Annurca peel extract: from the chemical composition, through the functional activity, to the formulation and characterisation of a topical oil-in-water emulsion

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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-β-glucoside). The isoquercitrin (quercetin-3-β-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 peel extract (APE); chemical analysis; free-radical scavenger; topical emulsion; formulation stability; in vitro permeation test

1. Introduction

‘Annurca’ is the most common variety of apple (Malus pumila Miller. cv Annurca) with a protected geographical indication (PGI), cultivated in the Campania region in southern Italy. Annurca fruit is a rich source of antioxidant polyphenols compounds, in particular flavonol glycosides, mainly found in the fruit peels (Mari et al. 2010; Tenore et al. 2013). Nowadays, the

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incorporation of plant extracts or food supplements rich in polyphenols into formulations for
topical application has attracted considerable attention because of their ability to provide both
protection against skin free-radical-induced damage (leading to skin aging, wrinkles appearance
and tone and resilience reduction) and product oxidation events (González et al. 2013). Based on
these evidences, the aim of the present research was to develop an antioxidant Annurca peel
extract (APE) and to support its potential use in a topical emulsion. The chemical composition of
the hydro-alcoholic APE was determined by HPLC-PDA-ESI-MS\textsuperscript{n} analysis. The free-radical
scavenging activity of APE was determined by DPPH test. Moreover, the effect of APE on
primary human epidermal keratinocyte (HEKa) and human dermal fibroblast (HDFa) cell lines
viability was evaluated by MTT test. The obtained results were encouraging both in term of
functionality as well as safety of use, stimulating the research to develop a topical oil-in-water
(O/W) emulsion suitable in delivering APE to the skin. The stability of the APE-loaded emulsion
with respect to the blank emulsion was proved by fluorescence microscopy (FM) analysis and
physico-chemical parameters evaluation. Finally, an in vitro permeation study was performed to
verify the release flow rate of APE from the formulation through synthetic membranes.

2. Results and discussion
2.1 Preparation and HPLC analysis of APE
The lyophilised apple peels of Annurca variety were extracted with EtOH:H\textsubscript{2}O (7:3), and dried
under vacuum to give a hydro-alcoholic extract (APE). The chemical profile of APE was
investigated by a fast chemical screening using a HPLC combined with online DAD detection
and tandem mass spectrometry (HPLC-DAD-ESI-MS\textsuperscript{n}). Major peaks of APE chromatographic
fingerprints (Figure S1) were characterised based on UV, MS and MS\textsuperscript{n} spectral information
(Table S1). The concentration of isoquercitrin (quercetin-3-O-glucoside) (2), one of the
characteristic flavonol of apple peels (He & Liu 2008) was determined by a HPLC-DAD
calibration method as 0.3% w/w of the dry matter. The total phenol content, determined by the
Folin–Ciocalteu method (Mencherini et al. 2011) and expressed as quercetin equivalent, was
120.3 µg/mg of APE. Phenols are powerful antioxidant plant components (Corradini et al. 2011)
and the polyphenol-rich APE as well as its major compound (2) showed a significant and
concentration-dependent free-radical scavenging activity, evaluated by a DPPH test (IC\textsubscript{50} 524.6
and 15.8 µg/mL, respectively). \textalpha-tocopherol and L-ascorbic acid were used as positive controls
(IC\textsubscript{50} 10.1 and 5.8 µg/mL, respectively) (Picerno, Mencherini, et al. 2011; Picerno, Sansone,
et al. 2011; Sansone, Mencherini, et al. 2014).

2.2 In vitro cytotoxicity evaluation
The results showed that APE does not affect the viability of both HEKa and HDFa cell lines
(Mencherini et al. 2009; Aquino et al. 2014) also at the highest tested concentration (6.0 mg/
/mL). This promising result indicates that APE can be used safely in as functional ingredient in
topical formulations, without posing any risk for the health of final consumer.

2.3 Oil-in-water (O/W) emulsion
On the basis of the recent increased research and commercial polyphenol applications as
ingredients in topical products (Sandeep et al. 2012), as well as the encouraging results obtained
in this work, an O/W emulsion was chosen as topical system to delivery APE to the skin. All the
ingredients were selected for their natural origin which gives them a higher dermo-compatibility
with respect to the synthetic ones. A lot of experimental formulative studies were carried out to
assess both the ratio of each selected ingredient (Table S2) as well as the appropriate preparation
method to reach a stable final emulsion (blank emulsion). An add value of the in house O/W emulsion was the ability to incorporate a high amount of APE (6.0 % w/w) without altering the formulation stability properties as discussed below. A so high APE concentration, chosen on the basis of the polyphenol content and antioxidant activity of the extract, should ensure an appropriate fortification of the emulsion in functional ingredients, providing the desired beneficial effect on the skin.

