SUPLEMENTARY MATERIAL

In vitro solar protection factor, antioxidant activity, and stability of a topical formulation containing Benitaka grape (*Vitis vinifera* L.) peel extract

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Abstract

A flavonoid enriched (FE) extract was obtained from grape peels, and the *in vitro* SPF, antioxidant activity, and effects on cell viability of this extract were tested with the intent to develop a cosmetic product. A formulation was developed with the FE, and the stability of this mixture was evaluated in terms of pH, density, viscosity, and SPF (90-days). FE showed no cytotoxicity to human keratinocytes and an *in vitro* SPF of 18.56 (UV-spectrophotometry). Further, FE showed a UVA protection factor of 3.17±0.2, a critical wavelength of 318.0±0.1 and a UVA/UVB of 0.9. Antioxidant activity assays resulted in 92.08% and 86.85% of activity against DPPH and ABTS (IC_{50} = 296.90±1.2 µg/mL and 643.13±0.9 µg/mL), respectively. Finally, SPF of formulation with FE was 12.45. Results from the *in vitro* SPF and product stability tests (especially storage under refrigeration), indicate that FE is a promising compound for use as an innovative sunscreen formulation.

Keywords: Benitaka grape; sunscreen development; flavonoids; rutin

Experimental

Flavonoid extraction from Benitaka grape peel

Flavonoids were extracted from Benitaka grape peels according to the method of Lees and Francis (1972). In brief, 100 g of dried grape peels were mixed with a 1000 mL ethanol (99.5% purity; Synth, Sao Paulo, Brazil) and the mixture was kept under magnetic stirring (Fisatom, Mod 753E, São Paulo, Brazil) for 3 hours at 50 ± 2°C, to extract the flavonoids. The supernatant was then filtered and analyzed for flavonoid content.

Flavonoid identification by high performance liquid chromatography

To identify the flavonoid content of the extract, the diluted extract was filtered through a 0.45-µm membrane (Merck, São Paulo, Brazil) and identified by HPLC-DAD (Agilent, Mod 1250 infinity, Barueri, Brazil) at 257 nm, using a C_{18} (Phenomenex, Sao Paulo, Brazil) monomeric chromatographic column, at a flow-rate of 0.3 mL/min, at 27 ± 1°C. The mobile phase consisted of a solution of methanol and 0.1% formic acid (Synth, São Paulo, Brazil) at isocratic conditions (80:20; (Seal 2016). Rutin (500 µg/mL) was included as a standard; the stock solution was prepared in HPLC grade methanol (Merck, São Paulo, Brazil) at 1000 µg/mL. All assays were performed in triplicate.

Validation of the analytical method by HPLC

The analytical method was validated using the analytical curve obtained from the rutin standard at 257 nm and the same conditions as for identification of the flavonoids.

The precision assay was performed using intra and inter-day repeatability according to the ANVISA guidelines (Anvisa 2003, Anvisa 2015). Six samples, with 500 µg/mL rutin added to each, were analyzed on the same day and on two consecutive days. The area of the standard's peaks was obtained and used to calculate the variation coefficient percentage (Table S2).

The exactness test was performed through the recovery assay, which consisted of adding a known concentration of the flavonoid standard to the extract. Then, different volumes (0.75 mL, 1.25 mL, and 1.75 mL) of rutin (500 µg/mL) were transferred to 5 mL volumetric flasks containing 0.625 mL of grape (100 mg/mL) extract; the remainder of the volume was constituted with HPLC grade methanol, resulting in different concentrations of rutin (Cf). The recovery percentage of rutin (R%) was determined according to Eq (1) (Table S3; (Anvisa 2003, Anvisa 2015):

$$\%R = [(Cf - Ca]/x100 \qquad (1)$$

Where: Cf is the extract concentration containing the rutin (µg/mL); Ca is the concentration of extract (µg/mL); and Cr is the volume of rutin (mL).

For robustness, six different concentrations of extract were analyzed by different analyzers (Anvisa 2003) and the data were compared. Detection (DL) and Quantification limits (QL) were evaluated to determine and quantify the lowest acceptable concentration of rutin in grape extract, according to Eq. (2) and (3) (Anvisa 2003):

$$DL = SDx3/SC \qquad (2)$$

$$QL = SDx10/SC \qquad (3)$$

Where: SD is the standard deviation of the Y-axis intercept from at least three analytical curves; and SC is the slope of the analytical curve.

To determine the selectivity of the method, extract chromatograms were compared to analytical standards to identify impurities in the extracts (Anvisa 2005).

