Industrial Crops & Products 111 (2018) 141–148

Contents lists available at ScienceDirect

Industrial Crops & Products

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

Research paper

Citrus reticulata Blanco peels as a source of antioxidant and anti-proliferative phenolic compounds

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ARTICLE INFO

Keywords:
Citrus reticulata
Peels
Polyphenols
Antioxidant activity
Anti-proliferative activity
BT-474 cells

ABSTRACT

Citrus reticulata Blanco industrial use worldwide generates millions of tons of by-products, mainly peels, with negative environmental impact. The main purpose of this work was to compare the extraction efficiency of water and 70% ethanol, for recovering *C. reticulata* peel phenolic compounds. A simple solid phase extraction method was used for obtaining enriched phenolic extracts. There were no significant differences in the extraction efficiency for the two solvents used. The main components were hesperidin, naringin, tangeritin, and rutin, that accounted for nearly 86% of the total phenolics extracted. Solid phase extraction allowed a 4.5-fold enrichment in phenolics and antioxidant activity of the extracts. The anti-proliferative activity of the extracts was found to be dose-dependent but also dependent on the cell line. The solid phase extraction enriched phenolic extracts after 48 h exposure presented an IC_{50} of 174.5 ± 5.8 μg/mL (BT-474), 391.9 ± 15 μg/mL (Caco-2) and > 500.00 μg/mL (HepG2). These results show that *C. reticulata* peels are a cheap and abundant source of antioxidant and potentially bioactive phenolic compounds.

1. Introduction

Citrus genus is one of the most economically relevant fruit crop in the world. It includes about 17 species of citrus fruits such as *Citrus reticulata* Blanco (mandarin orange, tangerine), *Citrus sinensis* L. (sweet orange), *Citrus aurantium* L. (bitter orange), *Citrus lemon* L. (lemon), *Citrus paradise* M. (grapefruit) (Chutia et al., 2009). These crops of Asian origin, are nowadays distributed throughout the world, mainly in the tropical, subtropical and temperate regions, like Brazil, Portugal, Spain, Italy, Greece, Morocco, Turkey and Egypt (Boluda-Aguilar et al., 2010; Chutia et al., 2009). Their worldwide annual production is near 88 million tons (Negro et al., 2016). The industrial processing of citrus fruits generates millions of tons of organic waste residues such as peels, seeds, pulp and membranes (Boluda-Aguilar et al., 2010; Hiasa et al., 2016; Negro et al., 2016). These industrial by-products are an environmental problem and a product waste. Increased awareness of food industries of the economic and environmental impacts of waste generation and disposal are calling for transition towards more sustainable practices and the adoption of the circular economy concepts (Jurgilevich et al., 2016). Circular economy means reuse, repair, refurbishing, and recycling of the existing materials and products, what was earlier considered to be waste becomes a resource. The recovery of phenolic compounds present in considerable amounts in agricultural by-products for pharmaceutical and cosmetic applications has been studied as an attractive way of industrial by-products valorization (Melgar et al., 2017; Silva et al., 2016). Recycling of citrus by-products has been mainly performed, fresh or after ensilage or dehydration, for cattle feed (Bampidis and Robinson, 2006; Caparra et al., 2007). Other potential re-uses of citrus fruit by-products, include bioethanol production due to its high carbohydrate content (Boluda-Aguilar et al., 2010), essential oil production/recovery, mainly composed by D-limonene (Negro et al., 2016), as a substrate for multienzyme production (Mamma et al., 2008). Due to their high content of phenolic acids, polyphenols (e.g., polymethoxylated flavones, glycosylated flavanones, flavonoids and limonoids), and carotenoids (Boluda-Aguilar et al., 2010; Hiasa et al., 2016; Ho and Lin, 2008; Huang and Ho, 2010; Moulehi et al., 2012), citrus peels can also constitute a source of potential pharmacological molecules. In fact, citrus peel medicinal use is known for centuries, as dried peels of *C. reticulata* “chen pi” have been used in the Traditional Chinese Medicine for treating indigestion,

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http://dx.doi.org/10.1016/j.indcrop.2017.10.009
Received 5 February 2017; Received in revised form 15 August 2017; Accepted 8 October 2017
Available online 16 October 2017
0926-6690/

bronchitis and asthma (Ho and Lin, 2008). Polymethoxylated flavones, such as tangeritin and nobiletin (Stuetz et al., 2010) have been reported to have anti-inflammatory (Zhang et al., 2016), antioxidant, anti-allergic, anti-proliferative, anti-atherogenic, antibacterial, antifungal and antiviral activities (Ho and Lin, 2008; Liu et al., 2012; Manthey and Guthrie, 2002). Moreover, the flavonoids hesperidin and diosmin, play an important role in the prevention of atherosclerosis as they reduce total blood cholesterol, presenting also anti-hypertensive, hypolipidaemic, diuretic and analgesic properties (Stuetz et al., 2010; Tounsi et al., 2011). Polyphenols may have potential uses as biobased phytosanitary products, able to control the incidence of crop diseases (Benouaret et al., 2014). The extraction of valuable polyphenols from by-product can reduce the environmental impact of some of these substances on the cultivated fields and on the irrigation water (Kuppusamy et al., 2015; Seabra et al., 2010) and on the other hand can constitute a valuable source of natural phenolics for the pharmaceutical and cosmetic industries. There are several evidences highlighting the relevance of citrus flavonoids as anti-cancer agents acting through several mechanisms, such as inhibition of cell proliferation and of oncogene and/or induction of tumor suppressor gene, induction of cell-cycle arrest and/or apoptosis, as a result of their capacity to modulate relevant cellular signal transduction pathways, and from several epidemiological studies there is a direct correlation between citrus fruit intake and a decrease in risk cancer, as recently reviewed by (Cirmi et al., 2016). Thus the main purpose of this work was to evaluate the *C. reticulata* industrial by-products as a cheap and abundant source of bioactive phenolic compounds by determining their *in vitro* antioxidant activity and anti-proliferative potential against three cancer cell lines (BT-474, Caco-2 and HepG2).

2. Materials and methods

2.1. Samples and preparation of extracts

Samples of *C. reticulata* peels (from fresh tangerines) were obtained from the food processing factory Douromel ("Fábrica de Confeitaria, Lda", at Barcos, Tabuaço; Trás-os-Montes region, Portugal) that acquired the fruits from local farmers. The confirmation of the species was carried out by botanists at UTAD Botanical Garden.

