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Antioxidant and cytoprotective activities of an ancient Mediterranean citrus (*Citrus lumia* Risso) albedo extract: microscopic observations and polyphenol characterization.

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ABSTRACT

Citrus fruits are a rich source of bio-functional compounds with various and well-proven health properties. We focused our attention on an ancient Mediterranean species, *Citrus lumia* Risso. The aim of this work was to investigate the polyphenol content and biological activities of *C. lumia* albedo extract by cell-free and cell-based assays. Fifteen polyphenols were quantified by LC-DAD-FLD analysis (flavonoids 89.34% and phenolic acids 10.66%) with eriocitrin and hesperidin as major components (52.81% and 31.31%, respectively). These results were corroborated also by micromorphological observations, which showed clusters of needle-shaped crystals of hesperidin highlighted by TBO staining. *C. lumia* albedo extract revealed marked antioxidant and free radical scavenging properties, and a significant cytoprotective activity on t-BOOH-treated lymphocytes. Results indicate that *C. lumia* albedo extract could be exploited as an antioxidant source, suggesting the use of albedo, currently considered a by-product, as a valuable raw material for nutraceutical employments.

KEYWORDS: *Citrus lumia* Risso; Albedo extract; Polyphenols; Flavonoids; Hesperidin crystals; Antioxidant properties; Cytoprotective activity.

1. Introduction

The genus *Citrus* L., belonging to the Rutaceae family, is a major fruit crop worldwide, whose production has been increasing strongly in the last decades, reaching over 100 million metric tons per year (Zou, Xi, Hu, Nie, & Zhou, 2016; Castro-Vazquez et al., 2016). Citrus fruits are well-accepted by consumers due to their colours, flavors and aroma, and also because they are a major source of bioactive compounds such as vitamins, carotenoids, fiber, flavonoids and other phenolics, as well as essential minerals (Zou, Xi, Hu, Nie, & Zhou, 2016; Barros, Ferreira, & Genovese, 2012).

Only a little part of this production is destined to the fresh market, while the remaining portion is used to make fresh juice, citrus-based drinks, marmalades, jams, dehydrated products, and flavoring ingredients. This complex of production chains generates large amounts of by-products, globally accounting for a 50% in weight of the original fruit crop (Castro-Vazquez et al., 2016; Barros, Ferreira, & Genovese, 2012). Wash water, solid residues, such as seeds, peels and membranes, as well as semisolid residue (centrifugation pulp), are major waste products, which, if not recycled, become a possible source of environmental pollution (Barros, Ferreira, & Genovese, 2012).

Solid residues, mainly composed by the fruit exocarp (flavedo and albedo) and endocarp residual membranes, have been popularly used as molasses for animal feed, as dietary fiber source (pectin), and for fuel production (Barros, Ferreira, & Genovese, 2012). More recently, this material has been reevaluated with the aim of obtaining value-added products (Sharma, Mahato, Cho, & Lee, 2017; Shan, 2016). Different studies conducted on various *Citrus* species have shown that peels are rich in natural antioxidants, suggesting possible food, nutraceutical and cosmetic applications (Liu, Heying, & Tanumihardjo, 2012). Anticancer, antimicrobial, antioxidant, anti-inflammatory, cardioprotective and neuroprotective effects have been associated with the consumption of *Citrus* fruit or derived products, as reported by *in vitro* and *in vivo* studies (Barreca et al., 2017). These beneficial effects are mainly attributable to the high flavonoid content and in particular, to flavanones, some of which typical of this plant genus, able to scavenge reactive oxygen species (ROS) and nitrogen species (RNS), inhibiting oxidant enzymes, interacting with redox signaling-pathways as well as chelating transition metals (Barros, Ferreira, & Genovese, 2012; Zou, Xi, Hu, Nie, & Zhou, 2016).

The antioxidant activity and polyphenol content of fruits and by-products of various *Citrus* species have been evaluated (Safdar et al., 2017; Pepe et al., 2017; Castro-Vazquez et al., 2016; Sharma et al., 2017; Diab, 2016; Esparza-Martínez, Miranda-López, Mata-Sánchez, & Guzmán-Maldonado, 2016), but up-to date, no data were available about *C. lumia* Risso, which is an old lumie cultivated since ancient times in the Mediterranean area. We have previously investigated the chemical

composition, and the antioxidant, anti-cholinesterase, and neuroactive properties of *C. lumia* essential oil, showing that the fruit peel is has marked antioxidants activities that could be exploited for the prevention of oxidative stress-related diseases, especially neurodegenerative ones (Smeriglio et al., 2017a). This work was aimed at investigating the polyphenol profile of a *C. lumia* albedo extract, through chromatographic and microscopic techniques, as well as antioxidant and cytoprotective properties.

2. Materials and Method

2.1 Chemicals

Reagents were from Sigma-Aldrich (Milan, Italy), HPLC-grade solvents from Merck (Darmstadt, Germany), and polyphenol standards from Extrasynthese (Genay, France).

2.2 Sample preparation

The fresh fruit albedo of *Citrus lumia* Risso, were harvested in March 2017 by a farmer in Agrigento (AG, Italy), manually separated from peel and pulp, and grounded to fine powder by a blade mill (IKA® A11 basic analytical mill), under liquid nitrogen, in order to block enzymatic activities and preserve organoleptic and nutritional characteristics.

Two grams of frozen powder were extracted 3 times with 40 mL of CH_3OH/H_2O (80:20 *v*/*v*), vortex-mixed for 3 minutes, and sonicated in an ice-cold bath for 5 minutes, using a 3 mm titanium probe, set to 200W power and 30% amplitude signal (Vibra CellTM Sonics Materials, inc., Danbury, Connecticut, U.S.A.) (Safdar et al., 2017). Thereafter, the sample was centrifuged at 3000 × *g* for 10 minutes, and the supernatant was filtered through filter paper and evaporated to dryness by a rotary evaporator at 37 °C. The dry extract (yield 34.10 %), was suspended in CH_3OH/H_2O (80:20 *v*/*v*), and used for polyphenol profile determination and subsequent analyses.