2.4 Morphological analysis
The characterisation of topic formulations by microscopic techniques is essential to obtain reliable data about the actual morphology of the system. Actually, scanning electron microscopy (SEM) techniques have been employed to characterise up to nanoemulsions (Klang et al. 2012). In this work, an original morphological analysis was conducted by FM. APE contains polyphenols that are naturally red fluorescent molecules (Sansone et al. 2009; Sansone, Mencherini, et al. 2011; Sansone, Picerno, et al. 2011) and during the FM experiment they can act as marker of the extract distribution through O/W emulsion phases (Sansone, Picerno, et al. 2014). The blank formulation resulted well structured (Figure S2a) having also a clear internal homogeneous distribution of the spherical oil droplets (size of about 5 µm) in the aqueous phase. Figure S2b shows the APE-loaded emulsion with the same structure of blank emulsion having in evidence, with an orange fluorescence, the presence of the polar extract well dispersed within the aqueous phase and around the oil droplets. The homogeneity in the distribution was probably due to the appropriate developed method used in producing the emulsion. Moreover, Figure S2c, which is a magnification of the internal structure of the APE-loaded emulsion, shows that the incorporation of APE did not cause structural changes to the formulation resulting in a good stability of the APE-loaded emulsion.

2.5 Accelerated stability test
Emulsions are inherently unstable systems usually splitting into two distinct phases. In addition, change in colour, smell and liquefaction could be observed over time (Masmoudi et al. 2005). Accelerated stability tests are internationally recognised as predicting new formulated product shelf-life under appropriate conditions of storage, transport and use (Guidelines COLIPA 2004).

2.5.1 Macroscopic analysis
Typically, the incorporation of plant extracts can lead to a rapid modification of the final emulsion. In our in-house formulation, due to the addition of APE, a slight variation in the colour, from white to pale yellow, and a low increase in the intensity of odour perception occurred. The constant maintenance of those characteristics during the storage period highlights that the emulsion balance was not upset by the APE incorporation step.

2.5.2 Centrifuge test, pH determination and viscosity evaluation
The emulsions shelf-life under normal storage conditions can be rapidly predicted by observing so the phase separation when the biphasic system is exposed to centrifugation, as supervising the pH value during the emulsion storage at different conditions. The obtained results showed that neither phase separation nor significant changes in pH values (skin compatible range from 5.36 to 5.57) (Georgetti et al. 2006) up to 3 months were observed for both blank and APE-loaded emulsion. Moreover, after production, time and temperature-dependent reduction in the system viscosity occurred (Herbert et al. 1998). The viscosity values of both emulsions (ranging from 21.131 to 27.684 cP) classified them as fluid O/W emulsions (Proserpio 2000).
During the storage time, no liquefaction events were observed. All the obtained results confirmed the chemical and physical stability of the emulsion both in the case of APE-loaded and blank ones.

2.5.3 Recovery of APE from O/W emulsion and quantitative analysis

In order to evaluate the APE chemical stability after the incorporation in the produced O/W emulsion, an extraction procedure, reported by Rolim et al. (2006), slightly modified, was performed. The actual isoquercitrin content (AIC) was evaluated by HPLC-DAD and the actual extract content (AEC) calculated as reported in Supplementary material. Results showed that percentage of recovery efficiency (RE%) both at $t_0$ (48 h) and after 3 months was of 46% with respect to the loaded extract (TEC%). In fact, no significant differences of AEC in term of stability (variations < 1% w/w) were detected. The functional components resulted stables in the O/W formulation because no significant AEC variations (< 1% w/w) were detected. The AIC recovery percentage was kept constant during all the storage period, but as the recovery was partial, it seems essential to optimize the extraction procedure. An improvement of method will serve to better follow the shelf-life of natural ingredients in a biphasic cosmetic delivery system.

2.6 In vitro permeation test

Many skin damages are treated topically with active ingredients dissolved in gel or emulsion vehicles. Permeation of active ingredients through the epidermis is a prerequisite for an effective topical formulation. An artificial membrane was used to study the rate of APE release from the produced O/W emulsion (Sansone et al. 2013). As shown in Figure S3, at the same time (180 min) it was obtained an increase in the extract release rate of 22% (5.45 mg/cm$^2$) from the formulation compared to the extract raw material (4.26 mg/cm$^2$) that permeates through the membrane. This behaviour is probably due to the effect of other ingredients, such as the emulsifier system that can act as APE release enhancers (Spernath et al. 2002). Moreover, according to the performed DPPH test (APE IC$_{50}$ 524.6 µg/mL), the amount of APE able to through the membrane in 10 min (1.02 mg/cm$^2$) should be sufficient to provide the free-radical scavenging functional effect.

3. Conclusions

The APE hydro-alcoholic extract exhibited a significant DPPH scavenging activity due to its high polyphenols content, mainly quercetin derivatives, such as isoquercitrin. APE did not affect viability of HEKa and HDFa cell lines, up to 6.0 mg/mL. In this work APE was used as a safe natural antioxidant ingredient in a new-formulated O/W emulsion. 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 aqueous phase was clearly demonstrated by FM analysis.

Our in-house O/W emulsion seems to be a suitable vehicle to deliver an active APE concentration, also potentially promoting its skin permeation.

Supplementary material

Supplementary materials associated with this article are available online at http://dx.doi.org/10.1080/14786419.2015.1062005.
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