The crude extracts, hydro-ethanolic (HEtOH) extract and aqueous (H_2O) extract and their solid phase extraction enriched phenolic extracts (HEtOH-C18-MeOH and H_2O-C18-MeOH) were obtained as following: the crude extracts were obtained by solvent extraction (ethanol:water 70:30 or water, HEtOH and H_2O, respectively) of *C. reticulata* fresh peels (50 g/L of solvent) by boiling during 60 min. The final suspension (HEtOH or H_2O) was filtered, concentrated by vacuum evaporation (35 °C) and freeze-dried (Dura DryTM μP), at −41 °C and ~180 mTorr. The freeze-dried HEtOH or H_2O extracts were then fractionated by solid phase extraction (SPE) using C18 cartridge (20 mL, 5 g packing, Supelco SPE pack, Sigma-Aldrich). The SPE stationary phase was initially activated using 3 × 5 mL of methanol and then conditioned with 3 × 5 mL of water before sample application. Five milliliters of a solution at 7 mg/mL of the crude extracts were then applied followed by washing with 3 × 5 mL of water and then the elution was performed with 3 × 5 mL of methanol (HEtOH-C18-MeOH and H_2O-C18-MeOH for the HEtOH and H_2O extracts, respectively). The extracts were concentrated by vacuum evaporation to remove methanol and then freeze-dried.

2.2. Determination of total phenols contents

Total phenol content of crude extracts and SPE enriched fractions were determined according to the method described by Bekedam et al. (2006) with modification. To 1 mL of the extract solution (1 mg/mL), 500 μL of Folin-Ciocalteu reagent, 1 mL of a saturated Na_2CO_3 solution and 7.5 mL of mQ water were added. After 1 h at room temperature, the absorbance was measured at 725 nm (Spectronic Genesys2PC). Gallic acid was used as phenolic compound reference. Results were expressed as mg gallic acid equivalents (GAE)/g of sample.

2.3. Trolox equivalent antioxidant capacity assay

The antioxidant activity against ABTS radical was determined according to Barros et al. (2011). Briefly, the ABTS radical was prepared after adding 7 mM ABTS solution and 2.45 mM potassium persulfate, and incubated in the dark at room temperature for 12–16 h. The ABTS radical formed was diluted with 20 mM sodium acetate buffer (pH 4.5) until an absorbance at 0.70 ± 0.02 at 734 nm was obtained. The reaction started by addition of properly diluted extracts with 2 mL of the diluted ABTS radical solution. After 15 min at room temperature, the absorbance was measured at 734 nm (PerkinElmer-Lambda 25, PerkinElmer, London, United Kingdom). The antioxidant activity of the extracts was expressed in Trolox equivalents (mmol Trolox/100 g fresh weight), using a Trolox calibration curve. Caffeic acid was used as a positive control.

2.4. Profiling of C. reticulata peel phenolic compounds by high performance liquid chromatography (HPLC)

The phenolic compounds present in extracts (crude extracts and SPE enriched extracts) were determined by HPLC using an Ultimate 3000, Dionex HPLC equipped with PDA-100, Dionex photodiode array detector and an Ultimate 3000 pump. A reverse phase C18 column was used (ACE 5, 250 mm × 4.6 mm, 5 μm; Advanced Chromatography Technologies, Scotland). The mobile phase consisted of solvent A – 5% formic acid and solvent B – methanol, the flow rate was 1.0 mL/min and the column temperature was maintained at 30 °C during the run. The elution program was: 5% B from zero to 2 min followed by a linear gradient up to 60% B until 68 min and from 76 to 80 min down to 5% B. Detection was performed in the 200–600 nm range. The injection volume was 50 μL. All analyses were performed in duplicate. For equipment control and data acquisition, the software Chromeleon version 7.1 (Dionex, USA) was used (Guise et al., 2014). Peak identification was confirmed by retention time and UV-spectra comparison with those of authentic commercial standards (chlorogenic acid; caffeic acid; ferulic acid; naringin; rutin; hesperidin; naringenin; quercetin; hesperitin and tangeretin). For the quantification of the polyphenolic compounds a calibration curve was used in the range of 5–100 mg/L for caffeic acid (R^2 = 0.9967; y = 8.9245 x); ferulic acid (R^2 = 0.9993; y = 8.5812 x); naringin (R^2 = 0.999; y = 1.3497 x); hesperidin (R^2 = 0.9558; y = 0.6793x); naringenin (R^2 = 0.9977; y = 1.9082 x); quercetin (R^2 = 0.9916; y = 1.3736 x); hesperetin (R^2 = 0.9994; y = 2.6778 x), and between 50–1000 mg/L for rutin (R^2 = 0.9995; y = 0.9919 x); chlorogenic acid (R^2 = 0.9998; y = 2.662 x) and tangeretin (R^2 = 0.9997; y = 2.4078 x). For those compounds which no standards were commercially available calibration curves of gallic acid (R^2 = 0.9964; y = 4.3579 x), caffeic acid, rutin and tangeretin were used for benzoic acid, cinnamic acid, flavonoid and flavone derivatives, respectively.

2.5. Cell culture and cell viability assay

In this study the adherent cell lines BT-474 (Human breast carcinoma; CLS, Eppelheim, Germany), Caco-2 (Human colon adenocarcinoma; CLS, Eppelheim, Germany) and HepG2 (Human liver hepatocellular carcinoma; from ATCC, kindly provided by Prof. Carlos Palmeira (CNC, UC, Portugal)) were used. BT-474 cells were maintained in DMEM/F12 medium (1:1; Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12) and Caco-2 and HepG2 cells in DMEM (Dulbecco's Modified Eagle Medium), both media supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (all cell culture reagents were from Gibco,

Alfagene, Portugal), in incubator at 37 °C in a 5% CO_2/95% air atmosphere, with saturated humidity.

For experiments, cells were manipulated as already reported (Severino et al., 2014) and seeded in 96-well plates (100 µL/well) at a density of 5×10^4 cell/mL. After 24 h, the culture medium was removed and replaced by extract solutions (diluted in FBS-free culture medium, at different concentrations 50, 100, 200 and 500 µg/ mL; 100 µL/well). The extract effects on cell viability were determined, 24 or 48 h after exposure, using the Alamar Blue assay, the extract solution was removed and replaced by Alamar Blue solution (10% v/v), diluted in FBS-free culture media, after 4–5 h incubation, absorbances at 570 and 620 nm were read, and the percentage of reduction was calculated (Andreani et al., 2014), and results are expressed as percentage of control (non-exposed cells).