2.3 Determination of polyphenol profile by RP-LC-DAD-FLU analysis

Polyphenol determination was carried out according to Barreca et al. (2016), using an Agilent high-performance liquid chromatography system (1100 series, Santa Clara, CA, USA), equipped with a photodiode-array detector (DAD) (G1315) and a fluorescence detector (FLD) (G1321). Chromatographic analysis was carried with a 5 μm ODS3 reversed-phase Prodigy column (250 mm x 4.6 mm; Phenomenex) with solvent A (water/acetic acid, 97/3 v/v) and solvent B (methanol) under the following gradient conditions: 0–3 min, 0% B; 3–9 min, 3% B; 9–24 min, 12% B; 24–30 min, 20% B; 30–33 min, 20% B; 33–43 min, 30% B; 43–63 min, 50% B; 63–66 min, 50% B; 66–

76 min, 60% B; 76–81 min, 60% B; 81–86 min, 0% B and equilibrated 4 min for a total run time of 90 min. Flow rate, injection volume and column temperature were 1.0 ml/min, 50 μL, and 25 °C, respectively. UV-Vis spectra of phenolics were acquired in the range 190-400 nm. Quantitative analysis was done at 260 nm for hydroxybenzoic acids, 292 nm for hydroxycinnamic acids, 330 nm for flavanones and 370 nm for flavonols. Detection of fluorescence was carried out at λ_{ex} 276 nm and λ_{em} 316 nm. Peaks were identified by comparing absorption spectra and retention times with those recorded for commercially-available reference compounds (purity ≥ 99%, concentration range 1–50 μg/mL).

2.4 *Light microscopy (LM)*

Frozen fruits of *C. lumia* were hand sliced with a knife and the peel separated from edible portions. Small pieces of peel, approximately 1 cm^2, were sectioned with a razor blade, fixed in FineFIX working solution (Milestone s.r.l., Bergamo, Italy) with 70% ethanol, and left overnight at 4 °C (Chieco, Rotondi, Morrone, Rapparini, & Baraldi, 2013). Samples were dehydrated for 1 h in ethanol: 80, 90, 95 and 100%, and then embedded in paraffin. Cross sections of 5-10 μm were cut with a rotary microtome (Thermo Scientific® HM 325). Paraffin was removed by multiple immersions in Histo-Clear®, followed by ethanol (100% and 75%, 5 min per step) and finally streaming water. Sections were mounted on slides, stained with toluidine blue in phosphate buffer, pH 4.4 (O'Brien & McCully, 1981), and observed with a Leica DM 2000 transmission-light microscope, equipped with a DFC 320 camera (Leica Microsystems, Wetzlar, Germany).

2.5 Scanning electron microscopy (SEM)

Pieces of peel measuring 1.5-2 cm^2 were cut with a razor blade to obtain longitudinal and tangential sections. Samples were then fixed in FineFIX, followed by dehydration through ethanol series as reported above. The specimens were subsequently dehydrated in CO_2 using a Critical Point Drier (K850 CPD 2M Strumenti S.r.l., Roma, Italy). Dried samples were mounted on stubs, coated with 10 nm gold, and observe with a Vega3 Tescan LMU scanning electron microscope (SEM) (Tescan USA Inc., Cranberry Twp, PA) at an accelerating voltage of 20 kV, coupled to an X-ray energy dispersive system (EDS) Apollo XSD (Tescan USA Inc., War-rendale, PA, USA).

2.6 Determination of total phenolics

Phenols were detected using the Folin–Ciocalteu method as reported by Smeriglio *et al.* (2016). Aliquots of 50 μL of sample (4-17 μg/mL) were mixed with Folin–Ciocalteu reagent (500 μL), then

with deionized water (450 µL), and allowed to react for 3 min. Thereafter, sodium carbonate 10 % (w/v) (500 µL) was added, samples were maintained in the dark at RT for 1 h, vortexing every 10 min, and absorbance was then read at 785 nm with an UV–VIS spectrophotometer (Shimadzu UV-1601).

The antioxidant activity, expressed as inhibition (%) was obtained from the following equation:

$$(1)\ \text{Inhibition}\ (\%) = \frac{A_0 - A_S}{A_0} \times 100$$

where A_0 is control absorbance and A_s is sample absorbance.

2.7 Ferric reducing antioxidant power (FRAP) assay

This assay was performed following Smeriglio *et al.* (2018). Working FRAP reagent, prepared daily by mixing acetate buffer (300 mM, pH 3.6), 2,4,6-Tris(2-pyridyl)-S-triazine (TPTZ) solution (10 mM in 40 mM HCl), and $FeCl_3 \bullet 6H_2O$ solution (20 mM) in 10:1:1 v/v ratio, was warmed at 37 °C, and used to read initial absorbance. After that, 50 µl of sample (1.5-8 µg/mL) were added to 1.5 mL of FRAP reagent and absorbance was measured after 4 min at 593 nm in the above spectrophotometer. Radical scavenging activity was obtained from equation (1), and expressed as inhibition (%).

2.8 Trolox equivalent antioxidant capacity (TEAC) assay

Antioxidant activity against ABTS radical was evaluated as reported by Smeriglio *et al.* (2017b). Briefly, a mix of 4.3 mM potassium persulfate and 1.7 mM ABTS solution 1:5, v/v was incubated for 12–16 h in the dark at RT, and then diluted with phosphate buffer (pH 7.4) until an absorbance at 734 nm of 0.7 ± 0.02 was obtained. An aliquot of 50 µL of sample (12-95 µg/mL) was then added to 1 mL of the above mix, incubated in the dark at RT for 6 min, and read at 734 nm in the spectrophotometer. Radical scavenging activity was derived as above using equation (1).

2.9 DPPH Assay

By following the protocol reported in Smeriglio et al. (2017b), fresh DPPH methanol solution (10^{-4} M) was mixed with sample (37.5 µL, 76-610 µg/mL), vortexed for 10 s at RT, and after 20 min was read at 517 nm against blank in the spectrophotometer. Radical scavenging activity was derived as above using equation (1).