In vitro cellular viability analysis by neutral red uptake (NRU)

Non-tumor cell line HaCat (human keratinocytes), donated by Professor Dr. Ricardo Della Coletta, FOP/ UNICAMP, was maintained in complete medium [RPMI 1640 supplemented with 5% fetal bovine serum and 1% of penicillin:streptomycin mixture (1000 U/mL, 1000 g/mL)] and incubated at 37ºC in humid atmosphere with 5% of CO_2. The in vitro experiments were conducted in the same conditions. FE extract and doxorubicin (positive control) were previously diluted in DMSO and then diluted in complete medium. The DMSO final concentration (>0.25%) did not affect cell viability.

Twenty-four hours after keratinocytes seedling (HaCaT, 3 x 10^4 cell/ml, 100 µl/well) in 96-well tissue culture, cells were treated with FE extract (final concentration: 0.25, 2.5, 25.0 and 250.0 µg/mL, in triplicate), and incubated for 48h. Doxorubicin (final concentration: 0.025, 0.25, 2.5 and 25.0 µg/mL, in triplicate) or complete medium (untreated cells) were used as positive and negative controls, respectively. After sample exposition, the cell viability was determined using the NRU method (OECD 2010). In brief, the neutral red (NR) work solution was prepared by dilution of NR stock solution (33 µg/mL, 1 mL) in complete medium (79 mL). After warming (37°C, 30 min) and centrifugation (1000 rpm, 10 min), cells were stained with NR work solution (200 µL/well) during 3 h at 37°C. Then, all supernatant were removed and replaced by acid alcohol (100 µL/well). The absorbance values were determined at 540 nm using a spectrophotometer (VersaMax, Molecular Devices). Cell viability was calculated considering the absorbance of untreated cells as 100% of viable cells. The sample concentration required to reduce in 50% cell viability was calculated by non-linear regression using Origin.

In vitro SPF determination

The *in vitro* SPF (290 to 320 nm) was determined by ultraviolet-visible spectrophotometry and calculated by the equation given by Eq (4) (Mansur et al. 1986):

$$SPF = CF \times \Sigma_{290}^{320} \times EE_{(\lambda)} \times I_{(\lambda)} \times Abs_{(\lambda)} \qquad (4)$$

Where: SPF is the solar protection factor; CF is the correction factor (10); $EE_{(\lambda)}$ is the erythemogenic effect of wavelength radiation (λ) nm which was calculated by Sayre *et al.* (1979); $I_{(\lambda)}$ is the intensity of the solar radiation in the wavelength (λ) nm; and $Abs_{(\lambda)}$ is the absorbance of the sunscreen solution at the wavelength (λ) nm.

Similarly, the *in vitro* UVAPF, critical wavelength (λc), and UVA/UVB rate were assessed according to the methods of Velasco *et al.* (2008) and Rai and Srinivas (2007) by spectral transmittance (Labsphere® UV-2000S Ultraviolet Transmittance Analyzer, Sao Paulo, Brazil) at 290-450 nm.

In vitro antioxidant activity analysis

To determine the *in vitro* antioxidant activity, DPPH and ABTS were used. For the DPPH assay, different extract concentrations (75, 125, 200, 400, 500, 750, and 1000 µg/mL) were placed in test tubes with 2.5 mL of 0.004% DPPH solution (w/v) in ethanol. The reaction was then carried out for 30-min in a dark place. The analysis was also performed using quercetin as a standard at concentrations of 0.25, 0.5, 1.0, 1.75, and 2.5 µg/mL. Ethanol was used as the blank for maximal absorbance determination at 531 nm (Rufino et

al. 2010). In the presence of DPPH, the absorbance intensity decreased, and the percentage of inhibition (% Inhibition) was calculated using Eq. (5):

$$\%\ Inhibition = \frac{Abs_{max} - Abs_{test}}{Abs_{max}} \times 100 \quad (5)$$

Where: Abs_{max} refers to the radical (DPPH or ABTS) absorbance; and Abs_{test} is the radical with sample absorbance.

For the ABTS analysis, 5.0 mL of aqueous ABTS (7 mM) was mixed with 0.088 mL of potassium persulfate (140 mM) and stored in dark place for 16-h. After, 3.0 mL of ABTS solution was added to test tubes containing different extract concentrations (75, 125, 200, 400, 500, 750, and 1000 μg/mL) and the reaction was carried out for 6-min. The free radical absorbance kinetics were determined at 734 nm and the inhibition percentage of the samples was calculated according to Eq. 5 (Rufino, et al. 2010). As in the DPPH assay, quercetin (0.5, 1, 2, 5, 10, and 20 μg/mL) as included as an antioxidant standard. All the assays were performed in triplicate. The sample concentration required to produce 50% radical inhibition (IC_{50}) was calculated by non-linear regression using Origin.

