30:70.

After conclusion of tests performed, the amount of formulation still remained on the upper part of the membrane was find out: membranes were gently washed out with 10 mL of methanol in order to completely dissolve emulsion traces not diffused onto the internal layers of the skin; solution was then evaporated in ventilated oven at 27 °C, the residuum was dissolved again in 500 μL of methanol and samples obtained were then submitted to flavonoids content analysis as previously described. After washing, membranes were cut out into pieces, dispersed into 10 mL of ethanol:water 30:70 and homogenized by Ultraturrax for 3 min to favorite the complete dissolution of the emulsion entrapped onto the epidermis. Homogenized obtained was then centrifuged at 14,000 rpm for 5 min, filtered with a syringe filter (0.45 μm pore size) and liquid obtained was analyzed by withdrawing 200 μL of sample that was additioned to 150 μL of 5% $NaNO_2$ water solution, after 5 min 10% of $AlCl_3 \cdot 6H_2O$ water solution was then added and, after further 5 min, mixture was centrifuged at 12,000 rpm for 5 min and analyzed by UV–vis spectrophotometry at 450 nm wavelength. Tests were done in triplicate in order to express data as mean ± SD.

2.12. In vivo permeation test (stripping test)

In order to evaluate the penetration capability of sylimarine through the *stratum corneum* further in vivo tests were performed. Stripping test permits to remove most external cells of *stratum corneum* by application of wax strips (Klang et al., 2011; Sgorbini et al., 2010). Also a percentage of sylimarine not deeper penetrated was removed with cells. Thanks to this technique it is possible to evaluate the capability of phytocomplex to favorite the diffusion of sylimarine through the skin layers and the distribution on the *stratum corneum*.

Test was done on 10 female volunteers comprised between 25 and 45 years that subscribed an informed consent to the personal data treatment. None of the volunteers had a known history of skin diseases. Formulations AC, BC and formulation AE or BE containing only MT extract without CD, were applied on a selected forearm zone (total surface: 2.83 cm^2) previously washed with 1 mL of ethanol, 1 mL of distilled water and subsequently dried with hydrophilic cotton. In each area about 100 mg of formulation was applied and massaged for 30 s in order to achieve its complete absorption through the skin. After a waiting time of 60 min *stratum corneum* was removed using wax strips having dimensions of 4.0 cm length, 1.7 cm breadth; strips were applied for three times and incubated for 100 s in 10 mL of methanol at 40 °C in order to dissolve sylimarine entrapped onto the wax. Afterwards, solvent was evaporated and sylimarine residuals were dissolved again in 500 μL in order to carry on flavonoids content analysis using the usual colorimetric method. Tests were done in triplicate in order to express data as mean ± SD.

3. Results

3.1. Preparation of MT dry extract and its complexation with HP

Significant variations of the flavonoids content after complexation process are observed: purified extract is constituted by about 85% of sylimarine, on the contrary complexation with HP causes the objective difficult to find all the amount of flavonoids effectively used for the complexation process. About 45% of sylimarine appears to be into the phytocomplex analyzed, this result is re-conductible to the diminution of enthalpy energy during complexation that positively influences the entrapment stability (Cal & Centkowska, 2008) and the possibility to evaluate the effective amount of MT included into the HP cavity. About 80% of sylimarine added as phytocomplex on the final formulation was found; therefore, flavonoid are not damaged by the ingredients of formulation.

Proton NMR spectroscopy was used to determine the complex formation; spectrum of phytocomplex is compared with the spectra of the single substances (MT or HP).

As already reported by Ficarra et al. (2002) the interaction between flavonoids with β-cyclodestrins results in the modification, particularly, of H3 and H5 protons of the hydrophobic internal cavity. Results obtained from NMR analysis show that H3 and H5 protons are appreciably shifted ($\Delta\delta$ in the range of 0.04–0.05 ppm). Moreover, the upfields shifts of the aromatic protons of MT ($\Delta\delta$ in the range of 0.02–0.025 ppm) were found. On the basis of literature data and NMR results, effective complex formation can be hypothesize.