2.10 β-Carotene bleaching

The assay was carried out by preparing an emulsion as described in Smeriglio et al. (2016). Aliquots of 8 mL of emulsion were mixed with sample (0.32 mL, 20-160 µg/mL), while emulsion without β-Carotene was used as control. The mixture was read at *t*=0, 470 nm in the spectrophotometer, incubated in a water bath at 50 °C for 120 min, and then read every 20 min. Data were % inhibition of β-carotene bleaching obtained from equation (1), using BHT (2 mg/mL) as reference compound.

2.11 Oxygen Radical Absorbance Capacity (ORAC) assay

Antioxidant activity against AAPH radical was measured according to Bellocco et al. (2016). Briefly, 20 µL of sample (0.8-6.2 µg/mL) were dissolved in 75 mM phosphate buffer (pH 7.4), pre-incubated for 15 min at 37 °C with 120 µL of fluorescein (117 nM, fresh-daily), combined with 60 µl of freshly-prepared 40 mM AAPH, and read every 30 s for 90 min (λ_{ex}: 485; λ_{em}: 520) in a fluorescence plate reader (FLUOstar Omega, BMG LABTECH). In each assay, blanks containing phosphate buffer instead of sample, and trolox standards (10–100 µM), were also present. Data were expressed as % inhibition of fluorescence decay, using equation (1).

2.12 Lymphocyte isolation

The isolation of lymphocytes was performed from heparinized blood collected from healthy volunteers, who were not subjected to anti-inflammatory treatment and whose medical histories had been recorded by standardized questionnaire. Blood samples were mixed 1:1 with balanced salt solution, layered over Histopaque-1077 (Sigma-Aldrich), and centrifuged at 400×*g* at 25 °C for 30-40 min. The peripheral blood mononuclear cell (PBMC) layer was recovered with a pipette, washed by centrifugation, and the fraction was enriched in lymphocytes through a Percoll gradient (Repnik, Knezevic, & Jeras, 2003). Cell viability (> 90%) was checked with a haemocytometer, and $1x10^5$ cells/ml were then suspended in a growth medium consisting of RPMI 1640, 10% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin G and streptomycin.

2.13 Cytotoxicity and Cytoprotective assays

Cells ($1x10^5$/ml) were incubated in growing medium for 24 h in the presence or absence of 5, 10, 15, 25, and 50 µg/mL of albedo extract, for cytotoxicity assay, and with or without 5-50 µg/mL extract in the presence of 100 µM tert-butyl hydroperoxide (t-BOOH) for cytoprotective assay.

Extract stock solutions were properly diluted with PBS in order to keep methanol solvent below 0.1% in cell incubation mixtures. After incubation, suspended cells were diluted 1:1 (v:v) with 0.4% trypan blue and counted with a haemocytometer. Cytotoxicity was also determined through lactate dehydrogenase (LDH) activity (BioSystems S.A.), caspase 3 activation according to the method described by Barreca et al. (2017), and quantification of protein carbonyl groups as reported by Bellocco et al. (2016). The extracts concentrations used in these experiments did not show any interference with the assays.

3. Results and discussions

3.1 Polyphenolic profile

Extract samples subjected to RP-LC-DAD-FLD analysis showed a very high content of polyphenols, 1,917.233 mg/100 g of albedo fresh weight (FW) (Table 1; Figure 1). Such a result was probably made possible by the efficient ultrasound-assisted extraction of raw material previously treated with liquid nitrogen to block enzymatic activity. Flavonoids were the most abundant fraction (1,712.896 mg/100 g of FW) followed by phenolic acids (204.337 mg/100 g of FW), mostly represented by clorogenic and ferulic acids (Table 1). Among flavonoids, flavanones were the most abundant class (98 %) followed by flavonols (2 %), similarly to what found in other *Citrus* species (Barreca et al., 2017; Testai and Calderone, 2017; Castro-Vazquez et al., 2016; Sharma et al., 2017).

In the *Citrus* genus, the major flavonoid is generally hesperidin, followed in many species by naringin or narirutin, such as *Citrus japonica* Thumb., *Citrus reticulata x C. sinensis, Citrus medica* L., *Citrus sinensis* (L.) Osbeck, and *Citrus reticulate* Blanco (Barreca et al., 2017; Sharma et al., 2017; Barros, Ferreira, & Genovese, 2012). In contrast, in our extract the most abundant flavonoid was eriocitrin followed by hesperidin, representing 53% (1,012.407 mg/100 g FW) and 31% of total flavonoids (600.377 mg/100 g FW), respectively (Table 1). In addition, naringin and narirutin were not detectable.

C. lumia albedo showed instead a flavonoid profile similar to that obtained from peels and other solid residues of lemon (*C. lemon* (L.) Burm. F.). Lemon solid waste contains mainly hesperidin and eriocitrin, whereas in the liquid residue naringin and eriocitrin are predominant (Sharma et al., 2017).; Species-specific patterns of flavanones make these compounds useful phytochemical markers for the *Citrus* genus (Sharma et al., 2017).

In light of our results, *C. lumia* albedo shows a higher polyphenols content compared with other *Citrus* fruits, and in addition a peculiar flavonoid profile. This suggests the possibility of using this by-product of *C. lumia* processing for nutraceutical, pharmaceutical and cosmetic applications.

3.2 Micro-morphological studies

TBO metachromatic staining allowed to detect hesperidin crystals in *C. lumia* peel. Hesperidin was observed as blue-green clusters of spherical crystals, gathering at the boundary between flavedo and albedo layers (Figure 2 A, red arrows). These crystals were very abundant in areas surrounding vascular bundles (Figure 2 B-C, red arrows). At higher magnification, spherical hesperidin crystals appeared as aggregates of needle-shaped crystals (Figure 2 D, red arrows). In addition, TBO-negative prismatic crystals of calcium oxalate were also visible (Figure 2 C, green arrows).

