

Nanoberries for topical delivery of antioxidants

JORGE MONTANARI, MARÍA VERA, EMANUEL MENSI,
MARÍA MORILLA, and EDER ROMERO, *Nanomedicine Research
Program, Universidad Nacional de Quilmes, B1876BXD Bernal, Pcia. de
Buenos Aires, Argentina.*

Accepted for publication May 20, 2013.

Synopsis

With the aim of improving the antioxidant activity of polyphenols from blueberries (*Vaccinium myrtillus*) on skin targets after topical application, ethanolic extracts from three blueberry varieties (named Millenia, O'Neal, and Blue Crisp) were loaded into ultradeformable liposomes. These nanocarriers are known to be capable of penetrating through the stratum corneum reaching its deeper layers and the viable epidermis. On the other hand, blueberries contain large amounts of polyphenols, whose antioxidant properties as tissue protectors against processes mediated by reactive oxygen species have been extensively proved. Blueberries are usually consumed as edible products, but their antioxidant compounds are poorly absorbed. The antioxidant properties of the extracts were screened before and after being loaded into ultradeformable liposomes made of soy phosphatidylcholine and sodium cholate, of nearly 100 nm in size at 0.223 extract/lipid w/w. The ethanolic extract-loaded ultradeformable liposomes (nanoberries) from Millenia variety retained an 85% of the antioxidant capacity of the free extract and showed low cytotoxicity on HaCaT cells (less than 20%) at active concentration against free radicals.

INTRODUCTION

Polyphenols include a great diversity of compounds, with more than one hydroxyl group on an aromatic ring among which flavonoids and several classes of nonflavonoids are usually distinguished (1). Polyphenols are highly reactive and also act as substrates for enzymes such as polyphenoloxidases, peroxidases, glycosidases and esterases. They can be found in fruits (2) and plant foods (3) to which they provide organoleptic properties such as color and flavor.

In particular, the added value related to blueberry (*Vaccinium myrtillus*) ingestion results from its high content of flavonoids, mainly positive-charged derivatives from anthocyanidines named anthocyanins (4). Anthocyanins are highly soluble in polar solvents such as water, and possess antioxidant (5–7), anti-inflammatory (8), antitumor (9), and cardio-protective (10,11) properties. Blueberries are rich in anthocyanins, with a mean total content in organic extracts of up to 6 g anthocyanins/kg of fresh fruit (12).

Address all correspondence to Jorge Montanari at jmontanari@unq.edu.ar.

In view of the potential carcinogenicity of synthetic antioxidants (13,14), the search for new sources of natural antioxidants has gained growing interest. Blueberries are usually consumed as fresh fruit or fruit juice and polyphenols undergo absorption and metabolism along the gastrointestinal tract, the liver, and the skin, to be finally eliminated via urine and bile (15). Compounds such as dietary flavonoids, with relative molecular mass over 500 which can form hydrogen bond interactions are generally unable to cross biological membranes by passive diffusion (16); thus, having practically no chance to be passively absorbed at the gastrointestinal level. This was confirmed by bioavailability studies showing levels lower to 1% of the administered dose (17). In addition, cell plasma membranes generally block the flavonoid diffusion into peripheral tissues. Anthocyanins—the main flavonoid compounds in blueberries—are rapidly absorbed in the stomach but also rapidly eliminated, showing poor efficiency in general (17). To improve the availability of natural antioxidants at their intended sites of action appears as a crucial issue.

Environmentally generated free radicals act on the skin at two levels: direct oxidation (damaging cell membranes, nucleic acids, and proteins) and indirectly by activating transcription factors for matrix metalloproteases including collagenases (18,19). An alternative strategy to increase the antioxidant effect of anthocyanins on the skin could be based on their topical application in suitable vehicles, as a key factor on the accurate reaction of these compounds with the damaging reactive oxygen species relies on their appropriate location (4,20).

With that aim, we prepared and characterized *V. myrtillus* extracts in ultradeformable liposomes (UL) (nanoberries) made of soy phosphatidylcholine and sodium cholate (NaChol). The elastic modulus of UL is nearly 20-fold lower than that of conventional liposomes (21). This enables UL to penetrate the stratum corneum (SC) presumably driven by the transepithelial humidity gradient (22), to shuttle their inner aqueous content within the viable epidermis (23) (several tens of micrometers of depth) instead of aggregate or coalesce on the skin surface as conventional liposomes do (24). Conventional liposomes for topical applications are mainly used as depots for sustained release on the skin surface. When they have been studied for the delivery of cosmetic actives into the skin, no evidence of vesicle penetration was found (25) even when actives could be effectively loaded into vesicles of different lamellarity and size (26). Nonocclusive application of UL could be a powerful tool for controlled/targeted delivery of cosmetic and/or skin-therapeutic actives (27) and it has been found that their depth of penetration beyond the SC depends on their applied amount per surface area (28). Although conventional liposomes fail to penetrate through the SC into the viable epidermis (29) where the antioxidant compounds should be delivered, UL provide a higher penetration/accumulation of hydrosoluble actives into the epidermis and potentially into the dermis, in absence of conventional permeation enhancers such as low molecular weight alcohols (30), dimethyl sulfoxide (31), or oleic acid in synergy with propylene glycol (32). Thus, although conventional liposomes cannot penetrate below the first layers of the SC after application on the skin (23), ultradeformable vesicles have been reportedly found in channel-like penetration pathways across the SC (33). Several studies of UL as delivery systems for small drugs have been reported in the last years (34–36). UL matrix composition only differs from conventional liposomes in the presence of a minor fraction of a common surfactant (e.g., NaChol, Tween 20) (29) added to the main phospholipid, whereas the process for obtaining them practically does not differ from the conventional technique for obtaining large unilamellar vesicles (23). UL have been reviewed between potentially important delivery systems for both cosmetic and cosmeceuticals (37).