2.6. Statistical analysis

All analyses were performed in duplicate (chemical analyses) or quadruplicate (cell viability studies). The data was expressed as means ± standard deviation and analyzed by *t*-Student test for comparison of two means and ANOVA with Tuckey post-hoc test for comparison of more than two means (Origin Software). A value of $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Total phenols and antioxidant activity of C. reticulata peels extracts

For extraction of polyphenols from *C. reticulata* peels water and ethanol (70% in water; HEtOH) were used (Table 1). As can be observed water extraction yield a higher amount of soluble solids than ethanol extraction, nevertheless the amount of total phenols extracted was not significantly different between solvents (Table 1). The same trend was observed for the antioxidant activity for the ethanol and water extracts (Table 1). The HEtOH extract yield was slightly lower when compared to other values found in the literature. Zia-ur-Rehman, 2006 reported a yield of 11% for an ethanolic extract from dried and grounded citrus peels, and Apraj and Pandita (2014) reported a yield of 9.7% from a dried powder of *C. reticulata* Blanco peels. On the other hand the total phenols extracted and the antioxidant activity observed in our work were higher than that found in the literature. Casquete et al. (2015), using 80% aqueous ethanol for extraction of mandarin (*Citrus reticulata*) peels obtained a total phenolic content of 0.530 ± 0.010 mg GAE/g fresh citrus peels and an antioxidant activity of 12.0 ± 1.8 mmol Trolox/kg of fresh peels. Zhang et al. (2014) analyzed lyophilized powdered peels of 14 Chinese wild mandarin (*Citrus reticulata* Blanco) genotypes after extraction with 80% methanol and obtained a total phenol content from 29.38–51.14 mg GAE/g dry weight and an antioxidant activity (ABTS assay) from 65.62–108.60 mmol Trolox/kg dry weight. In another study, Chen et al. (2010) using 70% ethanol and ultrasound extraction obtained a total phenolic content for *C. reticulata* Blanco *cv*. Ougan peel extracts from 15.6–19.0 mg GAE/g dry weight and an antioxidant activity (ABTS assay) from 226.5–255.8 mmol Trolox/kg dry weight. These differences can be due to the different extraction method details, region of production, cultivar and stage of ripeness. Wang et al. (2016) found a significant increase in the phenolic content of peel extracts (80% acetone) obtained from immature and mature *Citrus reticulata cv.* Chachiensis (Chachi), namely 14.6 ± 0.44 and 19.2 ± 0.62 mg GAE/g DW, respectively.

Reverse phase solid phase extraction (RP-SPE) was used as a simple method for obtaining enriched phenolic extracts from both crude extracts for further cytotoxic evaluation, reducing the interference from non-phenolic compounds present in the crude extracts, but without any concern about their quantitative recovery (Table 1). As can be observed, RP-SPE was efficient in obtaining a phenolic rich extract as this procedure allowed an increase in concentration of the extracts of 5.14 and 4.62 times for the total phenol content and 3.98 and 5.09 times for the antioxidant activity of the material obtained for the H_2O and HEtOH extracts, respectively. The fractions eluted with methanol from the SPE represented in average 34% of the total phenols of the initial extracts. This was due to the irreversible adsorption of some components present in the initial extract, as only 67% of the applied material were recovered in the elution step with water and methanol, and also because there was an overload of the column with the extract as the remaining 30% were recovered in the water eluted fraction.

Table 1
Yield, total phenol content and antioxidant activity (fresh weight basis) of crude extracts (hydro-ethanolic (HEtOH), aqueous (H_2O)) and solid phase extraction enriched fractions (HEtOH-C18-MeOH and H_2O-C18-MeOH).

Extracts	Yield (% w/w)	Total phenol content (mg GAE/g of sample)[z]	Antioxidant activity (mmol TE/kg of sample)[z]
Caffeic acid			$3.14 \times 10^4 \pm 4.75 \times 10^2$
HEtOH	3.30	12.2 ± 4.2 [a]	$3.22 \times 10^2 \pm 2.19 \times 10^1$ [a]
H_2O	4.87	10.4 ± 3.2 [a]	$3.10 \times 10^2 \pm 3.44 \times 10^1$ [a]
HEtOH-C18-MeOH	7.79	56.4 ± 1.6 [b]	$1.64 \times 10^3 \pm 1.35 \times 10^2$ [b]
H_2O-C18-MeOH	6.20	53.5 ± 5.3 [b]	$1.23 \times 10^3 \pm 2.94 \times 10^2$ [c]

[z] Values are expressed as mean ± standard deviation (n = 4). Means within a column for each sample followed by the same letter are not significantly different (t-Student test, $p < 0.05$).

3.2. Phenolic profile of the C. reticulata extracts and phenolic enriched fractions from SPE

Table 2 shows the polyphenol profile and content of the individual phenolic compounds present in the ethanol and water extracts obtained from *C. reticulata* peels. As can be observed in the crude extracts (Table 2 and Fig. 1), there were present the same components for both extraction solvents. For the SPE enriched extracts a total of 54 polyphenol compounds were found, that besides those present in the crude extracts also included narigenin (41), quercetin (43) and the unknowns 21, 34, 35, 39 and 45 (Table 2 and Fig. 2). The main components of both extracts were hesperidin (38), naringin (32), tangeritin (53), an unknown quercetin derivative (22) and rutin (33), that accounted for, in average, 86% for both water and ethanol extracts and 71% of the SPE enriched extracts (H_2O-C18-MeOH and HEtOH-C18-MeOH). Also identified, by comparison with the retention time and spectra of pure standards were: chlorogenic acid (13), caffeic acid (14), ferulic acid (26), naringenin (41), quercetin (43) and hesperitin (47). This phenolic composition is in accordance with previous studies on *C. reticulata* peels phenolic composition (Wang et al., 2016; Zefang et al., 2016), although the amount of each phenolic compound was slightly lower than those described in other works (Wang et al., 2008), and this can be due to the different extraction solvents used (Wang et al., 2008; Casquete et al., 2015), time of extraction (Wang et al., 2008) or to the variety used, maturity status and edapho-climatic conditions (Moulehi et al., 2012; Zou et al., 2016). The composition of the SPE enriched extracts was the same as the initial extracts although, as discussed previously, with a significantly higher concentration. As can be observed in Table 2 the water and ethanol extracts didn't differed significantly in the amount of the major phenolic compounds recovered from the peels, showing that both extraction methods can be applied for obtaining phenolic compound rich extracts from the *C. reticulata* peels.

The phenolic composition of the citrus peels extracts explains the values observed for the antioxidant activity (Table 1), as hesperidin, naringin, rutin, caffeic acid and chlorogenic acid, that account for 82%

Table 2
Amount of the polyphenolic compounds in *C. reticulata* peels water and 70% extracts and enriched extracts obtained by solid phase extraction and methanol elution determined by HPLC.