SEM observations confirmed the presence of many spherical clusters of hesperidin crystals (Figure 3 A-C, red arrows), together with calcium oxalate crystals (Figure 3 D green arrow). SEM-EDS analysis of the different crystal types revealed their chemical composition (Figure 3 E and F), showing the absence of mineral elements in spherical crystals (Figure 3 E), and a high level of calcium in prismatic crystals (Figure 3 F).

Organic crystals are infrequent in angiosperms, while hesperidin is a major flavanone of Rutaceae and Apiaceae. The compound was detected in *C. lumia* peel mainly around vascular bundles and at the limit between flavedo and albedo layers. Hesperidin and diosmin crystals have been previously reported in other Rutaceae, such as limon and buchu (*Barosma betulina*), both known for their high flavonoid content (Kanes, Tisserat, Berhow, & Vandercook, 1993). In peels of *C. unshiu* (satsuma mandarin), a distribution of hesperidin crystals similar to that observed by us in *C. lumia* was reported by Inoue et al. (2014). These authors carried out *in situ* detection of crystals by borax methylene blue staining, and used Raman microscopy to show the presence of hesperidin. In another study on the leaves of *Chritmum maritimum* (Apiaceae), crystals of hesperidin and diosmin were microscopically observed in vessels of the vascular bundles and near the phloem, and quantitatively characterized by HPLC analysis (Cornara et al., 2009).

3.3 Antioxidant activity

The *C. lumia* albedo extract showed dose-dependent antioxidant and free-radical scavenging activities with the following strengths among the performed assays: ORAC > FRAP> Folin-Ciocalteu > TEAC > β-carotene bleaching > DPPH (Figure 4). The results suggest that the extract acts as a powerful scavenger of different free radicals, with primary antioxidant activity probably depending on the reducing ability of hydroxylated phenolic structures and glycosylation degree (Barreca et al., 2016; Smeriglio et al., 2016).

Among polyphenols, major contributions to albedo extract antioxidant and free radical activities are probably due to eriocitrin and hesperindin. It is well known that dietary flavonoids retain high ROS

scavenging power, thereby counteracting lipid oxidation *in vitro* and improving the body's antioxidant enzyme activity (Zou, Xi, Hu, Nie, & Zhou, 2016). Among *Citrus* flavanones, the most studied from this point of view are naringin, hesperidin, and naringenin (Zou, Xi, Hu, Nie, & Zhou, 2016).

According to the literature, eriocitrin exerts the strongest antiradical activity among *Citrus* flavonoids. It has been shown that a major role is played by two hydroxy groups that in this flavonoid are bound to the B ring in ortho position with respect to each other (Diab, Shafik, & Yasuda, 2015). However, in C. lumia extract other flavonoids could contribute to total antioxidant activity, such as rutin, endowed with a catecholic B-ring and a 4-oxo-conjugated, 2,3 double bond in the C ring (Barreca et al., 2016). Flavonoids with the same hydroxylation pattern in the A and C rings and possessing a monohydroxylated B ring (i.e. naringenin, narirutin, naringin) generally show lower free radical scavenging and antioxidant activities compared to rutin, which is characterized by a catechol structure. This latter has approximately the same antioxidant activity of quercetin, one of the most active flavonoids, confirming that C-ring unsaturation plays an important role in radical stabilization through electron delocalization across the molecule (Bellocco et al., 2016).

Other compounds playing an antioxidant role in *C. lumia* albedo are phenolic acids, whose reducing ability is due to the number of free hydroxyl groups astrengthened by steric hindrance (Smeriglio et al., 2018). Among the phenolic acids identified in the extract, clorogenic acid plays a predominant role, followed by ferulic acid, both contributing significantly to the antioxidant pattern of *C. lumia* albedo.

The antioxidant power of *Citrus* flavonoids may exert a beneficial role to the human defence system, by protecting against the noxious effects of primary and relatively weak antioxidants that can lead to the formation of dangerous reactive species (Bellocco et al., 2016).

3.4 Analysis of cytotoxicity and cytoprotective activities

Tests aimed at evaluating the cytotoxicity of the *C. lumia* albedo extract, conducted by trypan blue staining and LDH release assay, revealed no significant variation in lymphocyte viability at doses ranging between 5-50 μg/mL.

Thereafter, cytoprotective effects of the albedo extract were analyzed on lymphocytes treated for 24 h with t-BOOH. Exposure to t-BOOH alone caused a significant fall in cell viability ($p < 0.05$), while the addition of 15, 20, 25 and 50 μg/mL of extract resulted in a dose-dependent decrease of cell mortality (Figure 5 A). Lower concentrations of extract were ineffective.

Lymphocytes treated with t-BOOH alone showed increased LDH release ($p < 0.05$), which was reduced by addition of 20, 25 and 50 μg/mL of extract (Figure 5 B). Concentrations lower than 20 μg/mL were not able to reduce significantly LDH release. The assays of caspase 3 activity and protein carbonyl groups showed significant increases in samples treated with t-BOOH alone ($p < 0.01$), and a significant reduction of such an effect in the presence of extract doses ranging between 20-50 μg/mL (Figure 5 C-D).

4. Conclusion

In this study, the albedo extract of *C. lumia* Risso, an ancient *Citrus* of the Mediterranean area, has been analyzed for the first time. Our results indicate that liquid nitrogen treatment followed by ultrasound-assisted method can be used to significantly enhance the polyphenols extraction with a consequent increase in the antioxidant activity. *C. lumia* albedo has proved to be an interesting source of bioactive compounds showing a very high polyphenol content, mainly flavanones, with eriocitrin and hesperidin as the most abundant compounds (52.81% and 31.31%, respectively). The presence of a high amount of hesperidin was confirmed by microscopic detection of crystal aggregates.