MATERIALS AND METHODS

CHEMICALS

Soybean phosphatidylcholine (SPC) (phospholipon 90 G, purity >90%) was a gift from Phospholipid/Natterman (Köln, Germany). NaChol, Sephadex G-50, butylated hydroxytoluene (BHT), Folin–Ciocalteu reagent, gallic acid dimethyl-thiazolyltetrazolium bromide (MTT), linoleic acid, b-carotene, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (St. Louis, MO). Tween 80 was purchased from Riedel-de Haën (Seelze, Germany). Other reagents were analytic grade from Anedra (Tigre, Argentina). Blueberries (Millenia, O'Neal, and Blue Crisp varieties) were donated by The Berry Store (San Pedro, Argentina).

EXTRACTION OF POLYPHENOLS

The varieties from *V.myrtillus* named Millenia, O'Neal, and Blue Crisp were harvested in November (springtime in the Southern hemisphere) 2010 at "The Berry Store" plantation in San Pedro, Argentina (latitude 33°45' S, longitude 59°45' W, elevation 36 m) and maintained at -18°C. Before extraction, 25 g of each variety were defrozen at room temperature and grounded with the help of scissors. Crushed fruit from each variety was transferred to an Erlenmeyer and then, 150 ml of ethanol (96%) and HCl 0.1% were added. Erlenmeyers were submitted to bath sonication for 75 min at 25°C, 40 kHz. The resultant extracts were filtered under vacuum through Whatman No. 1 filters. Organic solvent was eliminated under rotary evaporation at 30°C, 120 rpm, and the water was eliminated by increasing vacuum and rotation rate to 250 rpm, up to constant weight. Residues were weighted and redissolved in ethanol-to-water 5:1 v/v, to a final volume of 35 ml. The process rendered extracts of 715 mg of fresh fruit per milliliter. Three extracts were obtained by this process (38,39). Hydrodistillation was discarded as an extraction method after a 48-h distillation rendered no appreciable extract.

DETERMINATION OF TOTAL POLYPHENOLIC COMPOUNDS

Total polyphenolic compounds in each extract were measured by the method of the Folin–Ciocalteu reactive (40,41) using gallic acid as a standard. Aliquots containing 0.25 mg of each extract were mixed with 11.5 ml water and 0.25 ml Folin–Ciocalteu reactive under vigorous agitation. After 3 min, 0.75 ml of Na₂CO₃ (2%) was added. The samples were incubated for 2 h, and the tubes were periodically shaken at 15-min intervals. The same procedure was followed with gallic acid in the range from 0 to 0.25 mg to obtain a calibration curve. Absorbance of samples was then measured at 760 nm.

DETERMINATION OF ANTHOCYANINS

The content of total and monomeric anthocyanins of each extract was determined by the spectrophotometric pH differential method (42,43). Aliquots of each extract were added to two solutions of KCl 0.025 M and sodium acetate 0.4 M, respectively. The first one

was adjusted to pH 1.0, and the second one to pH 4.5 with HCl and their respective absorbance was determined at 520 and 700 nm in a Shimadzu UV-Vis 160-A spectrophotometer (Shimadzu Corp., Kyoto, Japan). For the calculations, cyanidin-3-glucoside (MW 449.2; $\epsilon = 26,900$) was used as an absorbance standard (44). The anthocyanin content was determined as:

$$\text{Anthocyanins (mg/l)} = A \times \text{MW} \times \text{Dilution Factor} \times 1,000 / (\epsilon \times l)$$

The absorbance (A) for the monomeric anthocyanins was then calculated as:

$$A = (A_{510} - A_{700})_{\text{pH}1.0} - (A_{510} - A_{700})_{\text{pH}4.5}$$

whereas for the total anthocyanins, it was calculated as:

$$A = (A_{510} - A_{700})_{\text{pH}1.0}$$

PREPARATION OF NANOBERRIES: BLUEBERRY-LOADED ULTRADEFORMABLE LIPOSOMES (UL-B)