3.2. Preparation and analyses of emulsions

Emulsions A and B highly differ each other to the excipients used. Nevertheless the same work parameters used permit to obtain creamy formulations characterized by white pale appearance. Both emulsions demonstrate good sensorial feelings thanks

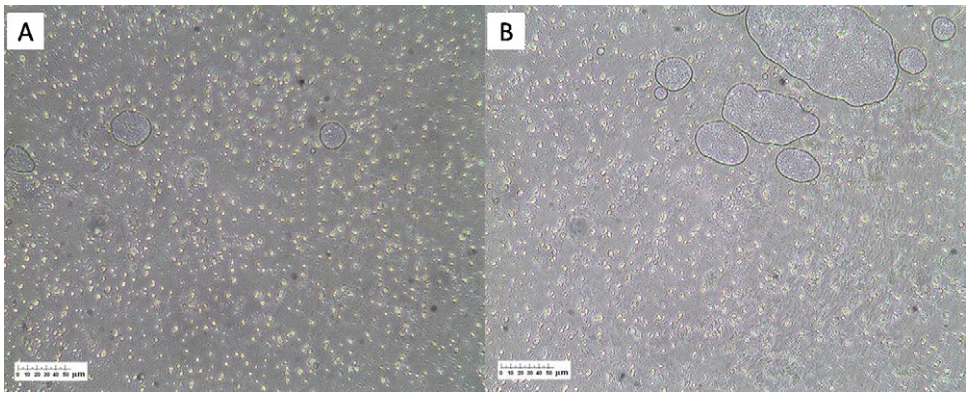

Fig. 1. AE emulsion (A) aspect compared with the correspondent aspect of emulsion AC containing Milk-Thistle-hydroxypropyl-β-cyclodextrin (MT-HP) complex (B). Pictures are obtained using an Optical Microscope at 20× magnification.

to fine spreadability and low dry time. Addition of phytocomplex on emulsions modifies the formulations appearance: they look both lightly yellow colored, phytocomplex powder mixture onto emulsions appears visible but impalpable; moreover softness and spreadability are not significantly varied. On the contrary, addition of MT dry extract causes the obtainment of a yellow cream, even if sensitive and microscopic characteristics of the emulsions are not significantly varied (Fig. 1).

3.2.1. Stability tests

Stability tests carried out demonstrate that both formulations A and B are stable after centrifuging at 3000 rpm for 30 min. Formulations are able to bear the strong mechanic stress imposed by centrifugation: about of 20% of emulsion A and 10% of emulsion B separate during tests; moreover, addition of phytocomplex of MT dry extract always increases the stability and no separated phases are observable.

3.2.2. Rheological tests

Rheological studies permit to individuate several information about topical emulsions and their performances after application. Preliminary analyses conduced at 10 rpm demonstrate that formulation A appears significantly more viscous than formulation B which is due to their different composition. From Fig. 2 it is possible to observe the viscosity variation due to the increasing or decreasing shear rate forces applied during the shear rate tests on formulations AE, BE, AC and BC.

Results obtained show that emulsions can be considered non-Newtonian fluids because of their viscosity is dependent by the shear rate applied. Constant increment and further decrement of shear rate demonstrate that variation of viscosity is dependent on the intensity of the rotating force applied; however sensibility of emulsion AE to the mechanic stress appears higher than emulsion BE as its viscosity undergoes to higher variation. At the end of the tests it is notable that viscosity of BE backs to starting values, while each viscosity measurement of AE, during increasing shear test, is higher than correspondent values recorded during decreasing shear rate test and, at the end of the test, final viscosity is significantly lower than the initial one. This behavior demonstrates that the mechanic stress causes the viscosity variation on emulsion AE.

Addition of the inclusion complex with CD does not significantly modify the viscosity of both formulations and they are able to maintain their initial viscosity after the shear stress conclusion.

3.3. Evaluation of antioxidant activity of flavonoids

Antioxidant activity of MT extract was tested by DPPH• reduction test, in particular this experiment was carried out in order to verify the variation in activity of the extract after complexation process and after vehiculation into the emulsions.

From Fig. 3 it is observable how complexation significantly decreases the antioxidant activity of phytocomplex respect to the pure dry extract. This phenomena is due to the complexation of the sylimarine into the HP cavity: the complex formation decreases the capability of flavonoids to react with DPPH• within 60 min; more time is probably necessary to permit the antiradical reaction. Therefore a lower antiradical effect but more prolonged activity could be obtained by using HP. Moreover, in vitro tests do not keep in consideration the effective drug delivery mechanism of cyclodextrins in case of topical applications in which the effective drug release is due to the drug uptake by the tissue and not by the dissociation due to the dilution fluids (Cal & Centkowska, 2008). Antiradical activity of formulation BC appears higher than its correspondent AC, which is due to the synergistic antioxidant effect of some excipients such Cocoa Butter, almond oil and hamamelis water with the extract.