The extract showed strong antioxidant and free-radical scavenging properties, as well as cytoprotective activity which can be related to the high content of flavanones, although a cooperative activity of other compounds, such as phenolic acids, cannot be ruled out. The complex of antioxidant compounds showed the ability of decreasing cell death and oxidative damage, with a clear dose-response relationship. These findings suggest that *C. lumia* albedo, could be exploited as a source of antioxidants for the formulation of nutraceutical supplements preventing oxidative stress-related diseases.

Conflict of interest

The authors declare no competing financial interest.

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Figure captions

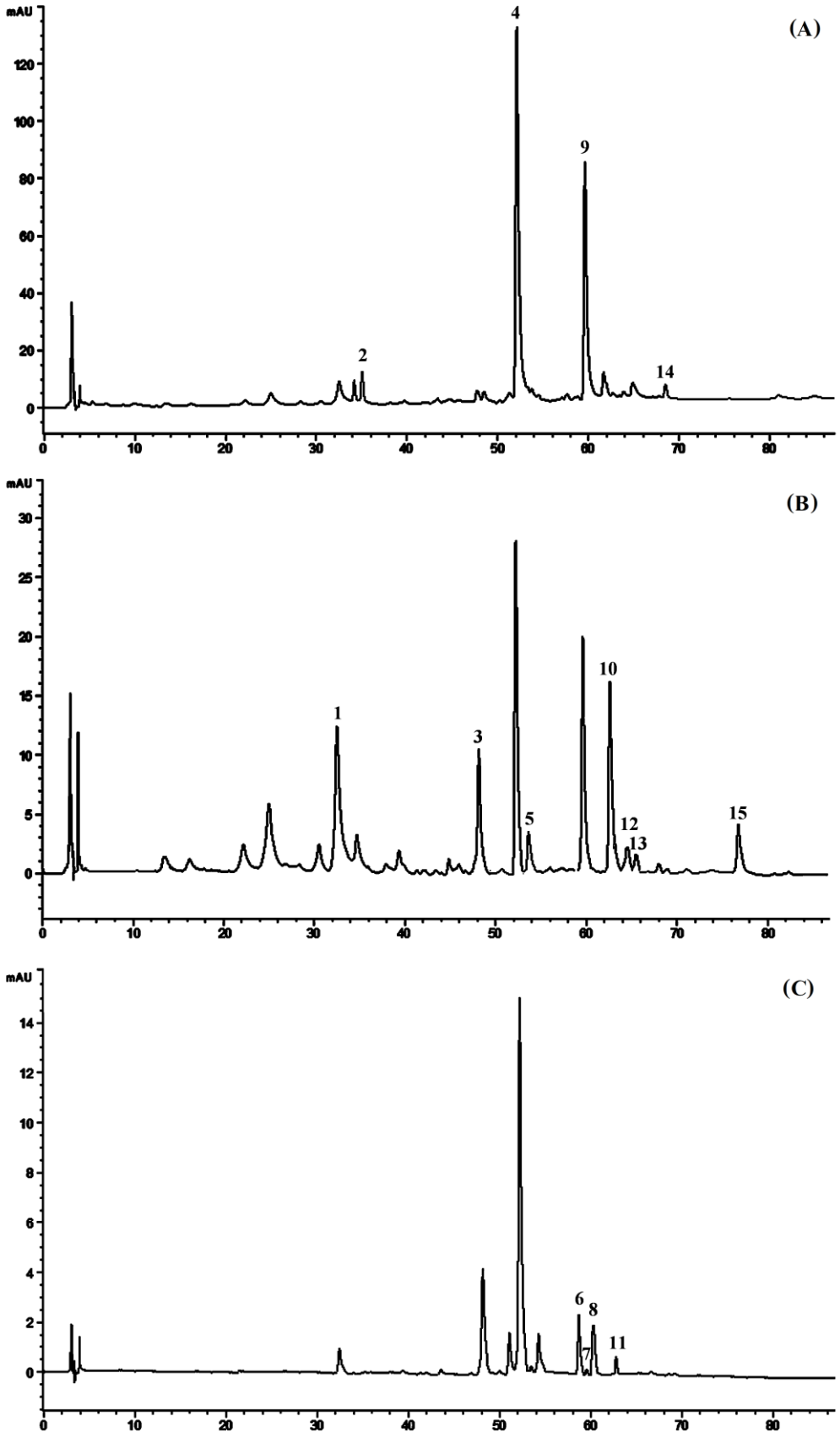
Figure 1. Representative RP-LC-DAD chromatogram of *C. lumia* albedo extract, acquired at 292 (A), 330 (B) and 370 (C) nm. Peak numbers correspond to compounds listed in Table 1.