Blueberry-loaded ultraformable liposomes (UL-B) were prepared according to Cevc (21). Briefly, UL composed of SPC and NaChol at 6:1 (w/w) ratio were prepared by mixing lipids from CHCl_3 and $\text{CHCl}_3 : \text{CH}_3\text{OH}$ (1:1, v/v) solutions, respectively, that were further flash-evaporated at 40°C in a round-bottom flask until all of the organic solvent was eliminated. The thin lipid film was flushed with N_2 , and hydrated in a 7.65 mg of extract/ml solution of the ethanol extract of *Millenia* variety in 10 mM Tris-HCl buffer plus 0.9% (w/v) NaCl, pH 7.4 (Tris buffer) (extract dilution-to-Tris buffer 1:19), up to a final concentration of 43 mg SPC/ml. The suspension was sonicated (45 min with a bath type sonicator 80W, 40 kHz) and extruded 12 times through two stacked 0.2 and 0.1 μm pore size polycarbonate filters using a 100 ml Thermobarrel extruder (Northern Lipids, Burnaby, Canada). After extrusion, the nonincorporated extract of *Millenia* variety was eliminated by gel filtration chromatography in a Shepadex G-50 column using the mini-column centrifugation method (45). Ultraformability was tested allowing the UL-B suspension to pass through a 50 nm pore membrane under low pressure (less than 0.8 MPa) and comparing it with a conventional liposomal suspension (L) (46). Liposomal phospholipids were quantified by a colorimetric phosphate micro assay (47). To quantify the content of extract of *Millenia* variety in liposomes, a calibration curve was prepared by recording the absorbance of aliquots at 583.5 nm of extract of *Millenia* variety in ethanol in the presence of phosphate, by diluting a phosphate salt to the same concentration (50 mmol/ml) as in the phospholipid mix. Mean particle size and zeta potential of the UL-B were determined by dynamic light scattering with a Nanozetasizer (Malvern Instruments, Malvern, Worcestershire, UK). The antioxidant capacity retained in the UL-B was evaluated by the free radical scavenging as stated earlier.

SPECTROSCOPIC ANALYSIS

The UV-Vis spectra of the ethanolic extracts alone, in UL-B or in presence of phosphate salts at the same phosphate concentration than that of the liposomal suspension, were

recorded in a Shimadzu UV–Vis 160-A spectrophotometer. On the basis of this, a calibration curve at 524 nm of extract of *Millenia* variety in ethanol was prepared.

DIFFERENTIAL SCANNING CALORIMETRY

The effect of the ethanolic extracts on the lipid matrix of the UL-B was determined by differential scanning calorimetry (DSC) on a MDSC Q-200 TA Instruments (New Castle, DE). For the determination of the (gel to liquid–crystalline) transition phase (T_m) and the related change in enthalpy (ΔH_{cal}), samples were placed in T-zero capsules, first in isothermal equilibrium at -40°C for 5 min, then heating to 20°C at a rate of $10^\circ\text{C}/\text{min}$.

ANTIOXIDANT ACTIVITY DETERMINATION METHODS

Free radical scavenging activity The ability of the extracts to donate electrons or a hydrogen atom to the stable free radical DPPH was determined as a measure of their radical scavenging antioxidant activity (48). In brief, aliquots between 0.057 and 0.113 mg of extract per milliliter were added by triplicating to a 0.0031% (w/v) DPPH solution in methanol. After a 30-min incubation at room temperature in the dark, the remnant DPPH was determined by reading the absorbance at 515 nm against a blank of methanol. The remnant DPPH is inversely proportional to the free radical scavenging activity of the antioxidant (49). BHT and DPPH solution were used as a positive control and inhibition blank, respectively. The inhibition percentage of DPPH ($I\%$) was calculated (50) as:

$$I\% = \left[\frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \right] \times 100$$

IC_{50} values, corresponding to concentrations inhibiting a 50% of the free radical were obtained by a linear regression. The activity was recorded 30 days later to evaluate the retention of the antioxidant capacity after storage at -18°C .

β -carotene–linoleic acid assay The total antioxidant capacity of the extracts was determined according to the following method adapted from (39,51,52). An emulsion was formed from a mixture of 0.5 ml β -carotene in chloroform at 1 mg/ml; 0.02 ml linoleic acid and 0.2 ml Tween 80. Chloroform was eliminated under rotary evaporation and then 50 ml of oxygen-saturated milliQ water was added under vigorous agitation. The emulsion was aliquoted in eppendorf tubes and samples from each extract (0.85 mg of extract per milliliter) were added. BHT was used as a positive control and absolute ethanol as a negative control. The absorbance of β -carotene at 460 nm determined against a blank (the same emulsion lacking β -carotene) was recorded as a function of time in the presence of the extracts and compared with that of the negative control. 42 h later (t), when the absorbance of the negative control was reduced to 10% of the absorbance at time zero, the percentage of antioxidant activity (% AA) was determined as follows:

$$\%AA = 100 \left[\frac{1 - (A_0 - A_t)}{A_0^0 - A_t^0} \right]$$

where A_0 and A_t are the absorbances at time zero and at time t , and A_0^0 and A_t^0 the absorbances of the negative control at time zero and time t , respectively.

CELLS

A cell line derived from human keratinocytes (HaCaT) was supplied by Dr. E. Salvatierra of Fundación Instituto Leloir (Buenos Aires, Argentina). Cells were cultured in MEM (Gibco, Life Technologies, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS), 1% antibiotic/antimycotic (PAA Laboratories GmbH, Pasching, Austria) (penicillin 10,000 U/ml, streptomycin sulphate 10 mg/ml, amphotericin B 25 µg/ml) and 2 mM glutamine, at 37°C in 5% CO₂ and 95% humidity.