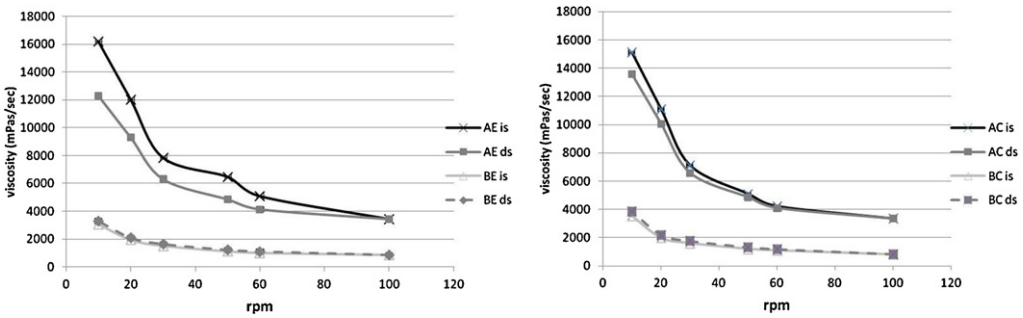

Fig. 2. Diagrams reporting the viscosity variations of emulsions containing milk thistle (MT) extract (AE and BE) or phytocomplex (AC and BC). Test indicates the viscosity values measured during the speed increment, coded as *is* (*increasing speed*) and the speed decrement, coded as *ds* (*decreasing speed*).

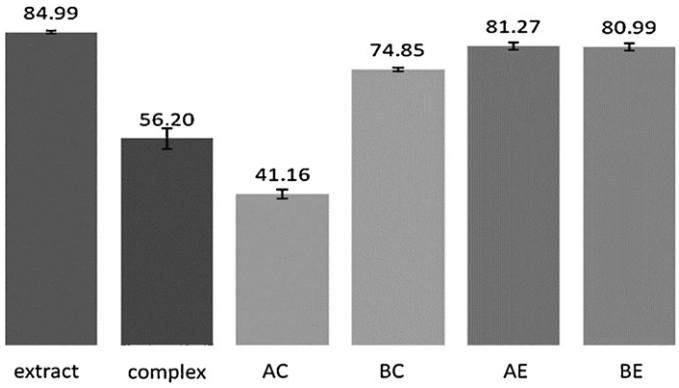

Fig. 3. Evaluation of antioxidant activity of milk thistle (MT) extract; phytocomplex (composed by milk thistle extract and hydroxypropyl-β-cyclodextrins) and loaded formulations AC and BC and AE and BE (different for composition in excipients and active ingredients formulations). Analyses were done in triplicate and expressed as DPPH• percentage of inhibition.

In vitro tests are surely not exhaustive in demonstration of antioxidant activity because they are not able to individuate all the phenomena occurring during in vivo administration but they give an adequate and satisfactory preliminary hypothesis about the effective antiradical activity of flavonoids widely used during research (Cabello-Hurtado, Gicquel, & Esnault, 2011; Rossi et al., 2003; Tabart et al., 2007; Zaouali, Bouzaine, & Boussaid, 2010). Antioxidant activity of formulations AE and BE is near to the value of MT extract alone: dispersion onto the creams does not alter the effective antioxidant activity of the extract.

3.4. In vitro dissolution tests

Tests were performed in order to establish the effective ability of the emulsions AE, BE, AC and BC to release the MT extract. Reaction medium used was selected between many others as the most useful as non-toxic dissolving agent. As it is possible to observe from Fig. 4, dissolution of emulsions and de-complexation processes are not immediate. After 2 h of test both formulations AC and BC still retain respectively more than 40 and more than 60% of extract, these results could be due to two different causes: complexation forces and dissolution rate of the emulsion into the medium. Comparison between data obtained from AC, BC, AE and BE tests shows that the most influent parameter on the release rate is the complexation linkages formed between HP and MT. Phytocomplex formation appears an useful method to guarantee the controlled delivery of sylimarine: during in vitro release tests is clearly observed a decrement of the delivery's kinetic respect to the comparison formulations AE and BE that can guarantee a release rate prolonged