Peak	RT (min)[w]	identification	λ_{max}[v]	HEtOH	H_2O	HEtOH-C18-MeOH	H_2O-C18-MeOH
				mg/g dry weight		mg/g of extract	
1	6.73 ± 0.03	Unk.[x]	266	0.120 ± 0.015[a]	0.163 ± 0.034[a]	0.335 ± 0.011[a]	0.265 ± 0.247[a]
2	7.26 ± 0.04	Unk.	312	0.005 ± 0.001[a]	0.007 ± 0.0[a]	0.180 ± 0.005[a]	0.197 ± 0.009[a]
3	8.62 ± 0.03	Unk.	324; 298sh[y]	0.015 ± 0.002[a]	0.020 ± 0.0[b]	0.375 ± 0.019[a]	0.473 ± 0.031[a]
4	10.25 ± 0.03	Unk.	324; 298sh	0.024 ± 0.003[a]	0.030 ± 0.001[a]	0.904 ± 0.022[a]	0.387 ± 0.031[b]
5	11.51 ± 0.03	Unk.	314	0.013 ± 0.001[a]	0.012 ± 0.001[a]	0.462 ± 0.023[a]	0.554 ± 0.019[b]
6	11.77 ± 0.03	Unk.	324; 298sh	0.012 ± 0.002[a]	0.018 ± 0.001[a]	0.517 ± 0.028[a]	0.633 ± 0.040[a]
7	12.30 ± 0.02	Unk.	327; 295sh	0.003 ± 0.0[a]	0.005 ± 0.0[a]	0.133 ± 0.028[a]	0.186 ± 0.011[a]
8	13.51 ± 0.03	Unk.	326; 296sh	0.023 ± 0.002[a]	0.034 ± 0.003[a]	0.802 ± 0.027[a]	0.992 ± 0.058[a]
9	14.56 ± 0.03	Unk.	314	0.016 ± 0.0[a]	0.019 ± 0.001[a]	0.646 ± 0.039[a]	0.739 ± 0.037[a]
10	14.85 ± 0.02	Unk.	330; 294sh	0.031 ± 0.003[a]	0.042 ± 0.003[a]	1.143 ± 0.004[a]	1.441 ± 0.165[a]
11	15.65 ± 0.02	Unk.	320	0.003 ± 0.0[a]	0.001 ± 0.0[b]	0.256 ± 0.016[a]	0.292 ± 0.007[a]
12	16.28 ± 0.01	Unk.	312; 288sh	0.002 ± 0.0[a]	0.003 ± 0.0[b]	0.065 ± 0.004[a]	0.065 ± 0.003[a]
13	16.77 ± 0.02	Chlorogenic acid	326; 295sh	0.011 ± 0.002[a]	0.025 ± 0.0[b]	0.548 ± 0.027[a]	0.660 ± 0.022[b]
14	18.09 ± 0.02	Caffeic acid	323; 300sh	0.032 ± 0.002[a]	0.045 ± 0.005[a]	1.359 ± 0.021[a]	1.624 ± 0.090[a]
15	18.94 ± 0.02	Unk.	329; 295sh	0.016 ± 0.0[a]	0.012 ± 0.002[a]	0.708 ± 0.027[a]	0.970 ± 0.059[b]
16	19.47 ± 0.01	Unk.	325; 295sh	0.003 ± 0.0[a]	0.004 ± 0.0[b]	0.116 ± 0.018[a]	0.160 ± 0.009[a]
17	20.61 ± 0.01	Unk.	330; 295sh	0.005 ± 0.0[a]	0.006 ± 0.0[a]	0.354 ± 0.046[a]	0.353 ± 0.078[a]
18	21.46 ± 0.02	Unk.	344; 270	0.068 ± 0.003[a]	0.094 ± 0.007[b]	2.934 ± 0.07[a]	3.657 ± 0.227[b]
19	22.37 ± 0.02	Unk.	324; 295sh	0.005 ± 0.001[a]	0.007 ± 0.0[a]	0.199 ± 0.009[a]	0.224 ± 0.017[a]
20	22.81 ± 0.01	Unk.	324; 294sh	0.004 ± 0.0[a]	0.007 ± 0.0[b]	0.181 ± 0.006[a]	0.206 ± 0.012[a]
21	23.54 ± 0.02	Unk.	320	n.d[z]	n.d	0.078 ± 0.008[a]	0.063 ± 0.004[a]
22	24.26 ± 0.02	Unk.	336; 271	0.140 ± 0.020[a]	0.299 ± 0.066[a]	5.942 ± 0.275[a]	6.743 ± 0.317[a]
23	25.01 ± 0.01	Unk.	335; 286	0.014 ± 0.001[a]	0.011 ± 0.0[b]	0.839 ± 0.037[a]	0.996 ± 0.085[a]
24	25.50 ± 0.01	Unk.	326; 298sh	0.003 ± 0.0[a]	0.005 ± 0.001[a]	0.138 ± 0.006[a]	0.151 ± 0.006[a]
25	26.35 ± 0.01	Unk.	345; 270	0.032 ± 0.002[a]	0.045 ± 0.003[b]	1.651 ± 0.008[a]	1.984 ± 0.150[a]
26	27.65 ± 0.01	Ferulic acid	323; 294sh	0.047 ± 0.006[a]	0.092 ± 0.008[b]	1.977 ± 0.178[a]	1.761 ± 0.226[a]
