

SUPPLEMENTARY MATERIAL

Annurca Apple Peel Extract: from the chemical composition, through the functional activity, to the formulation and characterization of a topical Oil-in-Water emulsion

Francesca Sansone, Tiziana Esposito, Teresa Mencherini*, Anna Lisa Piccinelli, Patrizia Gazzo, Patrizia Picerno, Paola Russo, Pasquale Del Gaudio, Massimiliano Essolito, Pietro Campiglia and Rita P. Aquino

Department of Pharmacy, University of Salerno, Via Giovanni Paolo II, 132, 84084 Fisciano (SA), Italy;

* Author to whom correspondence should be addressed; E-Mail: tmencherini@unisa.it; Tel. +39 (0)89 968294.

Abstract

The aim of this study was to produce a hydro-alcoholic safe antioxidant *Malus pumila* Miller cv Annurca peel extract (APE) useful as functional ingredient in an oil-in-water emulsion. Results showed that APE contains a hydroxycinnamic acid (chlorogenic acid), flavonol glycosides (quercetin derivatives) and a dihydrochalcone, phloridzin (phloretin-2-O-glucoside). The isoquercitrin (quercetin-3-O-glucoside) content was quantified in 0.3 % w/w of extract. APE showed a significant and concentration dependent free radical scavenging activity correlated to its polyphenols content. No cytotoxic effect was observed in Primary Human Epidermal Keratinocyte adults and Dermal Fibroblast cell lines. The formulative approach led to produce a stable emulsion able to load a high amount of APE, up to 6.0 % w/w. The homogenous distribution of APE in the emulsion was clearly demonstrated by fluorescence microscopy analysis. The emulsion resulted able to enhance the *in vitro* release rate of APE through synthetic membranes with respect to the raw material.

Keywords: Annurca apple peel extract (APE); chemical analysis; free-radical scavenger; topical emulsion; formulation stability; *in vitro* permeation test.

Experimental Section

Materials

HPLC grade methanol (MeOH), Acetic acid, Acetonitrile, ethanol 96% and distilled water from Carlo Erba (Rodano, Italy). HPLC grade water (18m Ω) was prepared using a Millipore Milli-Q purification system (Millipore Corp., Bedford, MA). Isoquercitrin, quercetin, Folin-Ciocalteu phenol reagent, and 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) from Sigma Chemical Co. (Milano, Italy). Cetearyl Alcohol and Cetearth-33 from Seppic (Puteaux, France). Coco-Caprylate/Caprates, and Caprylic/Capric Triglyceride from Group Basf (Ludwingshafen, Germany). C12-15 Alkyl Benzoate, Butyrospermum Parkii Butter, Cetearyl Alcohol, Persea Gratissima Oil, Dimethicone, Disodium EDTA, and Glycerin from A.C.E.F. (Piacenza, Italy). Xanthan Gum from Farmalabor (Barletta-Andria-Trani, Italy). The preservative solution containing Phenoxyethanol, Benzoic Acid, Dehydroacetic Acid, and Ethylhexylglycerin from Schulker & Mayr (Norderstedt, Germany).

Apple fruits material and extract preparation

Annurca (*M. pumila* Miller cv Annurca) apple fruits were acquired in a local supermarket. The apples were washed with water, wipe up and weighed (3.7 Kg). The peels were removed using a knife, and lyophilized after frozen in liquid nitrogen (110.0 g). A portion of the lyophilized peels (60.0g) was extracted for 24 h under continuous magnetic stirring at room temperature with ethanol:water (7:3). The solvent was removed with a rotavapor (Rotavapor R 200, Buchi, Switzerland) at 45 °C to give 20.0 g of residue (APE).