CYTOTOXICITY IN HaCaT CELLS

Cell viability was measured by the MTT assay. HaCaT cells were seeded at a density of 3×10^4 cells per well onto 96-well flat-bottom plates and grown for 24 h at 37°C. Then, the medium was replaced by 100 µl of fresh MEM with 5% FCS containing dilutions of the ethanolic extracts of each variety (3, 30, and 300 mg of fresh fruit per milliliter), UL-B suspension (2 and 14 mg of fresh fruit per milliliter), empty UL (at the same concentration in lipids than in UL-B) and untreated control. Cells were incubated at 37°C for 24 h. After incubation, 110 µl of 0.45 mg/ml MTT were added to cells attached to plates. After 3 h of incubation, MTT solution was removed, the insoluble formazan crystals dissolved with 100 µl of dimethylsulfoxide, and absorbance was measured at 570 nm using a microplate reader (Dynex Technologies, MRX tc, Chantilly, VA). Viability of cells was expressed as a percentage of the viability of cells grown in the medium.

RESULTS AND DISCUSSION

PHENOLICS AND ANTHOCYANIN CONTENT

The amount of polyphenolic compounds in each variety of blueberries was expressed as milligrams of equivalents of gallic acid in 100 g of extract. A calibration curve was obtained:

$$y = 0.004x - 0.0064 (R^2 = 0.9999)$$

Extracts of *Millenia* and O'Neal showed nearly twice the amount of phenolic compounds than that of the Blue Crisp extract (Fig. 1). Similarly, the anthocyanins content of *Millenia* and O'Neal extract were more than twice the amount in Blue Crisp, as presented in Fig. 2. More than 90% of the anthocyanins in all extracts were found in their monomeric

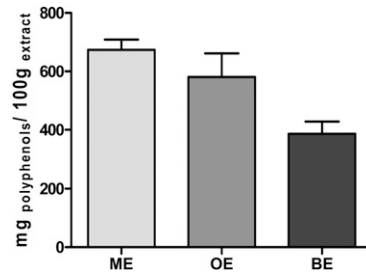


Figure 1. Content of total polyphenolic compounds in ethanolic extracts of Millenia (ME), O'Neal (OE), and Blue Crisp (BE) per 100 g of extract.

form. The monomeric fraction is responsible for the antioxidant activity of the anthocyanins, whereas the deductible amount of polymerized anthocyanins serves as a parameter for the degradation of their activity (42). Previous studies have shown higher yield and stability of anthocyanins when they are extracted in acidic media (53,54).

The amounts of anthocyanins were between 350 and 1000 mg/100 g of extract, into the expected range for the species (approximately 175–3500 mg/100 g extract) (55) and they were also proportional to the antioxidant capacity in the extracts. Previous studies have shown the correlation between the content of phenolic compounds and antioxidant activities (56).

Similar yields of phenolic compounds (and antioxidant capacity) were found in a pool of methanolic extracts obtained parallelly (data not shown, as the method was discarded due to potential toxicity of methanol traces).

PHYSICOCHEMICAL CHARACTERIZATION OF UL-B

NaChol was chosen as an edge activator to provide ultradeformability to the lipid matrix, toward the need for the delivery of the actives to deep layers into the skin as it has been remarked at the introduction of this work. The resultant UL-B were 100.6 ± 0.1 nm of diameter, with unimodal distribution (polydispersion index of 0.042) and a zeta potential of -10.57 mV. Their concentration in phospholipids was $46.85 \mu\text{mol/ml}$, and their extract content was 7.93 mg of extract per milliliter (the calibration curve for the extract showed linearity in the concentration range between 0 and 6 mg/ml with a linear correlation coefficient over 0.999). The efficiency of encapsulation was 52.5% and UL-B were

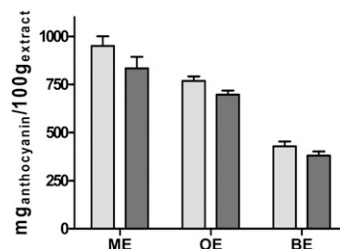


Figure 2. Total (light gray) and monomeric (dark gray) anthocyanin content (expressed as milligrams of anthocyanin in 100 g of extract) in ethanolic extracts of Millenia (ME), O'Neal (OE), and Blue Crisp (BE).

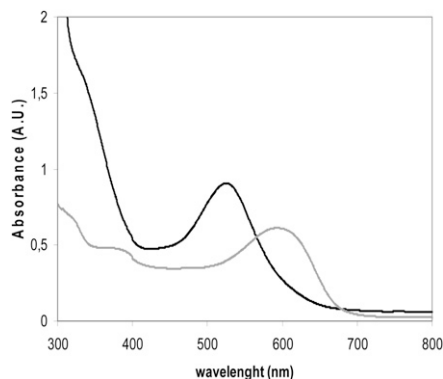


Figure 3. Spectra of the ethanolic extract of *Millenia* (dark plot) and bathochromic shift (gray plot) observed after loading the extract into UL-B. Similar shifts were observed when free extracts were mixed with phosphate salts.

3.5-fold more deformable than L when forced to pass through a 50-nm pored membrane under low pressure.