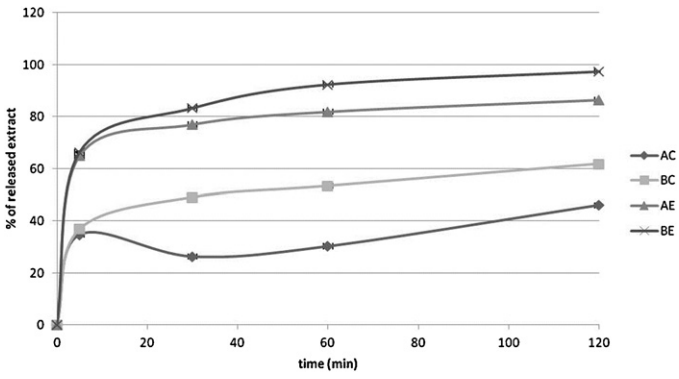

Fig. 4. In vitro drug release kinetics of both formulations containing free milk thistle (MT) extract and complexed extract. Tests were performed in triplicate ($n = 3 \pm SD$).

along several hours. Differences occurring between AC and BC profiles and between AE and BE show moreover that even composition of the emulsions, in particular their viscosity, influences the release rate: less viscous formulations BC and BE are able to release more MT extract respect to high viscous formulations AC and AE.

3.5. In vitro permeation tests

In vitro tests performed appear useful for preliminary studies establishing the drug transport through synthetic membrane simulating skin. Data obtained from AC, AE, BC and BE emulsions permit to say that all the formulations are not able to overcome synthetic barriers. None of the three kind of membranes used appears to influence the passage of the MT extract to the acceptor medium selected. Results, in fact, show that only a trifling quantity of MT extract is recovered into the acceptor medium after all the tests performed and there are no significant differences between the formulations tested. Results permit to conjecture that the passage of MT through a more complex membrane as the skin could be difficult.

3.6. Ex vivo permeation test

Permeation using pig ear skin were performed in order to simulate by an ex vivo method the absorption process of the MT through the epidermis. Nevertheless different acceptor mediums were used, results reported are obtained by using water:ethanol medium in which MT showed the highest solubility as required by USP. Literature reports that this kind of skin is the most similar to the human epidermis and for this reason is also the most used for evaluating percutaneous permeation of drugs. As expected and announced by the in vitro preliminary tests no traces of MT was found onto the acceptor medium; this first result constricts to deeper investigate about the very low permeability of all formulations. Therefore, the distribution of flavonoids in the tissue has been checked out. Results clearly demonstrate that into the samples washed from the skin about 75% of phytocomplex derived from AC and BC is present; on the contrary about 31% of MT derived from AE and BE is recovered in the washing medium. These data indicate that cyclodextrin is apparently unable to penetrate through the upper layers of the epidermis. However data obtained from the analyses of the cut out membranes about 45% of MT was found from formulations AE and BE and about 33% of sylimarine was recovered from AC and BC formulations. Test results clearly indicate that during simulated ex vivo tests HP does not act as penetration enhancer, in fact, it seems to inhibits the penetration process through the epidermis; however cyclodextrin appears as protective agents against degradation of extract (Mercader-Ros, Lucas-Abellán, Fortea, Gabaldón, & Núñez-Delicado, 2010), in fact 100% of complexed flavonoids are recovered, on the contrary about 30% of free MT extract is lost during the process from both formulations AE and BE. As it is possible to observe from Fig. 5 about 24% of MT is missed from both formulations AE and BE on the contrary the complete amount of flavonoids administered with AC and BC is recovered.

3.7. In vivo permeation test

Diffusion capacity of sylimarin from the formulation to the health skin was evaluated by the tape-stripping test. This common technique is widely used because of not-invasive and based exclusively by the removal of the most external layers of the epidermis (Tsai, Shen, Sheu, & Lu, 2003). The amount of active ingredient transferred from the skin to the strips indicates the penetration capability of the formulation through the upper layers of the skin. Data showed in Fig. 6 confirm that MT extract contained into AE and BE formulations does not easily permeate through the

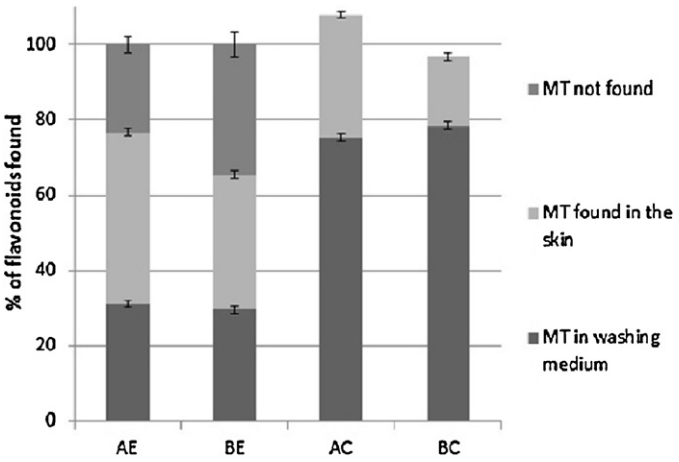

Fig. 5. Quantity of flavonoids recovered after ex vivo permeation tests (from the bottom) into the washing medium, into the homogenized pig ear epidermis and quantity of milk thistle (MT) not found (upper bar's fragment). Tests were performed in triplicate ($n = 3 \pm SD$).