27	28.90 ± 0.01	Unk.	345; 283	0.023 ± 0.004[a]	0.037 ± 0.0[b]	1.134 ± 0.051[a]	1.300 ± 0.123[a]
28	29.52 ± 0.01	Unk.	322; 300sh	0.004 ± 0.001[a]	0.006 ± 0.001[a]	0.166 ± 0.008[a]	0.173 ± 0.010[a]
29	30.30 ± 0.01	Unk.	326; 299sh	0.011 ± 0.003[a]	0.014 ± 0.001[a]	0.623 ± 0.032[a]	0.491 ± 0.016[b]
30	31.73 ± 0.01	Unk.	330; 298sh	0.004 ± 0.001[a]	0.006 ± 0.001[a]	0.192 ± 0.008[a]	0.214 ± 0.011[a]
31	33.07 ± 0.01	Unk.	326; 295sh	0.009 ± 0.0[a]	0.012 ± 0.001[b]	0.348 ± 0.010[a]	0.360 ± 0.021[a]
32	33.78 ± 0.01	Naringin	329; 284	0.177 ± 0.010[a]	0.291 ± 0.008[b]	9.446 ± 0.338[a]	10.432 ± 0.737[a]
33	34.00 ± 0.02	Rutin	347; 268	0.113 ± 0.008[a]	0.194 ± 0.008[b]	6.445 ± 0.100[a]	7.158 ± 0.473[a]
34	34.85 ± 0.01	Unk.	329; 296sh	n.d	n.d	0.092 ± 0.017[a]	0.070 ± 0.009[a]
35	35.67 ± 0.01	Unk.	32; 294sh	n.d	n.d	0.112 ± 0.015[a]	0.095 ± 0.0[a]
36	36.10 ± 0.01	Unk.	327; 299sh	0.007 ± 0.001[a]	0.012 ± 0.0[b]	0.344 ± 0.057[a]	0.380 ± 0.026[a]
37	36.49 ± 0.01	Unk.	350; 270	0.022 ± 0.001[a]	0.031 ± 0.003	1.402 ± 0.448[a]	1.529 ± 0.490[a]
38	37.35 ± 0.01	Hesperidin	328; 285	4.297 ± 0.042[a]	6.411 ± 0.023[b]	44.651 ± 0.796[a]	47.163 ± 1.652[a]
39	38.62 ± 0.01	Unk.	326; 300sh	n.d	n.d	0.098 ± 0.003[a]	0.117 ± 0.006[a]
40	39.67 ± 0.01	Unk.	330; 295sh	0.004 ± 0.0[a]	0.006 ± 0.0[b]	0.175 ± 0.011[a]	0.160 ± 0.005[a]
41	40.37 ± 0.01	Naringenin	340; 287	n.d	n.d	0.384 ± 0.017[a]	0.368 ± 0.026[a]
42	41.91 ± 0.01	Unk.	316	0.004 ± 0.0[a]	0.004 ± 0.002[a]	0.206 ± 0.017[a]	0.192 ± 0.008[a]
43	42.97 ± 0.02	Quercetin	374; 255	n.d	n.d	0.235 ± 0.102[a]	0.306 ± 0.026[a]
44	43.85 ± 0.04	Unk.	326; 290sh	0.003 ± 0.001[a]	0.004 ± 0.0[a]	0.057 ± 0.003[a]	0.052 ± 0.001[a]
45	44.74 ± 0.02	Unk.	335	n.d	n.d	0.039 ± 0.002[a]	0.042 ± 0.001[a]
46	46.35 ± 0.01	Unk.	325; 296sh	0.003 ± 0.0[a]	0.004 ± 0.001[a]	0.037 ± 0.011[a]	0.023 ± 0.002[a]
47	46.70 ± 0.01	Hesperetin	355; 280	0.005 ± 0.001[a]	n.d[b]	0.241 ± 0.006[a]	0.254 ± 0.002[a]
48	57.63 ± 0.01	Unk.	344; 271; 255	0.017 ± 0.002[a]	0.024 ± 0.002[a]	0.756 ± 0.033 [a]	0.880 ± 0.062 [a]
49	58.30 ± 0.00	Unk.	344; 271; 250	0.007 ± 0.002[a]	0.009 ± 0.0[a]	0.315 ± 0.015 [a]	0.307 ± 0.005 [a]
50	59.93 ± 0.00	Unk.	333; 254; 250	0.025 ± 0.001[a]	0.035 ± 0.001[b]	1.047 ± 0.046 [a]	1.135 ± 0.082 [a]
51	61.40 ± 0.00	Unk.	335; 250	0.012 ± 0.001[a]	0.017 ± 0.0[b]	0.526 ± 0.028 [a]	0.582 ± 0.045 [a]
52	63.39 ± 0.01	Unk.	350; 290; 260	0.019 ± 0.002[a]	0.024 ± 0.0[a]	0.339 ± 0.006 [a]	0.338 ± 0.007 [a]
53	64.98 ± 0.01	Tangeretin	334; 271; 251	0.167 ± 0.016[a]	0.237 ± 0.009[b]	7.655 ± 0.363 [a]	8.090 ± 0.50 [a]
54	66.35 ± 0.01	Unk.	344; 270; 254	0.084 ± 0.005[a]	0.133 ± 0.007[b]	3.329 ± 0.134 [a]	2.956 ± 1.093 [a]
Total of compounds identified (%)				85.6	85.6	70.7	70.1