The color of the UL-B was gray, whereas the blueberry extracts were violet. The spectra showed that the chromophore seen at 520 nm in the free extracts showed a bathochromic shift when incorporated to liposomes (Fig. 3). This effect has been previously reported when anthocyanins interact with other biological molecules (57). As it is shown in the work of Atrooz (58), this could be due to the association of anthocyanins with the phosphate groups of the lipids in the inner layer of the membrane. Evidence toward this direction was that the same chromatic shift was seen when samples of the free extracts were mixed with phosphate salts as described earlier to determine the extract content in liposomes.

DIFFERENTIAL SCANNING CALORIMETRY

Calorimetric measures were used to determine the interaction between molecules present in the extract and the membrane of UL-B. Thermotropic profiles of empty UL and UL-B are shown in Fig. 4. A shift of $+0.25^{\circ}\text{C}$ in the endothermic peak from the gel to liquid-crystalline transition (T_m) was seen in UL-B with respect to UL. In addition, a change in the phase transition enthalpy (from -59 to -25.6 J/ μmol of phospholipids) was observed.

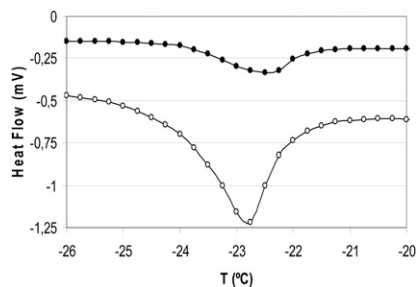


Figure 4. DSC analysis of UL (filled circles) and UL-B (empty circles), showing the shift and the lowering on the peak from the gel to liquid-crystalline phase transition.

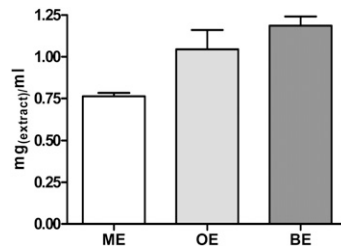


Figure 5. IC₅₀ of radical scavenging for ethanolic extracts of Millenia (ME), O'Neal (OE), and Blue Crisp (BE), expressed as milligrams of extract per milliliter.

These results could show that although the polar compounds in the extract are mainly dissolved in the inner aqueous space of the liposomes, some molecules interact with phosphate groups of the phospholipids (57) in lamellar phase that are oriented to the interior of these unilamellar liposomes (58), as suggested earlier. This interaction did not affect the bilayer deformability.

ANTIOXIDANT CAPACITY

The radical scavenging of each extract, measured as IC₅₀ is shown in Fig. 5. It was found that Blue Crisp IC₅₀ >> O'Neal IC₅₀ > Millenia IC₅₀; Millenia extract being the one with the highest antioxidant activity. The total antioxidant activity for all extracts in concentrations of 0.75 mg of extract per milliliter was higher than 90% (data not shown).

One month later, the Millenia and O'Neal extracts retained the 100% of their radical scavenging activity whereas the %AA for the Blue Crisp extract was a 25% lower (Fig. 6).

It was because of its high antioxidant capacity and high content in polyphenols and anthocyanins that the Millenia extract was selected for being loaded in UL. The antioxidant activity of radical scavenging of the UL-B was 85% of that of the same amount of free extract.

CYTOTOXICITY IN HaCaT CELLS

The extracts were not cytotoxic on HaCaT cells in concentrations close to their IC₅₀. Ten-fold higher concentrations were not cytotoxic either after a 24-h incubation. Only at

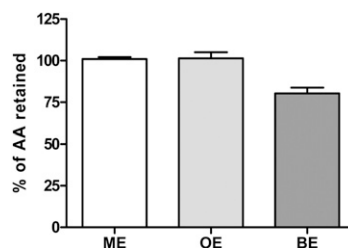


Figure 6. Retention of the antioxidant activity (AA) of the ethanolic extracts of Millenia (ME), O'Neal (OE), and Blue Crisp (BE) after 1-month storage at -18°C . Values have been obtained by measuring the AA on the 31st day on aliquots with the IC₅₀ concentration of day 1.

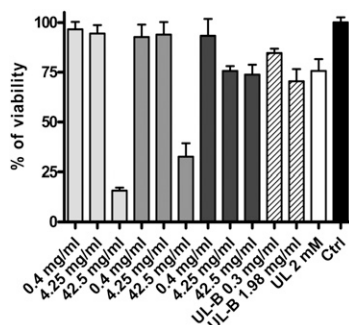


Figure 7. Cytotoxicity in HaCaT cell line after exposure to ethanolic extracts of *Millenia* (light gray), *O'Neal* (medium gray), and *Blue Crisp* (dark gray) in concentrations of 0.4, 4.25, and 42.5 mg of extract per milliliter; UL-B suspension in two concentrations expressed in terms of the loaded ethanolic extract of *Millenia*; empty UL (at 2 mM of phospholipids, intermediate between the concentration tested for UL-B); and the untreated control.

concentrations 100-fold higher than the IC_{50} , the cell viability was lowered under the 50% when incubated with *Millenia* or *O'Neal* extracts, as shown in Fig. 7.