Topical formulation development

The oil-in-water (O/W) emulsion was formulated according to Table S1, and the ethanol extract of grape peels was incorporated in the emulsion at a concentration of 5.0% (w/w), which is equivalent to 200 μg/mL of dry extract. To evaluate physical stability, approximately 5.0 g of each emulsion (with and without incorporated extract) were centrifuged three times at 3000 rpm for 30-min/cycle at 27±2 °C (Anvisa 2004, Isaac et al. 2008). Organoleptic characteristics (color, odor, and appearance), pH, density and viscosity values were also determined. To evaluate the density, an empty pycnometer with a 5 mL capacity and a pycnometer with 5 mL of the sample were weighed (Idson 1993). While the apparent viscosity determination was evaluated using a rotational viscometer (Brookfield, Mod LV-T, São Paulo, Brazil) with spindle number 4 and 1.5 rpm rotation for 30-sec at 27±2 °C (Corrêa et al. 2005). The viscosity results are expressed in centipoise (cP).

Formulation stability study

The formulation stability was evaluated according to ANVISA recommendations (Isaac, et al. 2008), with all tests performed in triplicate. Conditions for the stability study were as follows; formulations with and without grape peel extract were stored in glass containers and kept in the freezer (-5 ± 2 °C), the refrigerator (5 ± 2 °C), at room temperature (27 ± 2 °C), in a thermostatic oven (45 ± 2 °C) and exposed to indirect light. Samples were stored and tested over the course of 15 days (preliminary tests) and over 90 days for the accelerated stability test with assays performed on the 1st, 7th, 14th, 21st, 30rd, 45th, 60th, 75th, and 90th days. According to the recommendations, storage at these conditions should not induce variations higher than 10.0%.

In vitro SPF and antioxidant activity of formulation containing extract

The formulation was diluted in a chloroform and isopropanol solution (1:1), filtered, and submitted to the spectrophotometry techniques according Mansur's method (Mansur et al., 1986) for evaluation of SPF and antioxidant activity according Rufino et al. (Rufino, et al. 2010). The formulation without extract was used as a blank.

This formulation was evaluated for antioxidant activity against DPPH free radical and inhibition percentage was calculated using Eq. (5), as described above.

Tables

Table S1. Composition of oil-in-water emulsion.

Ingredients (INCI NAME)	Concentration (%)
Cetostearyl alcohol	5.0
Cetyl alcohol	6.0
Silicon oil	1.0
Polysorbate 80	7.0
Phenoxyethanol and parabens	0.2
Butylated hydroxytoluene	0.5
Carbormer (Dispersion of 2.0%)	20.0
Glycerin	5.0
Aqua	55.30

Table S2. Intra-day and inter-day precision values for analytical flavonoid standard.

	Peaks area		Total variation coefficient (%)
	Inter-day	Intra-day	
Rutin	269.65±0.8	270.30±0.5	0.89±0.4

Table S3. Recovery assay values referents to rutin concentration in extract.

Extract		Standard Volume(Cr) (mL)	Final Coef. of rutina (Cf)	%Rutin recuperated (R_{rutin}%)	Variation Coef. R_{rutin}%
Vitis vinifera L.	R1	0.75	81.69±0.1	106.02±0.2	0.41±0.3
	R2	1.25	139.92±0.4	102.20±0.2	
	R3	1.75	177.16±0.2	102.16±0.2	

Table S4. Absorbance and solar protection factor values obtained by spectrophotometry from the formulation with extract.

Wavelength (nm)	Absorbance	SPF values
290	1.732 ± 0.02	0.026
295	1.481 ± 0.01	0.121
300	1.330 ± 0.01	0.382
305	1.214 ± 0.02	0.398
310	1.127 ± 0.01	0.210
315	1.066 ± 0.01	0.089
320	1.024 ± 0.01	0.018

Figures

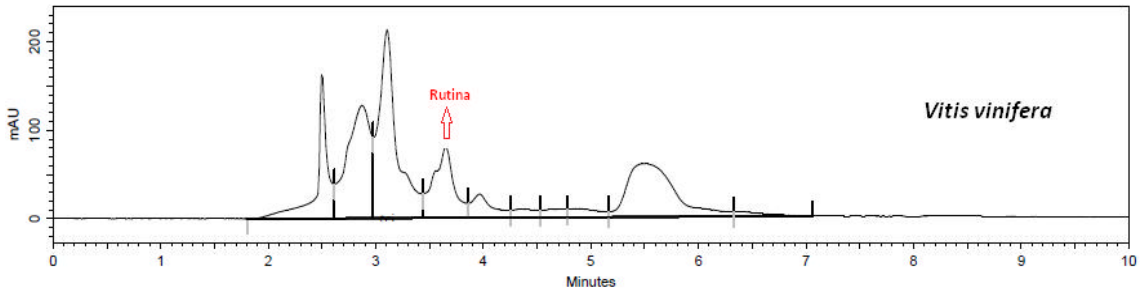

Figure S1. Chromatography representative of rutin presence in grape extract.