HPLC-DAD-ESI-MS Analysis

HPLC-DAD-MS analyses were performed with a HPLC system (ThermoFinnigan, San Jose, CA) including a Surveyor LC pump, Surveyor autosampler, Surveyor DAD detector, and LCQ Advantage ion trap mass spectrometer equipped with Xcalibur 3.1 software. Chromatographic separations were achieved with a Luna C18 (150 mm \times 2.0 mm, 5 μ m, Phenomenex) column protected by its Guard Cartridge (4 mm \times 2.0 mm i.d.). A linear gradient of water (solvent A) and acetonitrile/water 1:1 (solvent B), both containing 1% (v/v) acetic acid, from 10 to 55% B in 55 minutes, followed by a column washing (acetonitrile 100%) and re-equilibrating step, was adopted. Elution was performed at a flow rate of 300 μ L min⁻¹, and the volume of the injection was 10 μ L. Detection by diode array was performed simultaneously at two different wavelengths: 278 and 350 nm. The UV spectra were recorded in the range 200–600 nm. The mass analyses were performed with an ESI interface in the positive mode. The data were acquired in the full scan (range of m/z 200–1500) and tandem mass scanning modes (MS/MS and MS³, with a percentage of collision energy range of 32–45%). The setted instrumental parameters were: capillary temperature 250 °C; capillary voltage 34 V; spray voltage 5.10 kV; maximum injection time was 50 ms; number of microscans was three; sheath and auxiliary gas flow rates 30 and 5 (nitrogen, arbitrary units), respectively.

Quantitative HPLC-DAD analysis of APE

Quantitative HPLC analysis was carried out on an Agilent 1100 series system equipped with a Model G 1312 pump, Rheodyne Model G-1322A loop (20 μ l), and a DAD G-1315 A detector. Peaks area was calculated with an Agilent integrator. HPLC conditions were the same used for the HPLC-DAD-ESI-MS analysis. Detection by diode array was performed at 278 nm. The reference standard solutions of isoquercitrin were prepared at three concentration levels in the range 25.0-125.0 μ g/mL. The standard curve was analyzed using the linear least-square regression equation derived from the peak area (regression equation $y = 1335.8 x + 760.01$, $r = 0.9994$ where y is the peak area and x the concentration). The peak associated with isoquercitrin was identified by its retention time and confirmed by co-injection. UV and MS spectra were compared with those of standard compound. APE was dissolved in MeOH and analyzed under the same chromatographic conditions.

Quantitative determination of total phenols

MeOH dissolved APE was analyzed for its total phenolic content according to the Folin-Ciocalteu colorimetric method (Picerno et al. 2011 a). Briefly, an aliquot of APE (50 μ L) was added to 450 μ L of distilled water in the test tube, followed by 500 μ L of Folin-Ciocalteu reagent. After 3 min, 500 μ L of 10% water solution sodium carbonate was added. Tube contents were vortexed and the reaction mixture adsorbance was read at 723 nm after 1 h. The total polyphenol content was expressed as quercetin equivalents (μ g/mg APE, means \pm S.D. of three determinations).

Bleaching of the Free-radical 1,1-Diphenyl-2-picrylhydrazyl (DPPH Test)

The antiradical activity of APE and its major compound was determined using the stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), α -tocopherol and L-Ascorbic acid as positive controls, and the procedures previously described by Mencherini et al., 2011. Briefly an aliquot (37.5 μ L) of the MeOH solution containing different amounts of APE was added to 1.5 ml of daily prepared DPPH solution (0.025 g/L in MeOH); the maximum concentration employed was 600 μ g/mL. An equal volume (37.5 μ L) of the vehicle alone was added to control tubes. Absorbance at 517 nm was measured on Evolution 201 UV/VIS Spectrometer (Thermo Scientific, Waltham, MA, USA) 10 min after starting the reaction. All experiments were carried out in triplicate and the mean effective scavenging concentrations (IC₅₀) were calculated by using the Litchfield & Wilcoxon protocol (Picerno et al. 2011 b; Sansone et al. 2014 a).