epidermis: in fact about 65% of MT from both formulations is recovered in the strips; therefore, composition of creams does not favorite the active ingredient absorption. On the contrary, the presence on HP enhances the penetration of more than 80% of MT extract. During this test no lost in MT extract was observed neither in AE and BE neither in AC and BC formulations: this phenomena could be due to the absence of the physical stress (e.g. temperature higher than the room one and contact with simulated fluids for several hours) in which formulations are submitted during ex vivo tests. Opposite behavior observed between ex vivo and in vivo tests could be explained by the different treatment and viability of skin used for. However, this work confirm the need to perform in vivo tests in order to assess the influence of cyclodextrins on the substance penetration capability from formulations, as already pointed out by other authors

4. Discussion

Aim of this paper was to investigate the effect of HP on the topical administration of natural active ingredients contained into plant extracts. In particular the formation of the inclusion complex formed between HP and MT extract and the antioxidant activity of phytocomplex HP-MT were studied. Phytocomplex appears easy to reach; it can be characterized as already demonstrated by Ficarra et al. (2002) by analyzing complex formation among β-cyclodestrin and several flavonoids.

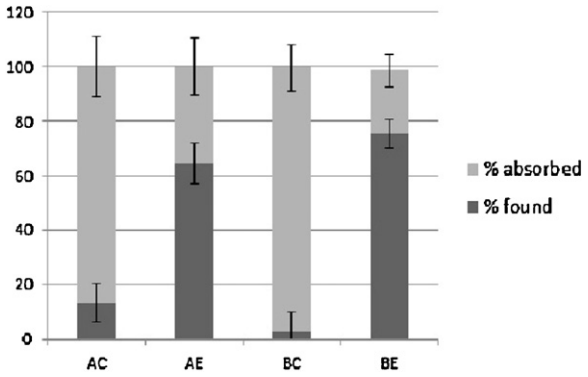

Fig. 6. Stripping test results in which are underlighted the quantities of Sylimarine effectively absorbed through the fore-harm skin (upper fragment of the bar) and the quantity adherent to the tape strip (lower fragment of the bar).

In vitro antioxidant activity of sylimarine is apparently influenced by the inclusion process of MT extract into the HP cavity. Radical reduction is, in fact, inhibited by the presence of HP that does not permit the immediate and complete reaction between sylimarine and DPPH•. This data clearly indicates that the Ka (association constant) between MT and HP is high and partially slows up the recovery capability of the free active ingredient. Several authors (Barbato, Cappello, La Rotonda, Miro, & Quaglia, 2003; Bogdan, Caira, Bogdan, Morari, & Farcas, 2004; Challa, Ahuja, Ali, & Khar, 2005; Loftsson & Olafsson, 1998) already described the effects of the constant association (Ka) between some drugs and cyclodextrins; in any case Ka influences the disruption velocity of the formed linkages and the consequent liberation of the entrapped molecules.

The high stability of the phytocomplex is furthermore demonstrated by the in vitro release tests, in which only a small amount of sylimarine was released from the phytocomplex, despite of the faster release rate of not complexed MT extract. However, a controlled release of the extract can be obtained.

Result obtained from in vitro and ex vivo permeation tests seem to emphasize the theory already described by some authors that cyclodextrins are not able to act as effective penetration enhancers of lipophilic substances through the epidermis (Matsuda & Arima, 1999; Simeoni et al., 2004). In fact, the most part of flavonoids is recovered above the epidermis after several hours of test; moreover formulations AE and BE appears more able to permit the active ingredient penetration than their corresponding formulations AC and BC. It is also important to highlight the effective activity of HP as stabilizer of MT demonstrated during ex vivo tests against degradation of MT exposed to climatic agents and emulsions components during the tests.