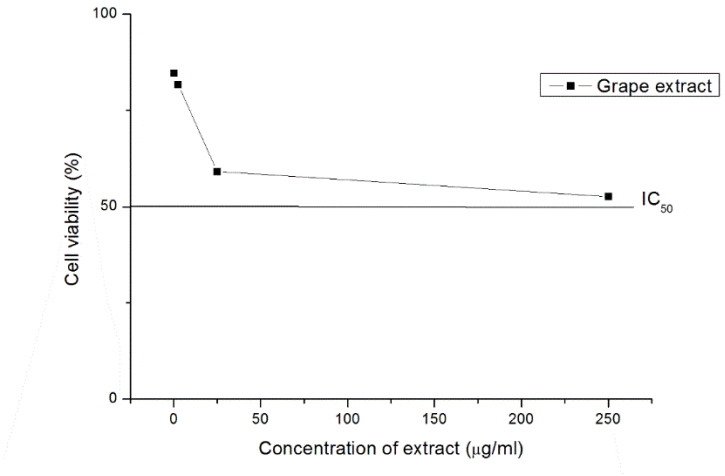

Figure S2. *In vitro* cell viability assay of grape extract. The errors for the cell viability percentage are about 1% (within the size of the symbols).

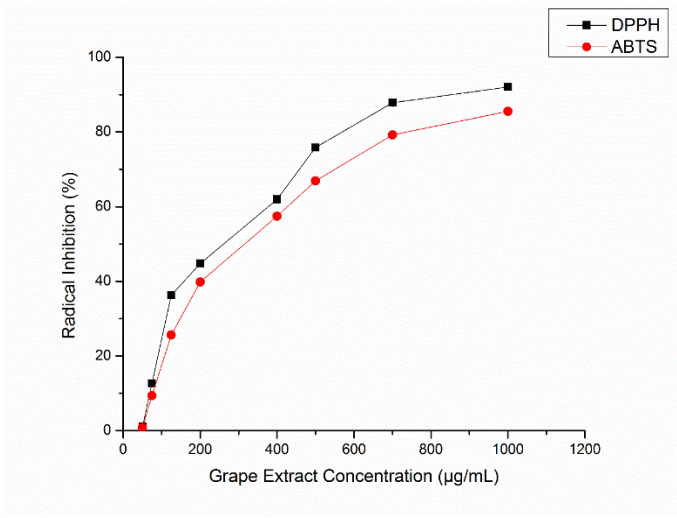

Figure S3. *In vitro* antioxidant activity of grape peel extract, using DPPH (IC_{50} = 296.90±1.2 µg.mL^{-1}) and ABTS (IC_{50} = 643.13±0.9 µg.mL^{-1}) radicals. The errors for the antioxidant activity are about 1% (within the size of the symbols).

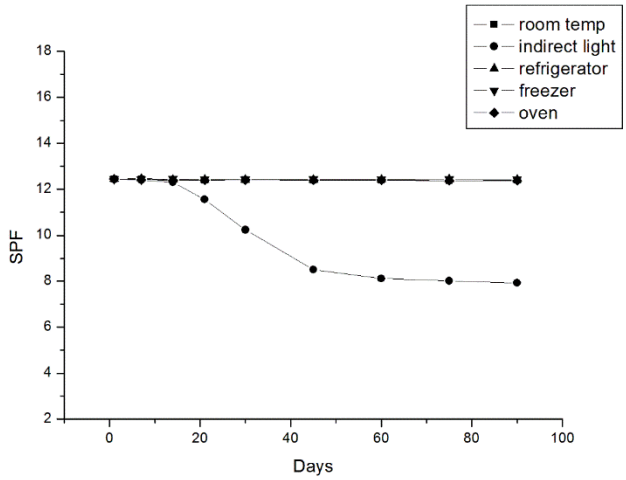

Figure S4. Stability study of formulation containing grape extract. The errors for the SPF values are about 1% (within the size of the symbols).

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