Values are expressed as mean ± standard deviation (n = 4); Means between two column for each polyphenol followed by the same letter are not significantly different (t-Student test, $p < 0.05$).

[w] RT – Retention time.
[v] λ_{max} – maximum wavelength.
[x] Unk – unknown.
[y] sh – shoulder.
[z] n.d. – not detected.

for H_2O and HEtOH extracts and 61% for the H_2O-C18-MeOH and HEtOH-C18-MeOH extracts, present a strong $ABTS^{+\bullet}$ radical scavenging activity (Fernandez-Panchon et al., 2008).

3.3. Effect of peel extracts on cell viability and anti-proliferative activity

As the bioactivity of the extracts depend on their composition, relative abundance of the different components, and on the cell type, three different cell lines were used to evaluate the anti-proliferative effect of HEtOH and HEtOH-C18-MeOH extracts. BT-474, Caco-2 and HepG2 cells were exposed for 24 h or 48 h to different concentrations of each extract, as shown in Fig. 3. As observed, exposure of cells to HEtOH extract (Fig. 3A–C) produce different effects on cell viability, with the BT-474 (human breast carcinoma) being the most affected

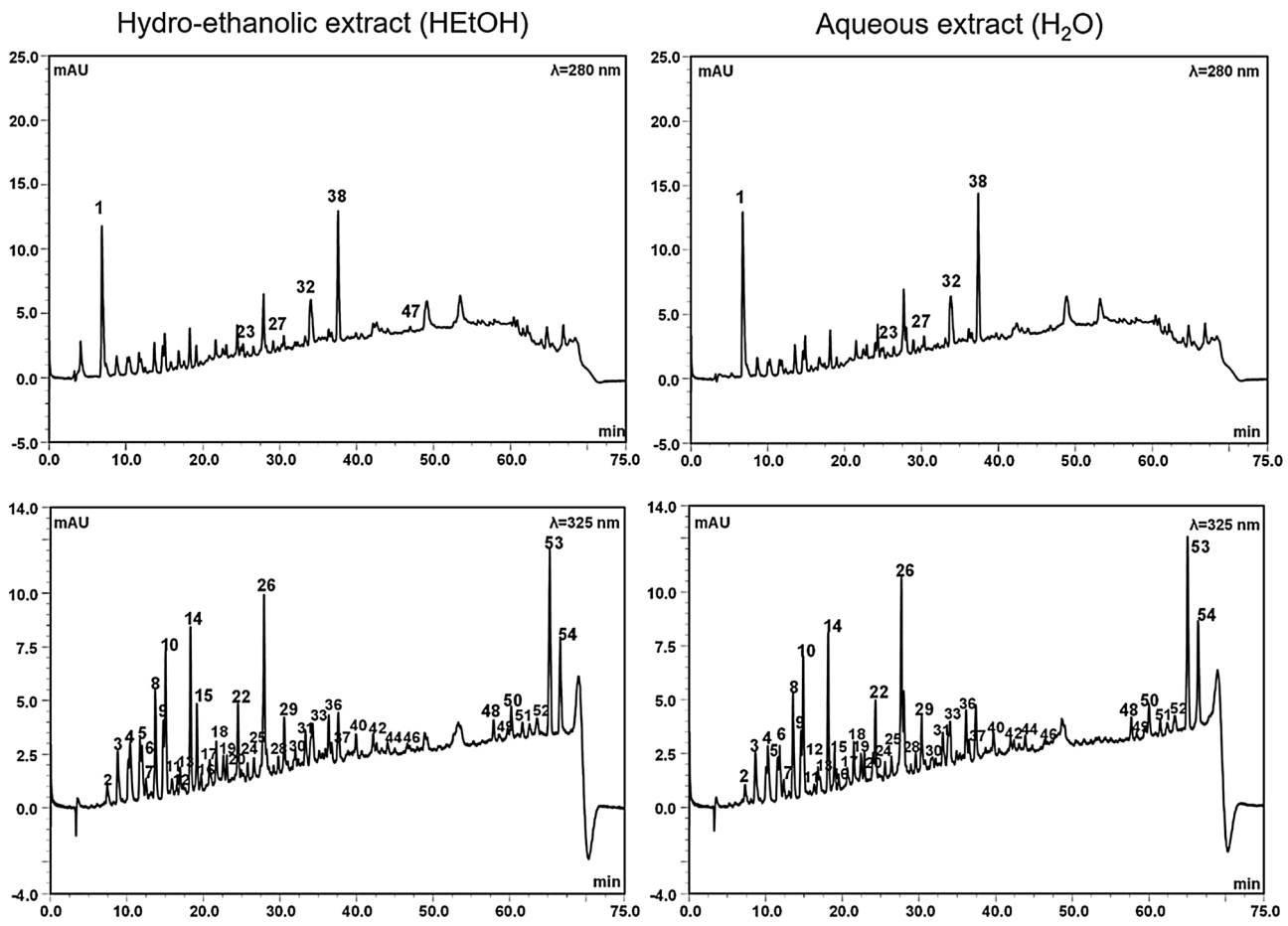

Fig. 1. Chromatograms at 280 nm and 325 nm of crude extracts (hydro-ethanolic and aqueous) obtained from *C. reticulata* peel. Peak identification: 13 – chlorogenic acid; 14 – caffeic acid; 26 – ferulic acid; 32 – naringin; 33 – rutin; 38 – hesperidin; 47 – hesperetin; 53 – tangeretin; 1–12, 15–20, 22–25, 27–31, 36, 37, 40, 42, 44, 46, 48–52 and 54 unknown compounds.

(reduction on the cell viability of about 47% and 60% after 24 or 48 h of exposure, respectively, to 500 μg/mL of HEtOH extract, Fig. 3A). For the Caco-2 (Fig. 3B) and HepG2 (Fig. 3C) cells only a slight reduction on cell viability was observed that seems to be reverted with increased exposure time. As HEtOH-C18-MeOH extract enriched by RP-SPE had a higher concentration of phenolics and less contaminants (Table 1), an increase of the anti-proliferative effect was expected. Indeed a reduction on cell viability was observed for the three cell lines (Fig. 3D–F), although not in the same extent as the increase in phenolic compounds in the extract (~4.6-fold increase of phenolics, Table 1). Again BT-474 cells were the most affected with reduction on cell viability of about 80 and 85%, after 24 or 48 h of exposure to the highest concentration (cell viability was 20.1 ± 4.9% and 15.6 ± 6.2% of control, at 24 or 48 h of exposure). Exposure to 500 μg/mL of HEtOH-C18-MeOH extract reduced Caco-2 (Fig. 3E) and HepG2 (Fig. 3F) cell viability to values close to 50% which were not significantly increased with exposure time (at 24 h exposure cell viability of Caco-2 was 54.0 ± 7.0% and of HepG2 was 57.7 ± 12.5% and at 48 h Caco-2 viability was 46.2 ± 6.7% and HepG2 was 62.1 ± 11.1%, of respective controls). We observed a dose dependent effect (for the three cell lines) but not a time-dependent exposure effect (Fig. 3).

To the best of our knowledge this is the first report of the effect of citrus peel extract on cell viability using BT-474 cells. However, some studies have been made using other breast cancer cell lines, such as MCF-7, SK-BR-3. Although all have mammary origin, BT-474, MDA-MB-361 and SK-BR-3 are known to overexpress the HER2/*neu* (Human Epidermal growth factor Receptor 2) oncogene (also known as ErB2 proto-oncogene) while its expression in MCF-7, MDA-MB-231, MDA-MB-435, and MDA-MB-468 is residual or absent (Hermanto et al., 2001; Huang et al., 2005). Overexpression of HER2/*neu* is correlated with aggressive tumors, including metastatic potential, and resistance to chemotherapy (Hermanto et al., 2001). The anti-proliferative activity of an extract from *C. reticulata* Blanco cv. Chachiensis peel against the cells MCF-7, HepG2 and SiHa (cervical cancer cell line) cells exposed for 72 h, revealed IC_{50} values of 2.35 ± 0.16, 2.08 ± 0.09 and 2.78 ± 0.14 mg/mL, respectively (Wang et al., 2016), these values are about 4–5.5 ~ fold higher than the highest concentration used by us (500 μg/mL). Our results indicate that after 48 h of exposure to HEtOH extract, the IC_{50} was 349.3 ± 13.2 μg/mL to BT-474 cells and higher than 500 μg/mL to the other cell types (Table 3), and 48 h exposure to HEtOH-C18-MeOH extract reduced the IC_{50} to all cell lines (BT-474: 174.5 ± 5.8 μg/mL; Caco-2: 391.9 ± 15 μg/mL; HepG2 > 500.0 μg/mL), showing that our extract presented a higher anti-proliferative activity. Scherbakov and Andreeva (2015) exposed MCF-7 cells and SKBR3 cells to individual phenolic compounds that were present in the citrus peel extract such as naringenin, quercetin and apigenin, which are phytoestrogens, and observed similar anti-proliferative results in both cell lines, being the best molecule apigenin with an IC_{50} ~30 μM, indicating that the target could not be directly linked to ErB2. Another component of citrus peel extract, hesperidin (Natarajan et al., 2011), was studied concerning its bioactivity against MCF-7 cells (24 h exposure, 20–100 μM), and was demonstrated to decrease cell proliferation in dose-dependent manner, showing an IC_{50} of 80 μM. The cytotoxicity effect was shown to be associated with apoptosis (expression of caspase-3 and accumulation of p53) and DNA damage (Natarajan et al., 2011). The *C. reticulata* HEtOH-C18-MeOH extract, obtained in this work, was rich in hesperidin (47 mg/g of extract, Table 2). In the IC_{50} value obtained for the BT-474 exposed to this extract (Table 3)