Despite to in vitro and ex vivo results, data obtained during in vivo tests demonstrate the effective activity of HP as penetration enhancer and the impossibility of penetration of the not complexed MT extract. In order to explain the orthogonal results obtained it appears reasonable to conjecture that the treatment of ears at 60 °C and the subsequent freezing process could cause the structural modification of the epidermis and the variation of lipid components of the tissue submitted to the ex vivo test; this pretreatment consequently can negatively influence the results from ex vivo penetration tests. Hypothesis should be reinforced by already obtained data cited by Matsuda and Arima: often active ingredient penetration through the skin is due to the cyclodextrin interaction with some skin component (Matsuda & Arima, 1999; Polyakov, Leshina, Konovalova, Hand, & Kispert, 2004). Furthemore, as already cited, the active ingredient release from cyclodextrins is regulated by several factors: during in vitro tests it is caused by dilution in fluid and on the contrary during in vivo studies it is regulated by the skin components as enzymes, proteins and lipids.

As concerning the different composition of the two emulsions produced (AC and BC), HP seems to have e determinant role in influencing release and permeation process; on the contrary emulsions AE and BE show similar performances indicating that the different ingredients do not affect release and penetration behavior. However, emulsions BE and BC, enriched in vegetal lipid substances, containing flavonoids, can act a synergistic effect with MT and at the same time protect the skin. Moreover the presence of these components improve the feeling of the emulsions during application compared to AE and AC.

5. Conclusions

Sylimarin appears one of the most useful natural active ingredients, the several potential uses known enhance to develop new

drug delivery systems able to improve its therapeutic activities as prevention of skin aging and skin diseases due to pollutant factors and sun.

These studies highlighted cyclodextrins appear good excipients for sylimarine complexation and administration; in particular, work demonstrated that flavonoids degradation can be efficiently avoided thanks to the phytocomplex formation. The apparent decrement of in vitro antioxidant activity could be compensated by the possibility to have slow flavonoids release regulated by the disruption of the binary complex; this aspect can open the possibility to fill the gap about in vivo studies of antioxidant activity. Despite of in vitro and ex vivo results, cyclodextrins act in vivo as valid penetration enhancers for topical administration respect to reference formulations. Moreover, use of two different emulsions as carriers for MT demonstrated their influence on the antioxidant activity as well as the patient compliance during administration.

References

Anchisi, C., Maccioni, A. M., Sinico, C., & Valenti, D. (2001). Stability studies of new cosmetic formulations with vegetable extracts as functional agents. *Il Farmaco*, *56*, 427–431.

Baert, B., Vansteelandt, S., & De Spiegeleer, B. (2011). Ion mobility spectrometry as a high-throughput technique for in vitrotransdermal Franz diffusion cell experiments of ibuprofen. *Journal of Pharmaceutical and Biomedical Analysis*, *55*, 472–478.

Barbato, F., Cappello, B., La Rotonda, M. I., Miro, A., & Quaglia, F. (2003). Diclofenacβ-cyclodextrin binary systems: A study in solution and in the solid state. *Journal of Inclusion Phenomena and Macrocyclic Chemistry*, *46*, 179–185.

Bogdan, M., Caira, M. R., Bogdan, D., Morari, C., & Farcas, S. (2004). Evidence of a bimodal binding between diclofenac-Na and β-cyclodextrin in solution. *Journal of Inclusion Phenomena and Macrocyclic Chemistry*, *49*, 225–229.

Cabello-Hurtado, F., Gicquel, M., & Esnault, M. A. (2011). Evaluation of the antioxidant potential of cauliflower (*Brassica oleracea*) from a glucosinolate content perspective. *Food Chemistry*, *132*, 1003–1009.

Cal, K., & Centkowska, K. (2008). Use of cyclodextrins in topical formulations: Practical aspects. *European Journal of Pharmacy and Biopharmacy*, *68*, 467–478.

Caon, T., Oliveira Costa, A. C., Leal de Oliveira, M. A., Micke, G. A., & Oliveira Simoes, O. S. (2010). Evaluation of the transdermal permeation of different paraben combinations through a pig ear skin model. *International Journal of Pharmacy*, *391*, 1–6.

Challa, R., Ahuja, A., Ali, J., & Khar, R. K. (2005). Cyclodextrins in drug delivery: An updated review. *AAPS PharmSciTech*, *6*, 329–357.

Dewanto, V., Xianzhong, W., Kafui, K., Adom, R., & Hai, L. (2000). Termal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. *Journal of Agricultural and Food Chemistry*, *50*, 3010–3014.