***In vitro* cytotoxic activity**

Primary Human Dermal Fibroblasts (HDFa) and Primary Human Epidermal Keratinocytes (HEKa), isolated from adult skin, were obtained from Gibco, Life Technology Corporation. HDFa cells were routinely grown in M106 medium supplemented with 100 U/mL penicillin, 100 μ g/mL streptomycin, fetal bovine serum (2% v/v), hydrocortisone (1 μ g/mL), human epidermal growth factor (10 ng/mL) and basic fibroblast growth factor/heparin (10 μ g/mL), in monolayer cultures at 37°C in a humidified atmosphere containing 5% CO₂ in air. HEKa cells were routinely grown in M154 basal medium supplemented with 100 U/mL penicillin, 100 μ g/mL streptomycin, bovine

pituitary extract (0.2% v/v), bovine insulin (5 µg/mL), hydrocortisone (0.18 µg/mL), bovine transferrin (5 µg/mL) and human epidermal growth factor (0.2 ng/mL), in monolayer cultures at 37°C in a humidified atmosphere containing 5% CO₂ in air. All reagents and supplements for cell cultures were purchased from Life Technologies.

HDFa (4 x 10³ cells per well) and HEKa (6 x 10³ cells per well) were cultured in triplicate in 96 well plates in a final volume of 100 µL of M106 complete medium and in M154 complete medium, respectively. DMSO dissolved APE was added to the cells to achieve final concentrations ranging from 0.06 mg/mL to 6 mg/mL. After 20 h or 44 h of growth at 37 °C, MTT (3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide) was added to each well (0.5 mg/mL, final concentration) and plates were incubated for 4 h at 37°C. Formazan product was solubilized with 100 µL of lysis buffer (10% SDS-0.01M HCl) and the absorbance of each well was measured at 570 nm using a plate reading spectrophotometer (Mencherini et al. 2009; Aquino et al. 2014).

Topical Oil-in-Water (O/W) emulsion containing APE

The oily (Cetearyl Alcohol, Cetareth-33, Caprylic/Capric Triglyceride, Coco-Caprylate/Caprate, C12-15 Alkyl Benzoate, Cetearyl Alcohol, Butyrospermum Parkii Butter, Persea Gratissima Oil, and Dimethicone) and the aqueous phase (Water, Glycerin, Xanthan Gum, and Disodium EDTA) (Table S2) were separately heated at 70°C. The oily phase was added to the aqueous phase under mechanical agitation by Silverson model SL2T (CRAMI Group srl, Milano, Italy) at 8000 rpm for 10 min. The obtained emulsion was manually stirred until 35°C, then the preservative solution was added. Finally, 6.0 % w/w of APE, previously solubilized in a small amount of ethanol: water (7:3) in order to facilitate its incorporation in the emulsion, was slowly introduced as active ingredient at room temperature. A formulation without APE (blank emulsion) was prepared with the same procedure, and used as control.

O/W emulsion morphological analysis

Morphology of the blank and APE-loaded emulsions was examined by fluorescent microscopy (FM). The samples were spread on a slide and dried under inert nitrogen flow. The dried sample was imaged by FM using a Zeiss Axiophot fluorescence microscope, with a 40x and 63x ± 1.4 NA plan Apochromat oil immersion objectives (Carl Zeiss Vision, München–Hallbergmoos, Germany) using standard DAPI (40,6-diamidino-2-phenylindole) optics that adsorb violet radiation (max 372 nm) and emit a blue fluorescence (max 456 nm).

O/W emulsion accelerated stability test

The formulations stability was evaluated according to Colipa guidelines (2004). During 3 months, both APE-loaded and blank emulsions were stored in glass jars at room temperature (24.0 ± 2.0 °C), lower temperature (4.0 ± 1.0 °C), and higher temperature (40.0 ± 1.0 °C). The macroscopic properties, physiochemical characteristics, as well as the centrifuge stability were evaluated after 48 h, 7, 15, 30, 60 and 90 days of storage. At the pre-determined times, samples were removed from the storage conditions and allowed to achieve room temperature (24.0 ± 2.0 °C) prior to the analysis.

Macroscopic analysis

Each emulsion was evaluated to detect visible modifications of organoleptic characteristics (color, odor, appearance) or instabilities such as creaming, coalescence, and/or separation of phases.

Centrifuge test

10 g of emulsion were placed in a graduated tube and underwent to centrifugation test at 25 °C using a Labofuge 200 Centrifuge (Thermo Scientific, Waltham, MA, USA). The test was performed at 4000 and 5300 rpm for 30 and 15 minutes, respectively.

pH Determination

The pH was measured with a digital pHmeter (MP220, Mettler Toledo, OH, USA) at $22 \pm 2^\circ\text{C}$.