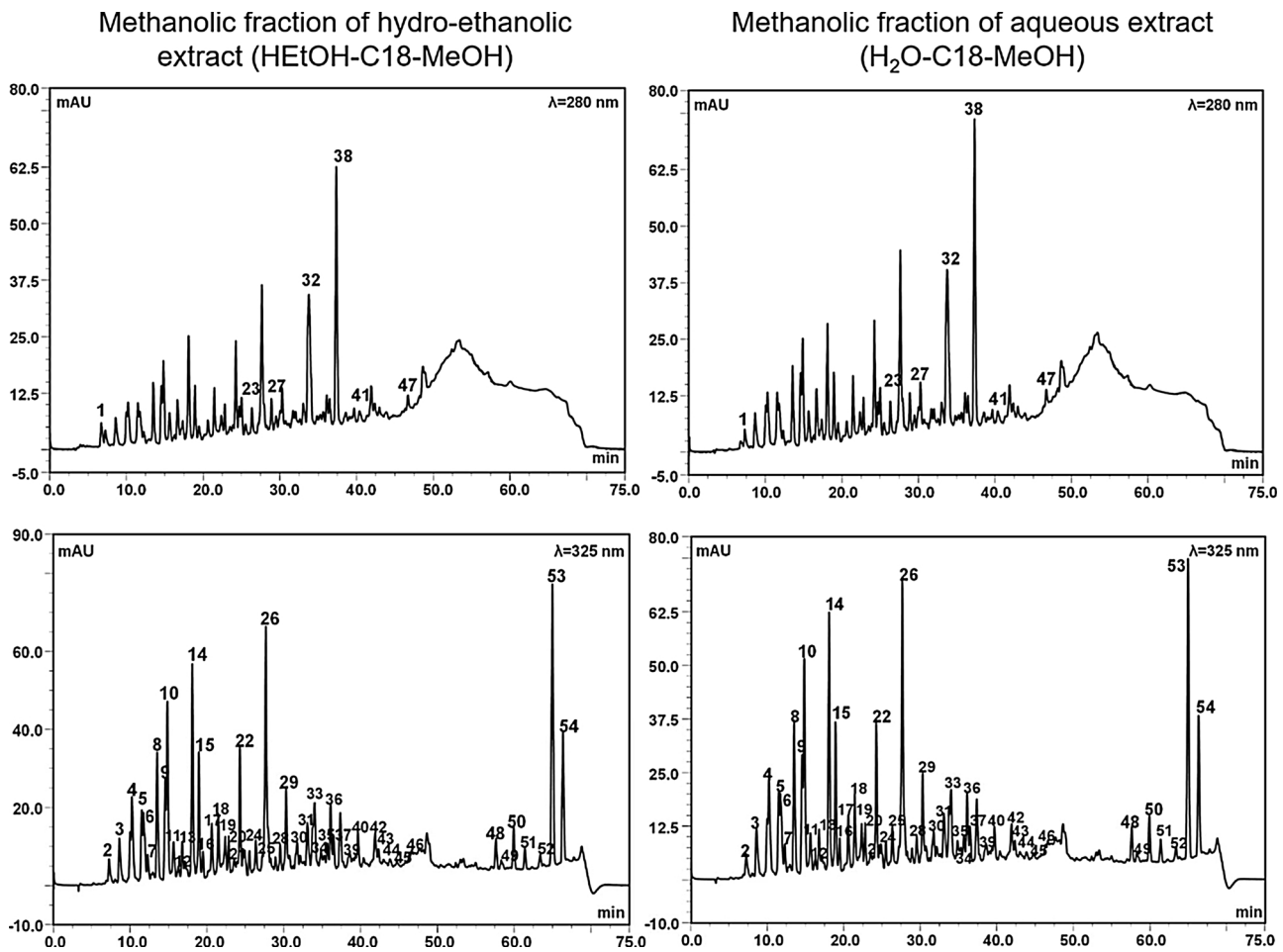

Fig. 2. Chromatograms at 280 nm and 325 nm of phenolic enriched extracts obtained by solid phase extraction of the *C. reticulata* peel. Peak identification: 13 – chlorogenic acid; 14 – caffeic acid; 26 – ferulic acid; 32 – naringin; 33 – rutin; 38 – hesperidin; 41 – naringenin; 43 – quercetin; 47 – hesperetin; 53 k tangeretin; 1–12, 15–25, 27–31, 34–37, 39,40,42, 44, 45, 46, 48 − 52 and 54 unknown compounds.