Ficarra, R., Tommasini, S., Raneri, D., Calabro', M. L., Di Bella, M. R., Rustichelli, C., et al. (2002). Study of flavonoids/β-cyclodextrins inclusion complexes by NMR, FT-IR, DSC, X-ray investigation. *Journal of Pharmaceutical and Biomedical Analysis*, *29*, 1005–1014.

Frum, Y., Eccleston, G. M., & Meidan, V. M. (2007). Evidence that drug flux across synthetic membranes is described by normally distributed permeability coefficients. *European Journal of Pharmacy and Biopharmacy*, *67*, 434–439.

Gabbanini, S., Matera, R., Beltramini, C., Minghetti, A., & Valgimigli, L. (2010). Analysis of in vitro release through reconstructed human epidermis and synthetic membranes of multi-vitamins from cosmetic formulations. *Journal of Pharmaceutical and Biomedical Analysis*, *52*, 461–467.

Gavini, E., Spada, G., Rassu, G., Cerri, G., Brundu, A., Cossu, M., et al. (2011). Development of solid nanoparticles based on hydroxypropyl-β-cyclodextrin aimed for the colonic transmucosal delivery of diclofenac sodium. *Journal of Pharmacy and Pharmacology*, *63*, 472–482.

Gillet, A., Lecomte, F., Hubert, P., Ducat, E., Evrard, B., & Piel, G. (2011). Skin penetration behaviour of liposomes as a function of their composition. *European Journal of Pharmacy and Biopharmacy*, *79*, 43–53.

Josef, E., Zilberman, M., & Bianco-Peled, H. (2010). Composite alginate hydrogels: An innovative approach for the controlled release of hydrophobic drugs. *Acta Biomaterialia*, *6*, 4642–4649.

Kanikkannan, N., Burton, S., Patel, R., Jackson, T., Shaik, M. S., & Singh, M. (2001). Percutaneous permeation and skin irritation of JP-8_100 jet fuel in a porcine model. *Toxicology Letters*, *119*, 133–142.

Katiyar, S. K., Korman, N. J., Mukhtar, H., & Agarwal, R. (1997). Protective effects of silymarin against photocarcinogenesis in a mouse skin model. *Journal of the National Cancer Institute*, *89*, 556–565.

Katiyar, S. K. (2007). UV-induced immune suppression and photocarcinogenesis: Chemoprevention by dietary botanical agents. *Cancer Letters*, *255*, 1–11.

Klang, V., Schwarz, J., Lenobel, B., Nadj, M., Auböck, J., Wolzt, M., et al. (2011). In vitro vs in vivo tape stripping: validation of the porcine ear model and penetration assessment of novel sucrose stearate emulsions. *European Journal of Pharmacy and Biopharmacy*, *80*, 604–614.

Křen, V., & Walterová, D. (2005). Silybin and silymarin – new effects and applications. *Biomedical Papers*, *149*, 29–34.

Loftsson, T., & Duchene, D. (2007). Cyclodextrins and their pharmaceutical applications. *International Journal of Pharmaceutics*, *329*, 1–11.

Loftsson, T., & Olafsson, J. H. (1998). Cyclodextrins: New drug delivery systems in dermatology. *International Journal of Dermatology*, *37*, 241–246.

Matsuda, H., & Arima, H. (1999). Cyclodextrins in transdermal and rectal delivery. *Advanced Drug Delivery Review*, *36*, 81–99.

Meghreji Moin, A., Patel, C., Dave, J. B., Badmanaban, R., & Patel, J. (2010). Validated method for silymarin by spectrophotometry in bulk drug and pharmaceutical formulations. *Journal of Chemical and Pharmaceutical Research*, *2*, 396–400.

Mercader-Ros, M. T., Lucas-Abellán, C., Fortea, M. I., Gabaldón, J. A., & Núñez-Delicado, E. (2010). Effect of HP-β-cyclodextrins complexation on the antioxidant activity of flavonols. *Food Chemistry*, *118*, 769–773.

Mitri, K., Shegokar, R., Gohla, S., Anselmi, C., & Müller, R. H. (2011). Lipid nanocarriers for dermal delivery of lutein: Preparation, characterization, stability and performance. *International Journal of Pharmacy*, *414*, 267–275.

Monti, D., Tampucci, S., Chetoni, P., Burgalassi, S., Saino, V., Centini, M., et al. (2011). Permeation and distribution of ferulic acid and its α-cyclodextrin complex from different formulations in hairless rat skin. *AAPS PharmSciTech*, *12*, 514–520.