Viscosity evaluation

The viscosity measurement was carried out by a Visco Basic plus viscosimeter (FungiLab s.a., Barcelona, Spain) at 25°C, with a Spindle L4 at 12 rpm. Each reading was done 60 seconds after the rotation speed started. The results were expressed as centiPoise (cP).

Recovery of APE from O/W emulsion and Quantitative Analysis

The recovery of APE from emulsion was carried out according to Rolim et al. 2006 with slight modifications. Both after 48h and 3 months from preparation, APE-loaded emulsion was accurately weighed (1.0 g) and transferred to a 50 mL centrifuge tube. The volume was brought to 25 mL with ethanol:distilled water (7:3). The suspension was centrifuged two times at 5300 rpm for 5 min at room temperature. The supernatants were gathered up, dried with rotavapor and the concentration of isoquercitrin was determined by HPLC-DAD analysis in the same condition previously reported (paragraph 3.4). The Theoretical Extract Content (TEC) was the percentage (6 % w/w) of APE in formulation. The Actual extract Content was derived by Actual Isoquercitrin Content (AIC) and calculated as (eq. 1)

$$\text{eq. 1: } \text{AEC\%} = \text{AIC}_{\text{RECOVERY}} / \text{AIC}_{\text{APE}} \times 100$$

The Recovery efficiency (RE%) (eq. 2) was the ratio of the actual to the theoretical extract content

$$\text{eq. 2: } \text{RE\%} = \text{AEC/TEC} \times 100$$

In vitro permeation study

The experiments were conducted according to Sansone et al., 2013 with slight modifications. The release/permeation behavior of APE from the formulation was measured in Franz-type vertical diffusion cells (Hanson research corporation, CA, USA) with permeation areas of 1.7671 cm^2 , applying a nitrocellulose membrane (size pores: $0.45 \mu\text{m}$) between the donor and the receiving compartment, assuring the absence of air bubbles. Each cell was filled with 7ml of previously degassed receptor fluid (ethanol: water 7:3) and heated by a thermostatic recirculating water bath at 37°C . During the experiments (up to 3h), the receptor fluid was continuously stirred (170 rpm) by a magnetic stirring. Prior to the application of the test sample, the system was stabilized for 15 min. In order

to increase the volume of the donor compartment, three dosage wafers (each of 15 mm diameter and 1mm height) were stacked together. Thus, 1 g of APE-loaded emulsion with an extract content of 0.06 g was uniformly applied on the membrane surface and subsequently sealed with a spring clips and laboratory parafilm (Parafilm®). At appropriate time intervals, aliquots of the receptor fluid (200 µL) were taken and immediately replaced by the fresh receptor mixture. The samples were filtered and directly analysed by HPLC-DAD for the determination of isoquercitrin used as marker of APE, as above reported. The amount of APE permeated per area (Q) for each time interval was calculated by means of the following equation:

$$Q \cdot \left(\frac{mg}{cm^2} \right) = \frac{V_R \times C_N + \sum_{i=n}^{n-1} V_P \times C_i}{A}$$

where V_R is the receiver volume; C_N is isoquercitrin concentration in the receiver at the time n ; V_P is the volume of the removed sample; C_i is isoquercitrin concentration in the receiver at the time $n - 1$. Permeation data were reported as the quantity of permeated isoquercitrin per permeation area related to time. All the permeation tests were made in triplicate; only the mean values are reported (standard deviations <1%). As control, the release/permeation test was also performed for APE raw material.

Table S1. Retention times, UV and ESI Mass Data of Annurca apple extract

Compound	Rt (min)	λ_{\max} (nm)	$[M+H]^+$ (m/z)	Diagnostic product ions (m/z) ^a
1 chlorogenic acid	14.4	330	355	145, 163
2 isoquercitrin	32.7	360	465	303
3 hyperin	33.6	360	465	303
4 reynoutrin	35.2	360	435	303
5 guajaverin	36.2	360	435	303
6 avicularin	37.5	360	435	303
7 quercitrin	38.4	360	449	303
8 isoramnethin glucoside	39.3	355	479	317
9 phloridzin	41.8	295	435	273

(APE).