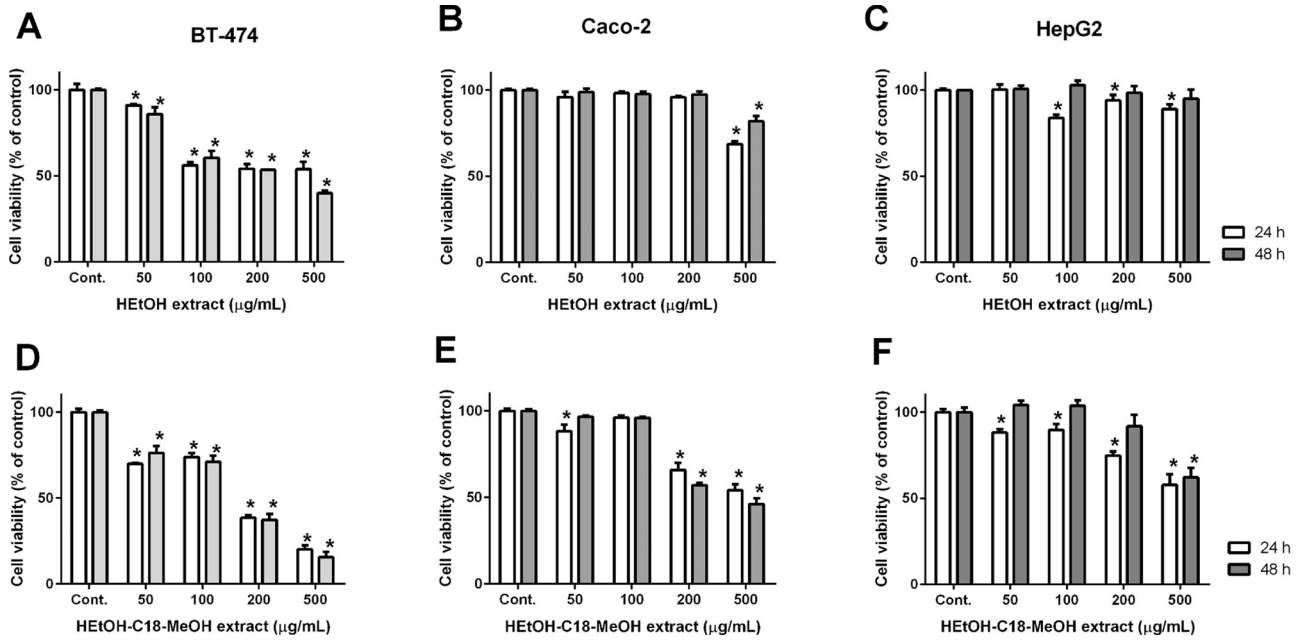

Fig. 3. Cell viability and anti-proliferative activity of BT-474 (A and D), Caco-2 (B and E) and HepG2 (C and F) cells exposed to the crude hydro-ethanolic extract (HEtOH, upper row) and its methanolic fraction (HEtOH-C18-MeOH, lower raw) obtained from *C. reticulata* peel, for 24 (white bars) and 48 (grey bars) hours. Data are expressed as percentage of control, untreated cells, as mean ± standard deviation (see methods). * means statistically different from control $p < 0.05$.

Table 3
Anti-proliferative activity against BT-474, Caco-2 and HepG2 cells, after exposure to HEtOH or HEtOH-C18-MeOH extracts for 24 or 48 h, as indicated. The IC_{50} values are the mean ± standard deviation of n = 4 experiments.

IC_{50} (μg/mL)	BT-474	Caco-2	HepG2
HEtOH (24 h)	> 500.0 (~550)	> > 500.0	> > 500.0
HEtOH (48 h)	349.3 ± 13.2	> > 500.0	> > 500.0
HEtOH-C18-MeOH (24 h)	167.2 ± 4.4	> 500.0 (~660)	> 500.0
HEtOH-C18-MeOH (48 h)	174.5 ± 5.8	391.9 ± 15.0	> 500.0

represents a concentration of 13.4 μM of hesperidin, below the IC_{50} obtained for the pure hesperidin reported by Natarajan et al. (2011) in MCF-7 cells. These results suggest that a synergistic effect of hesperidin (the major extract component, Table 2) with other polyphenols present in the extract might be occurring. Other major components of the extract were naringin (10.43 mg/g) and tangeretin (8.09 mg/g), that at BT-474 cell' IC_{50} were 2.83 and 4.67 μM, respectively. Naringin and tangeretin have been screened for anti-tumoral activity on other mammary cell lines showing IC_{50} of 50–200 μM (MDA-MB-435, MCF-7) and ~54 μM (MDA-MB-231, MDA-MB-468, and BT-549 cells), respectively (Cirmi et al., 2016). We also could speculate that the higher bioactivity of the HEtOH-C18-MeOH extract on BT-474 cells could result from a direct interaction of the extract components with specific receptors present on BT-474 cells and absent on MCF-7 cells. Taking into account our results and the characteristics of BT-474 cells, such as ER+, PR+, HER2+ (Hermanto et al., 2001; Willmann et al., 2015), shows that extracts from *C. reticulata* peel may have a therapeutic potential and chemopreventive effect against the breast cancer while only showing a small cytotoxic effect on HepG2 and Caco-2 cells. This may also imply a low hepatotoxicity and colon-toxicity of the extract and a cell specific selectivity of molecules in the extract. The American National Cancer Institute (Suffness and Pezzuto, 1990) and South-American Office for Anti-Cancer Drug Development (Mans et al., 2000) recommend to consider crude extracts promising for further purification, those with IC_{50} values less than 30–50 μg/mL. *C. reticulata* HEtOH-C18-MeOH extract presented a higher IC_{50} value (Table 3), nevertheless the application of the simple RP-SPE procedure allowed to decrease significantly the IC_{50} value of the extract for the BT-474 cells (~3.3 and 2 times for 24 h and 48 h exposure Table 3). Even after this enrichment, the extracts were not completely purified (Table 2) with various phenolic compounds being present in the extract (54 compounds), therefore further purification of this extract could render even more active fractions. Also the anti-proliferative activity presented by *C. reticulata* HEtOH-C18-MeOH extract to BT-474 cells could be explored in combined therapy with conventional anti-cancer drugs, as it has been reported that several of its main components such as tangeritin (25–150 μmol/L range; Arafa el al., 2009) and hesperidin (10–100 μM range; Nazari et al., 2011; El-Readi et al., 2010) have sensitizing and potentiating effects when combined with conventional anti-cancer drugs like cisplatin (Arafa el al., 2009) and doxorubicin (Nazari et al., 2011; El-Readi et al., 2010). This aspect should be further explored in future studies.

4. Conclusions

C. reticulata peel by-products can be re-used as a cheap and abundant rich source of antioxidant polyphenols for the pharmaceutical, cosmetic and related industries. The low hepatotoxicity and colon-toxicity of the obtained extracts and the anti-proliferative activity of the polyphenol extract obtained by the simple RP-SPE enrichment method show that polyphenols from *C. reticulata* peels may present therapeutic potential and chemopreventive effect against breast cancer. Re-use of citrus peel by-products by recovering its bioactive compounds can be a good strategy for implementation of the circular economy concept in citrus fruit transformation industries.

Acknowledgments

The authors want to acknowledge QREN, ADI, Programa Operacional do Norte and FEDER for the financial support of Project NutriDouro n. 13311. We also acknowledge the European Investment Funds by FEDER/COMPETE/POCI under Projects POCI-01-0145-FEDER-006958 (CITAB) and POCI-01-0145-FEDER-007728 (CQ-VR) and Funds from the Portuguese Foundation for Science and Technology (FCT), to CITAB (UID/AGR/04033/2013) and to CQ-VR (PEst-OE/QUI/UI0616/2014).

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