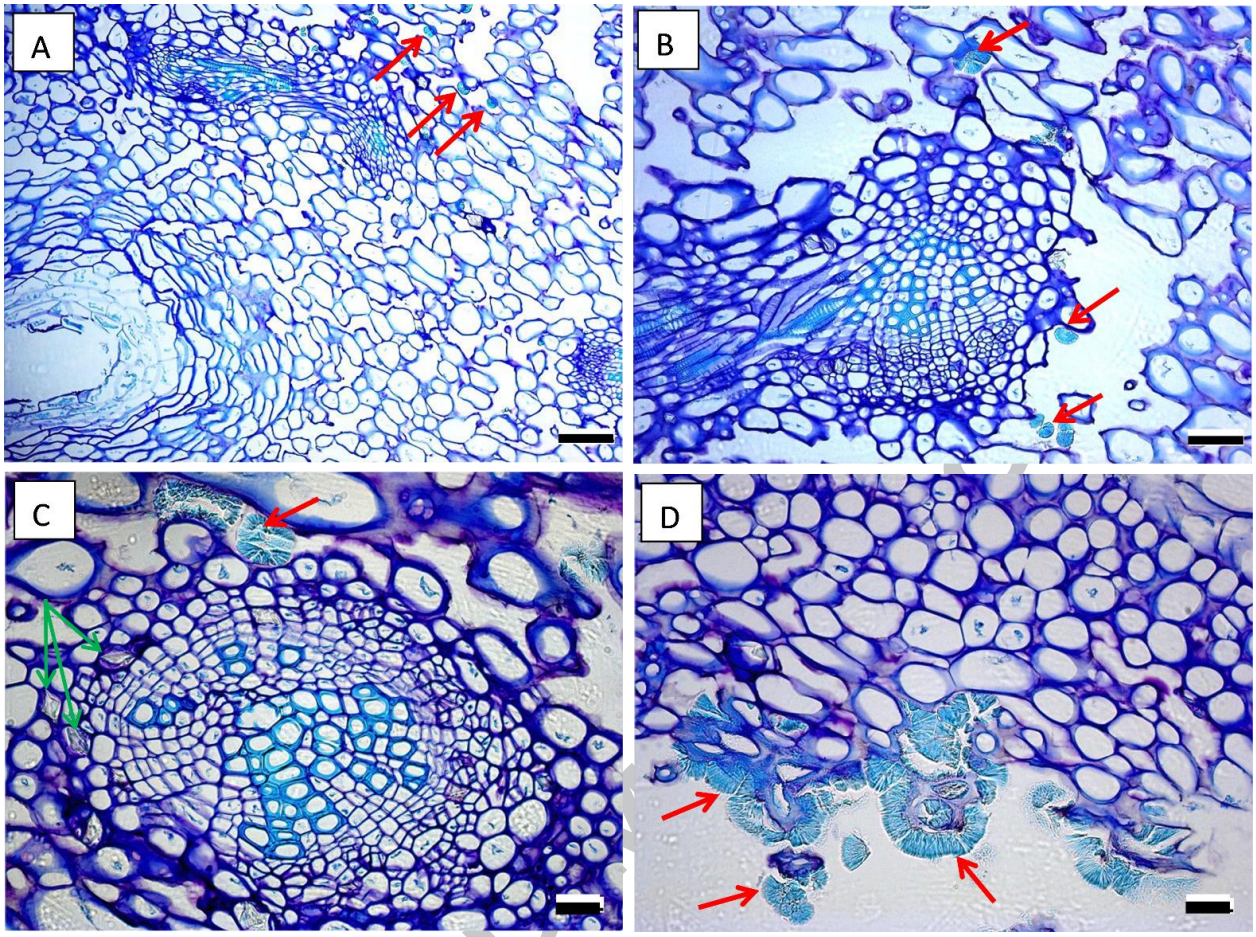
Figure 2. (A-D) In situ detection of hesperidin crystals in *C. lumia* by light microscopy. TBO staining reveals the presence of crystals as blue-green spherical clusters (A-D), especially around the border between flavedo and albedo layers (1 A), and around vascular bundles (B-C, red arrows). Prismatic crystals of calcium oxalate, unstained by TBO, are also visible (C, green arrows). At higher magnification, hesperidin crystals stained in blue-green by TBO appear as spherical aggregated of needle-shaped crystals (Fig 1 D, red arrows). Bars, A: 100 μm; B: 50 μm; C: 20 μm; D: 20 μm.

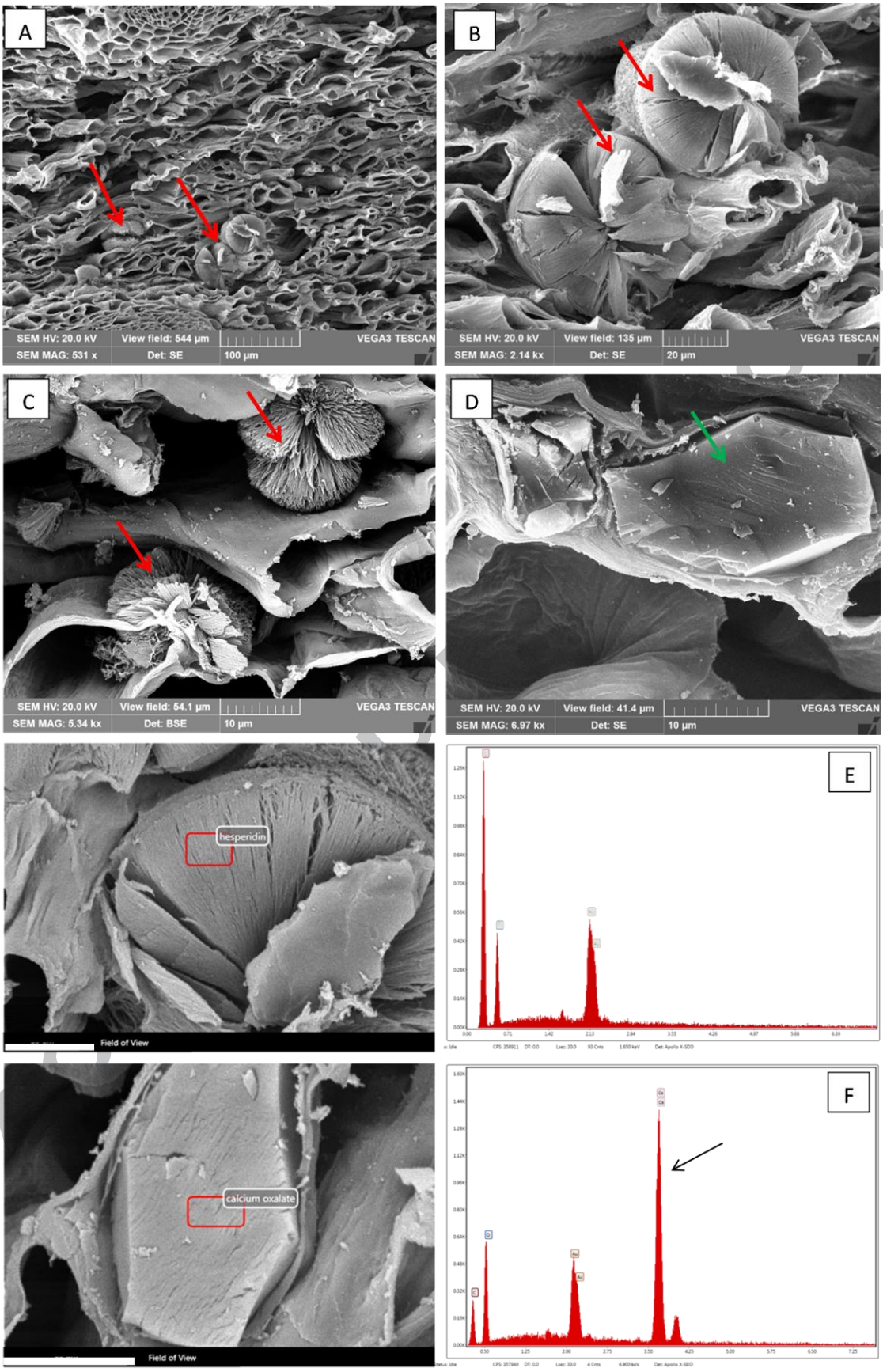
Figure 3. Scanning electron microscopy analysis showing needle-shaped crystals of hesperidin, forming spherical clusters (A-C, red arrows), and massive prismatic crystals of calcium oxalate (D, green arrow). SEM-EDS analysis showing the absence of mineral elements in spherical crystals of hesperidin (E), and a high level of calcium in the prismatic crystal of calcium oxalate (F, arrow). Bars 10 μm.