Table S2. Ingredients rate (% w/w) of the O/ W APE-loaded emulsion

Inci name	Amount (%)
Oil phase	
Cetearyl Alcohol & Cetareth-33	5.0
Coco-Caprylate/ Caprate	4.0
Caprylic/Capric Triglyceride	5.0
C 12-15 Alkyl Benzoate	4.0
Butyrospermum Parkii Butter	1.0
Cetearyl Alcohol	1.5
Persea Gratissima Oil	1.0
Dimethicone	1.0
Aqueous phase	
Aqua	68.8
Disodium EDTA	0.1
Glycerin	2.0
Xanthan Gum	0.1
Preservative solution	
Phenoxyethanol, Benzoic Acid, Dehydroacetic Acid, Ethylhexylglycerin	0.5
APE	6.0

^a Collision
Rt, retention
relative bundance

energy 30%;
time; RA,

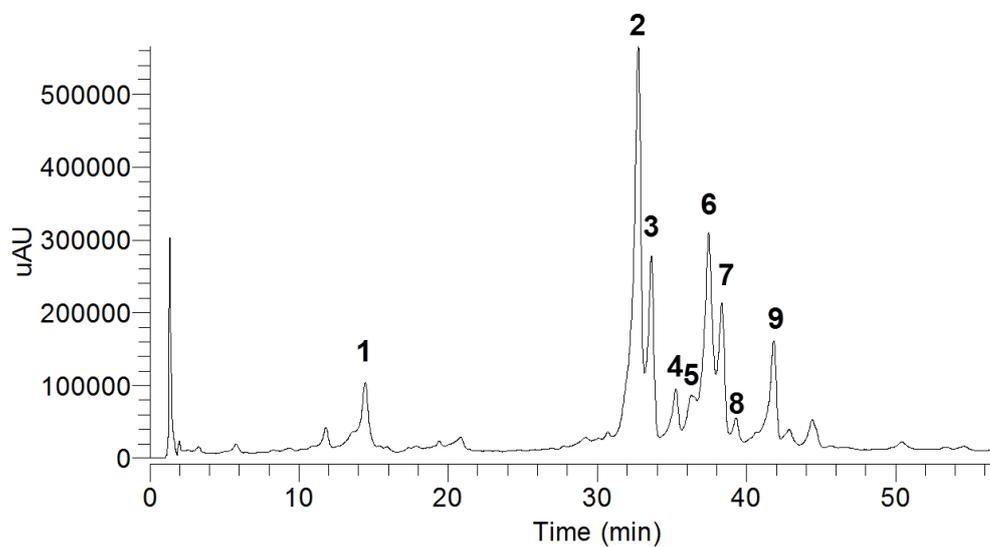


Figure S1. HPLC fingerprint (280 nm) of the hydro-alcoholic Annurca apple extract (APE). The identification of all peaks is reported in Table S1.

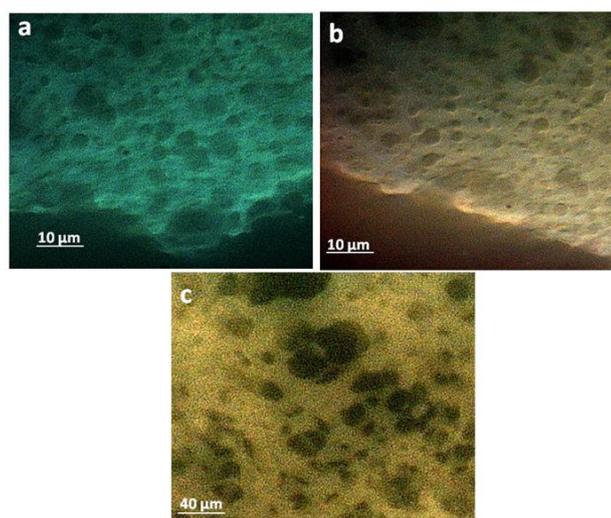


Figure S2. FM images of blank emulsion (without APE) (a), and APE-loaded emulsion (b-c)