Cell viability was around 80% when incubated with UL-B. Similar values were obtained with empty ultradeformable matrices in the same range of lipid concentration. Free extracts in the same concentration than into UL-B did not affect cell viability. These results showed that the low cytotoxic effect of UL-B could be related to the lipid matrix more than to the loaded extracts. In total, the high cell viability at working concentrations of both extracts and liposomes on a corneocytic-originated cell line is a good advice with regard to the biocompatibility of this system intended for topical application.

CONCLUSIONS

Nanoberryes were obtained by loading ethanolic extract of blueberry into UL. UV-Vis spectroscopy showed an interaction between the anthocyanins and the phosphate groups of lipids in the internal layer of the liposomes, and this was later confirmed by DSC. This interaction could lead to the high retention of the antioxidant activity determined in UL-B. The antioxidant activity depended on the variety of blueberries. The cell viability after incubation with UL-B was over 80%. This nanosystem for topical delivery could be an important tool for providing high levels of antioxidant activity from a natural product beyond the barrier of the SC.

ACKNOWLEDGMENTS

Jorge Montanari, María Morilla, and Eder Romero are members of the Carrera del Investigador del CONICET (National Council of Science and Technology Research in Argentina), which granted this work and gave approval to the "Native vegetal species extract-based nanocosmetics" research plan. Biotechnology students María Vera and Emanuel Mensi have been granted with the fellowship Beca Bicentenario of the Ministerio de Educación of the Argentine government. Jorge Montanari thanks Dr. Silvia Del Valle Alonso for her invaluable help in revising this work. The authors also thank

Phospholipid/Natterman (Köln, Germany) for providing the SPC and “The Berry Store” (San Pedro, Argentina) for kindly supporting our research by donating the blueberries.

REFERENCES

1. C. S. Evans, “Methods in Plant Biochemistry: 1. Plant Phenolics,” in *Phytochemical Analysis*, J. B. Harborne, Ed. (Academic Press, New York and London, 1991), Vol. 2, Issue 1, p. 48.
2. J. J. Macheix, A. Fleuriet, and J. Billot, *Fruit Phenolics* (CRC Press, Boca Raton, FL, 1990).
3. F. Shahidi and M. Naczk, *Food Phenolics: Sources, Chemistry, Effects, Applications* (Technomic Publishing Company, Lancaster, 1995).
4. R. L. Prior, G. Cao, A. Martin, E. Sofic, J. McEwen, C. O'Brien, N. Lischner, M. Ehlenfeldt, W. Kalt, G. Krewer, and C. M. Mainland, antioxidant capacity as Influenced by total phenolic and anthocyanin content, maturity, and variety of *Vaccinium* species. *J. Agr. Food Chem.*, 46(7), 2686–2693 (1998).
5. C. A. Rice-Evans and N. J. Miller, ChemInform abstract: Structure—antioxidant activity relationship of flavonoids and isoflavonoids. *ChemInform*, 29(19) (1998).
6. S. Y. Wang and H. S. Lin, Antioxidant activity in fruits and leaves of blackberry, raspberry, and strawberry varies with cultivar and developmental stage. *J. Agr. Food. Chem.*, 48(2), 140–146 (2000).
7. M. P. Kahkonen, A. I. Hopia, and M. Heinonen, Berry phenolics and their antioxidant activity. *J. Agr. Food Chem.*, 49(8), 4076–4082 (2001).
8. N. P. Seeram, Berry fruits: Compositional elements, biochemical activities, and the impact of their intake on human health, performance, and disease. *J. Agr. Food Chem.*, 56(3), 627–629 (2008).
9. B. N. Ames, Dietary carcinogens and anticarcinogens. Oxygen radicals and degenerative diseases. *Science*, 221(4617), 1256–1264 (1983).
10. M. G. Hertog, E. J. Feskens, P. C. Hollman, M. B. Katan, and D. Kromhout, Dietary antioxidant flavonoids and risk of coronary heart disease: The Zutphen Elderly Study. *Lancet*, 342(8878), 1007–1011 (1993).
11. P. Knekt, R. Jarvinen, A. Reunanen, and J. Maatela, Flavonoid intake and coronary mortality in Finland: A cohort study. *BMJ*, 312(7029), 478–481 (1996).
12. M. P. Kähkönen, J. Heinämäki, V. Ollilainen, and M. Heinonen, Berry anthocyanins: Isolation, identification, and antioxidant activities. *J. Sci. Food. Agr.*, 83(14), 1403–1411 (2003).
13. B. Sun and M. Fukuhara, Effects of co-administration of butylated hydroxytoluene, butylated hydroxyanisole, and flavonoids on the activation of mutagens and drug-metabolizing enzymes in mice. *Toxicology*, 122(1–2), 61–72 (1997).
14. M. Hirose, Y. Takesada, H. Tanaka, S. Tamano, T. Kato, and T. Shirai, Carcinogenicity of antioxidants BHA, caffeic acid, sesamol, 4-methoxyphenol, and catechol at low doses, either alone or in combination, and modulation of their effects in a rat medium-term multi-organ carcinogenesis model. *Carcinogenesis*, 19(1), 207–212 (1998).
15. C. Manach, A. Scalbert, C. Morand, C. Rémésy, and L. Jiménez, Polyphenols: Food sources and bioavailability. *Am. J. Clin. Nutr.*, 79(5), 727–747 (2004).
16. C. A. Lipinski, F. Lombardo, B. W. Dominy, and P. J. Feeney, Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug. Deliver. Rev.*, 46(1–3), 3–26 (2001).
17. C. Manach, G. Williamson, C. Morand, A. Scalbert, and C. Rémésy, Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am. J. Clin. Nutr.*, 81(1), 230S–242S (2005).
18. G. Murphy and H. Nagase, Progress in matrix metalloproteinase research. *Mol. Aspects. Med.*, 29(5), 290–308 (2008).
19. M. Cascales Angosto and J. A. Álvarez-Gómez, Metaloproteinasas, matriz extracelular y cáncer. *An. R. Acad. Nac. Farm.*, 76(1), 59–84 (2010).
20. D. Burdulis, A. Sarkinas, I. Jasutiene, E. Stackevicene, L. Nikolajevs, and V. Janulis, Comparative study of anthocyanin composition, antimicrobial and antioxidant activity in bilberry (*Vaccinium myrtillus* L.) and blueberry (*Vaccinium corymbosum* L.) fruits. *Acta Pol. Pharm.*, 66(4), 399–408 (2009).
21. G. Cevc and G. Blume, Lipid vesicles penetrate into intact skin owing to the transdermal osmotic gradients and hydration force. *Biochim. Biophys. Acta.*, 1104(1), 226–232 (1992).
22. G. Cevc and D. Gebauer, Hydration-driven transport of deformable lipid vesicles through fine pores and the skin barrier. *Biophys. J.*, 84(2 Pt 1), 1010–1024 (2003).