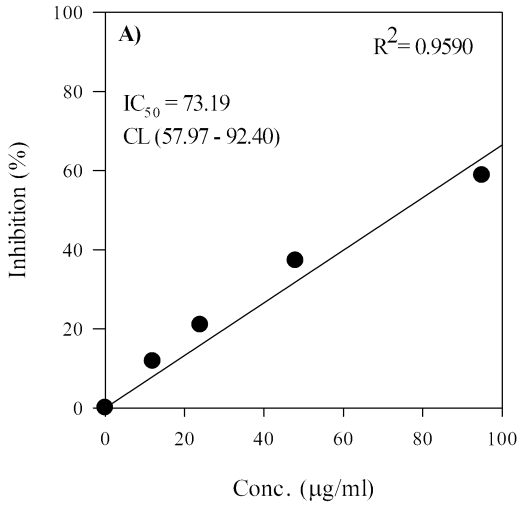
Figure 4. Dose-response curves of antioxidant and free radical-scavenging activities of *C. lumia* albedo extract towards TEAC (A); DPPH (B); ORAC (C); Folin-Ciocalteu (D); β-Carotene bleaching (E) and FRAP (F) assays.

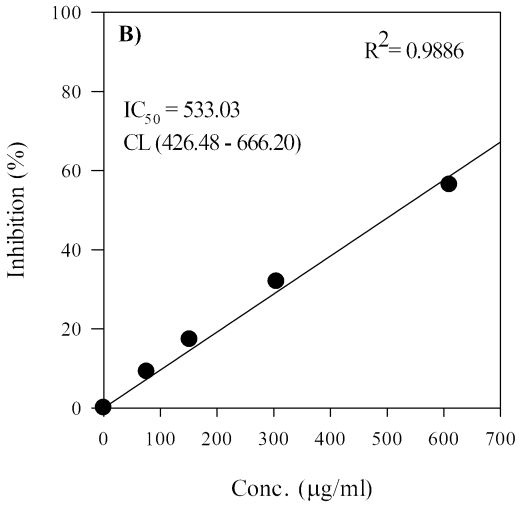
Figure 5. Cytoprotective effects of the *C. lumia* albedo extract on t-BOOH treated lymphocytes. Lymphocytes plus were incubated for 24 h under control conditions (a), in the presence of 100 μM of t-BOOH alone (b), and in the presence of t-BOOH combined with 50, 25, 20, 15, 10 and 5 μg/mL of extract (c, d, e, f, g, and h, respectively). Cell viability and integrity were assessed by trypan blue staining (A), LDH release (B), caspase 3 activation (C), and quantification of protein carbonyl groups (D). The asterisks (**) indicate significant differences with respect to t-BOOH alone ($P < 0.05$).