Moser, K., Kriwet, K., Yogeshvar, N. K., & Guy, R. H. (2001). Enhanced skin permeation of a lipophilic drug using supersaturated formulations. *Journal of Controlled Release*, *73*, 245–253.

Nichols, J. A., & Katiyar, S. K. (2010). Skin photoprotection by natural polyphenols: Anti-inflammatory, antioxidant and DNA repair mechanisms. *Archive of Dermatological Research*, *302*, 71–83.

Organization For Economic Cooperation And Development OECD, Guideline for the testing of chemicals skin absorption in vitro method. 428 Adopted: 13 April 2004.

Polyakov, N. E., Leshina, T. V., Konovalova, T. A., Hand, E. O., & Kispert, L. D. (2004). Inclusion complexes of carotenoids with cyclodextrins: 1H NMR, EPR and optical studies. *Free Radical Biology and Medicine*, *36*, 872–880.

Realdon, N., Perin, F., Morpurgo, M., & Ragazzi, E. (2002). Influence of processing conditions in the manufacture of O/W creams. I. Effect on dispersion grade and rheological characteristics. *Il Farmaco*, *57*, 341–347.

Reichling, J., Landvatter, U., Wagner, H., Kostka, K. H., & Schaefer, U. F. (2006). In vitro studies on release and human skin permeation of Australian tea tree oil (TTO) from topical formulations. *European Journal of Pharmacy and Biopharmacy*, *64*, 222–228.

Rossi, M., Giussani, E., Morelli, R., Lo Scalzo, R., Nani, R. C., & Torreggiani, D. (2003). Effect of fruit blanching on phenolics and radical scavenging activity of highbush blueberry juice. *Food Research International*, *36*, 999–1005.

Sgorbini, B., Ruosi, M. R., Cordero, C., Liberto, E., Rubiolo, P., & Bicchi, C. (2010). Quantitative determination of some volatile suspected allergens in cosmetic creams spread on skin by direct contact sorptive tape extraction–gas chromatography–mass spectrometry. *Journal of Chromatography A*, *1217*, 2599–2605.

Shimamura, T., Tairabune, T., Kogo, T., Ueda, H., Numajiri, S., Kobayashi, D., et al. (2004). Investigation of the release test method for the topical application of pharmaceutical preparations: release test of cataplasm including nonsteroidal anti-inflammatory drugs using artificial sweat. *Chemical and Pharmaceutical Bulletin*, *52*, 167–171.

Simeoni, S., Scalia, S., & Benson, H. A. (2004). Influence of cyclodextrins on in vitro human skin absorption of the sunscreen, butylmethoxydibenzoylmethane. *International Journal of Pharmaceutics*, *280*, 163–171.

Simon, Gad A., & Maibach, Howard I. (2000). The pig as an experimental animal model of percutaneous permeation in man: Qualitative and quantitative observations – An overview. *Skin Pharmacolology and Applied Skin Physiology*, *13*, 229–234.

Singh, R. P., & Agarwal, R. (2009). Cosmeceuticals and silibinin. *Clinical Dermatology*, *27*, 479–484.

Tabart, J., Kevers, C., Sipel, A., Pincemail, J., Defraigne, J. O., & Dommes, J. (2007). Optimisation of extraction of phenolics and antioxidants from black currant leaves and buds and of stability during storage. *Food Chemistry*, *105*, 1268–1275.

Toklu, H. Z., Tunalı-Akbay, T., Erkanl, G., Yuksel, M., Ercan, F., & Sener, G. (2007). Silymarin, the antioxidant component of *Silybum marianum*, protects against burn-induced oxidative skin injury. *Burns*, *33*, 908–916.

Tsai, J. C., Shen, L. C., Sheu, H. M., & Lu, C. C. (2003). Tape stripping and sodium dodecyl sulfate treatment increase the molecular weight cutoff of polyethylene glycol penetration across murine skin. *Archive of Dermatological Research*, *295*, 169–174.

United States Pharmacopeia Forum, 2009, 725.

Von Gadow, A., Joubert, E., & Hansmann, C. F. (1997). Comparison of the antioxidant activity of aspalathin with that other plant phenols of rooibos tea (*Aspatalus linearis*), α-tocopherol, BHT, and BHA. *Journal of Agricultural and Food Chemistry*, *45*, 632–638.

Zaouali, Y., Bouzaine, T., & Boussaid, M. (2010). Essential oils composition in two *Rosmarinus officinalis* L. varieties and incidencefor antimicrobial and antioxidant activities. *Food and Chemical Toxicology*, *48*, 3144–3152.