23. J. Montanari, C. Maidana, M. I. Esteva, C. Salomon, M. J. Morilla, and E. L. Romero, Sunlight triggered photodynamic ultradeformable liposomes against *Leishmania braziliensis* are also leishmanicidal in the dark. *J. Control Release*, 147(3), 368–376 (2010).
24. D. D. Verma, S. Verma, G. Blume, and A. Fahr, Liposomes increase skin penetration of entrapped and nonentrapped hydrophilic substances into human skin: A skin penetration and confocal laser scanning microscopy study. *Eur. J. Pharm. Biopharm.*, 55(3), 271–277 (2003).
25. C. Sinico, M. Manconi, M. Peppi, F. Lai, D. Valenti, and A. M. Fadda, Liposomes as carriers for dermal delivery of tretinoin: In vitro evaluation of drug permeation and vesicle–skin interaction. *J. Control Release*, 103(1), 123–136 (2005).
26. S. Golmohammadzadeh, M. R. Jaafari, and N. Khalili, Evaluation of liposomal and conventional formulations of octyl methoxycinnamate on human percutaneous absorption using the stripping method. *J. Cosmet. Sci.*, 59(5), 385–398 (2008).
27. J. Montanari, M. J. Morilla, and E. L. Romero, “Ultradeformable Liposomes for Topic Application of Cosmetic Actives can Modulate Their Destination Across the Stratum Corneum,” in *26th IFSCC Congress 2010 Innovation and Responsibility: Cosmetics Forever*, Buenos Aires, Argentina (International Federation of Societies of Cosmetic Chemists, 2010).
28. G. Cevc, Lipid vesicles and other colloids as drug carriers on the skin. *Adv. Drug. Deliver. Rev.*, 56(5), 675–711 (2004).
29. M. M. Elsayed, O. Y. Abdallah, V. F. Naggar, and N. M. Khalafallah, Lipid vesicles for skin delivery of drugs: Reviewing three decades of research. *Int. J. Pharm.*, 332(1–2), 1–16 (2007).
30. L. Denoble, K. Knutson, and T. Kurihara-Bergstrom, Enhanced skin permeability by ethanol: Mechanistic studies of human stratum corneum measured by DSC and FTIR. *Pharm. Res.*, 4(59s) (1987).
31. G. Embery and P. H. Dugard, The isolation of dimethyl sulfoxide soluble components from human epidermal preparations: A possible mechanism of action of dimethyl sulfoxide in effecting percutaneous migration phenomena. *J. Invest. Dermatol.*, 57(5), 308–311 (1971).
32. J. Hadgraft, Passive enhancement strategies in topical and transdermal drug delivery. *Int. J. Pharm.*, 184(1), 1–6 (1999).
33. P. L. Honeywell-Nguyen, A. M. de Graaff, H. W. Groenink, and J. A. Bouwstra, The in vivo and in vitro interactions of elastic and rigid vesicles with human skin. *Biochim. Biophys. Acta.*, 1573(2), 130–140 (2002).
34. M. Trotta, E. Peira, M. E. Carlotti, and M. Gallarate, Deformable liposomes for dermal administration of methotrexate. *Int. J. Pharm.*, 270(1–2), 119–125 (2004).
35. M. M. Elsayed, O. Y. Abdallah, V. F. Naggar, and N. M. Khalafallah, Deformable liposomes and ethosomes as carriers for skin delivery of ketotifen. *Pharmazie*, 62(2), 133–137 (2007).
36. R. R. Boinpally, S. L. Zhou, S. Poondru, G. Devraj, and B. R. Jasti, Lecithin vesicles for topical delivery of diclofenac. *Eur. J. Pharm. Biopharm.*, 56(3), 389–392 (2003).
37. D. Li, Z. Wu, N. Martini, and J. Wen, Advanced carrier systems in cosmetics and cosmeceuticals: a review. *J. Cosmet. Sci.*, 62(6), 549–563 (2011).
38. A. Căta, M. Ștefănu, C. Tănăsie, and R. Pop, Comparative analysis of bilberries alcoholic extracts regarding to anthocyanins content, total phenolics and antioxidant activity. *Ovidius Univ. Ann. Chem.*, 21(1), 15–19 (2010).
39. M. Gulluce, F. Sahin, M. Sokmen, H. Ozer, D. Daferera, A. Sokmen, M. Polissiou, A. Adiguzel, and H. Ozkan, Antimicrobial and antioxidant properties of the essential oils and methanol extract from *Mentha longifolia* L. ssp. *longifolia*. *Food Chem.*, 103(4), 1449–1456 (2007).
40. K. Slinkard and V. L. Singleton, Total phenol analysis: automation and comparison with manual methods. *Am. J. Enol. Viticult.*, 28(1), 49–55 (1977).
41. S. F. Chandler and J. H. Dodds, The effect of phosphate, nitrogen, and sucrose on the production of phenolics and solasidine in callus cultures of *Solanum laciniatum*. *Plant Cell Reports*, 2, 105–108 (1983).
42. E. E. Nicoue, S. Savard, and K. Belkacemi, Anthocyanins in wild blueberries of Quebec: Extraction and identification. *J. Agr. Food Chem.*, 55(14), 5626–5635 (2007).
43. M. M. Giusti and R. E. Wrolstad, “Characterization and Measurement of Anthocyanins by UV-Visible Spectroscopy,” in *Current Protocols in Food Analytical Chemistry* (John Wiley & Sons Inc, Hoboken, NJ, 2001).
44. F. J. Francis and P. C. Markakis, Food colorants: Anthocyanins. *Crit. Rev. Food Sci.*, 28(4), 273–314 (1989).