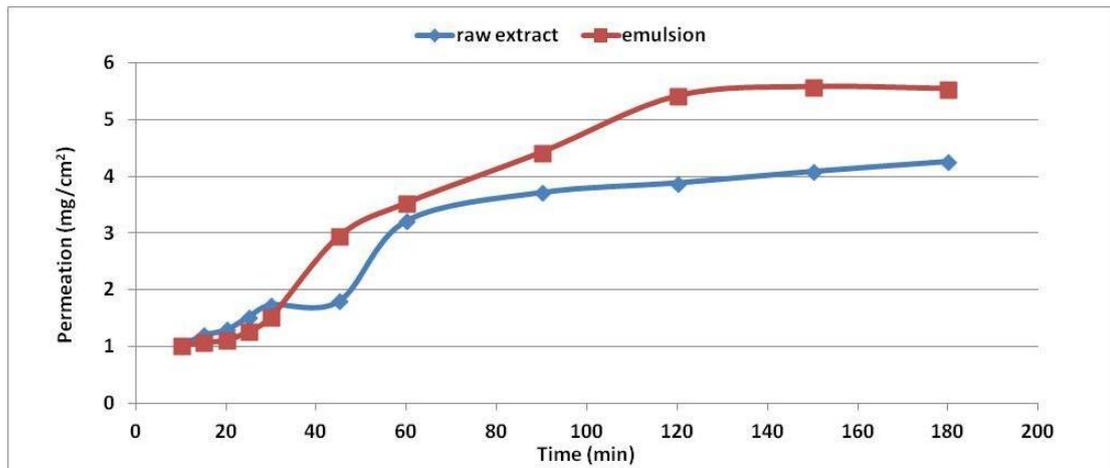


Figure S3. *In vitro* release/permeation rate of APE raw material (blue line) with respect to the APE from the emulsion (red line) (standard deviation for each point < 1%)

References:

- Aquino R P, Santoro A, Prota L, Mencherini T, Esposito E, Ursini M V, Picerno P, Nori S, Sansone F, Russo P. 2014. Composition and anti-inflammatory activity of extracts from three *Paeonia* species. *Pharmacologyonline*. 1:137-147.
- Mencherini T, Picerno P, Festa M, Russo P, Capasso A, Aquino R. 2011. Triterpenoid Constituents from the Roots of *Paeonia rockii* ssp. *rockii*. *J. Nat. Prod.* 74:2116-2121.
- Mencherini T, Picerno P, Russo P, Meloni M, Aquino R. 2009. Composition of the fresh leaves and stems of *Melissa officinalis* and evaluation of skin irritation in a reconstituted human epidermis model. *J. Nat. Prod.* 72:1512-1515.
- Picerno P, Mencherini T, Sansone F, Del Gaudio P, Granata I, Porta A, Aquino R.A. 2011a. Screening of a polar extract of *Paeonia rockii*: composition and antioxidant and antifungal activities. *J. Ethnopharmacol.* 138:705-712.
- Picerno P, Sansone F, Mencherini T, Prota L, Aquino R P, Rastrelli L, Lauro M R. 2011b. Citrus bergamia fresh juice: phytochemical and technological studies. *Nat. Prod. Commun.* 6: 951-955.
- Rolim A, Oishi T, Maciel C P M, Zague V, Pinto C A S O, Kaneko T M, Consiglieri V O, Velasco M V R. 2006. Total flavonoids quantification from O/W emulsion with extract of Brazilian plants. *International Journal of Pharmaceutics.* 308:107–114.
- Sansone F, Mencherini T, Picerno P, d'Amore M, Aquino R P, Lauro M R. 2011a. Maltodextrin/pectin microparticles by spray drying as carrier for nutraceutical extracts. *J. Food Engin.* 105:468-476.
- Sansone F, Picerno P, Mencherini T, Russo P, Gasparri F, Giannini V, Lauro M R, Puglisi G, Aquino R.P. 2013. Enhanced technological and permeation properties of a microencapsulated soy isoflavones extract. *J. Food Eng.* 115:298–305.