45. D. W. Fry, J. C. White, and I. D. Goldman, Rapid separation of low molecular weight solutes from liposomes without dilution. *Anal. Biochem.*, 90(2), 809–815 (1978).
46. G. Cevc, “Material Transport Across Permeability Barriers by Means of Lipid Vesicles,” in *Handbook of Biological Physics*, Lipowsky and Sachman, Eds. (Elsevier Science BV, Amsterdam, 1995), pp. 465–489.
47. C. J. F. Bötcher, C. M. Van Gent, and C. Pries, A rapid and sensitive submicro phosphorus determination. *Anal. Chim. Acta*, 24, 203–204 (1961).
48. K. Shimada, K. Fujikawa, K. Yahara, and T. Nakamura, Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. *J. Agr. Food Chem.*, 40(6), 945–948 (1992).
49. M. Burits and F. Bucar, Antioxidant activity of *Nigella sativa* essential oil. *Phytother. Res.*, 14(5), 323–328 (2000).
50. A. Adiguzel, H. Ozer, M. Sokmen, M. Gulluce, A. Sokmen, H. Kilic, F. Sahin, and O. Baris, Antimicrobial and antioxidant activity of the essential oil and methanol extract of *Nepeta cataria*. *Pol. J. Microbiol.*, 58(1), 69–76 (2009).
51. A. Dapkevicius, R. Venskutonis, T. A. van Beek, and J. P. H. Linssen, Antioxidant activity of extracts obtained by different isolation procedures from some aromatic herbs grown in Lithuania. *J. Sci. Food Agr.*, 77(1), 140–146 (1998).
52. Y. S. Velioglu, G. Mazza, L. Gao, and B. D. Oomah, Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. *J. Agr. Food Chem.*, 46(10), 4113–4117 (1998).
53. R. E. Wrolstad, “Anthocyanins,” in *Natural Food and Colorants Science and Technology*, G. J. Lauro and F. J. Francis, Eds. (Marcel Dekker, New York, 2000), pp. 237–252.
54. W. Kalt, J. E. McDonald, and H. Donner, Anthocyanins, phenolics and antioxidant capacity of processed lowbush blueberry product. *J. Food Sci.*, 65, 390–393 (2000).
55. H. Böhm, G. Mazza, and E. Miniati, Anthocyanins in Fruits, Vegetables and Grains,, 362 Seiten, zahlr. Abb. und Tab. (CRC Press, Boca Raton, Ann Arbor, London, Tokyo, 1994), *Food/Nabrung*, Vol. 38, Issue 3, p. 343.
56. C. C. Huang, H. F. Wang, C. H. Chen, Y. J. Chen, and K. H. Yih, A study of four antioxidant activities and major chemical component analyses of twenty-five commonly used essential oils. *J. Cosmet. Sci.*, 62(4), 393–404 (2011).
57. A. D. Sarma and R. Sharma, Anthocyanin-DNA copigmentation complex: Mutual protection against oxidative damage. *Phytochemistry*, 52(7), 1313–1318 (1999).
58. O. M. Atrooz, The incorporation effects of methanolic extracts of some plant seeds on the stability of phosphatidylcholine liposomes. *Pak. J. Biol. Sci.*, 10(10), 1643–1648 (2007).