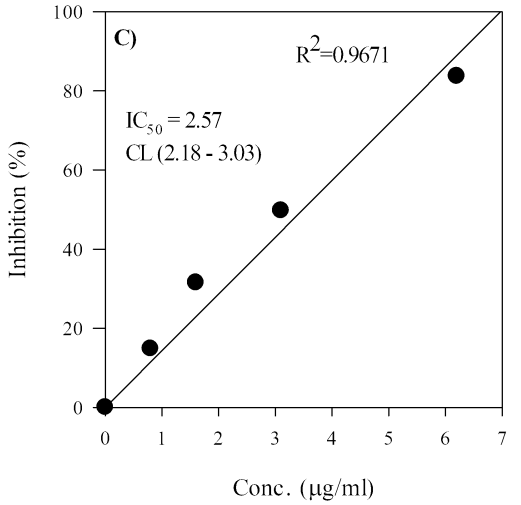


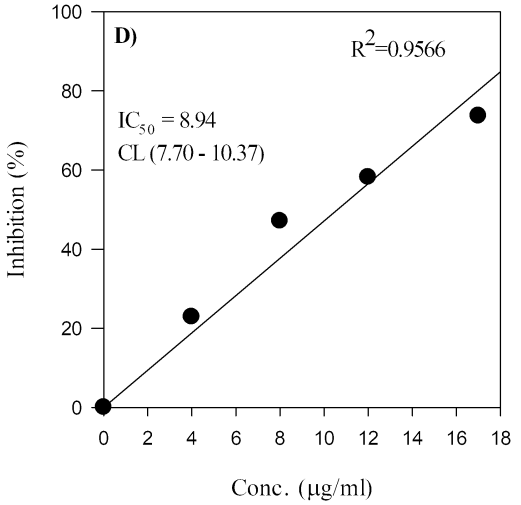


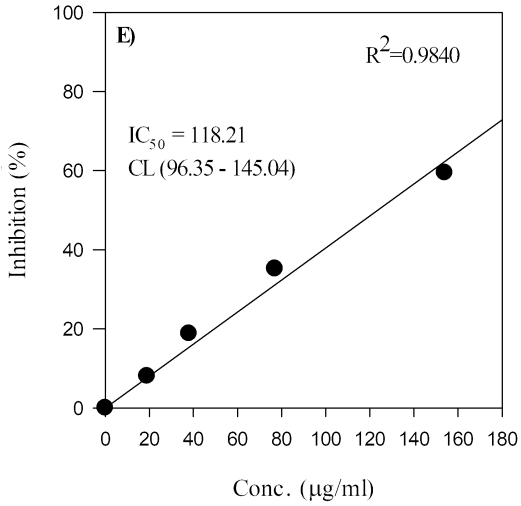


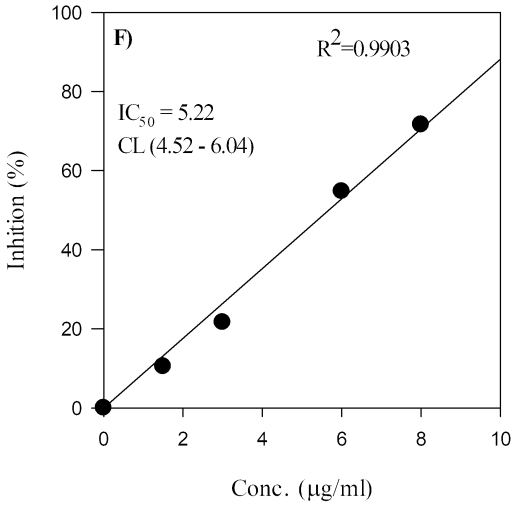












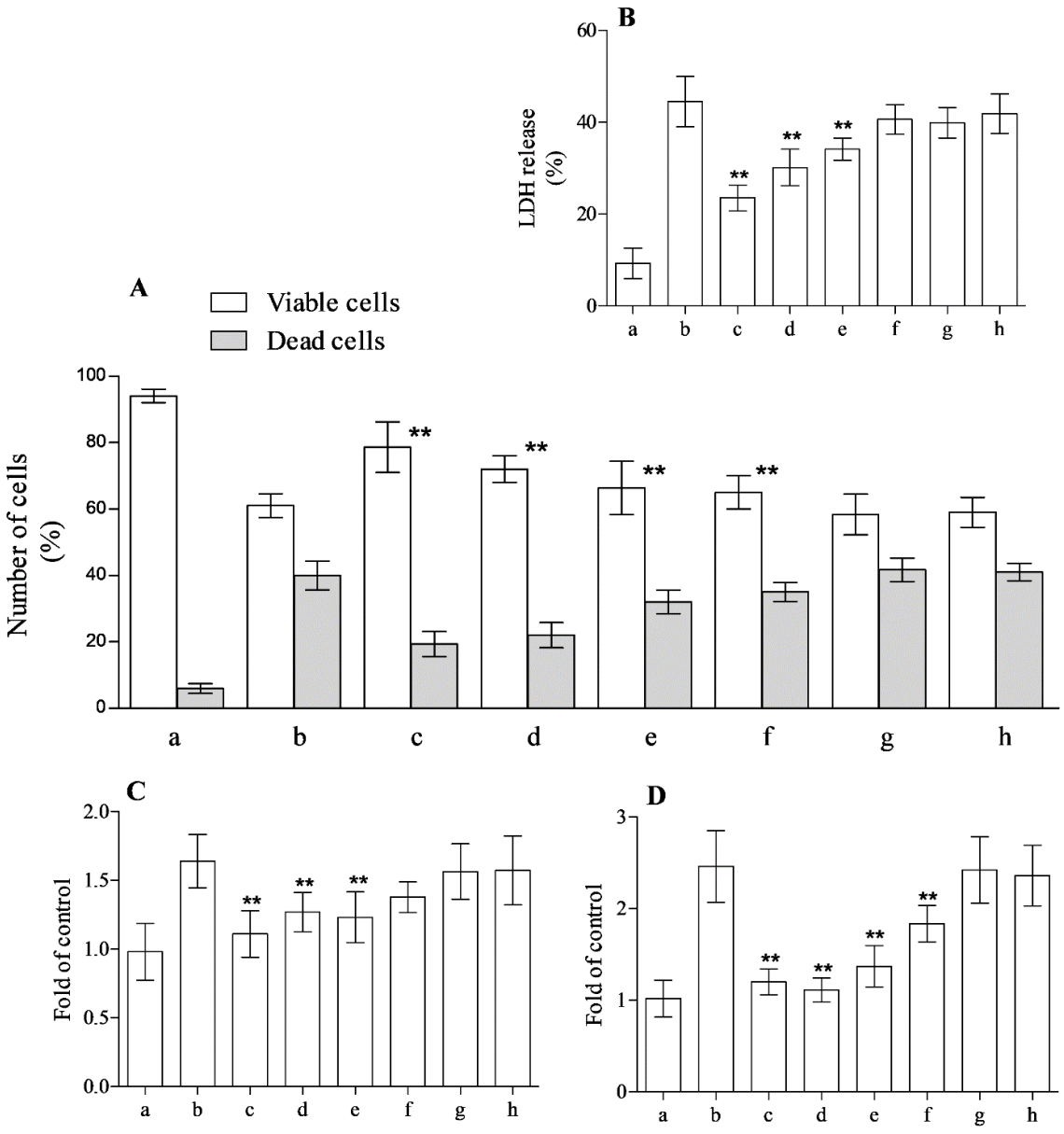


Table 1. Polyphenol compounds identified and quantified in *C. lumia* albedo extract. Data were expressed as mg/100 g of FW and as means ± SD (n = 3) of three independent experiments. Bold values correspond to the peak numbers showed in figure 1.

Compounds	**Peak n.**	**R_t (min)**	**λ (nm)**	**mg/100 g FW[§]**
Phenolic acids				
Chlorogeic acid	**1**	32.493	298; 326	151.512 ± 4.842
Vanillic acid	**2**	35.264	260; 292	1.839 ± 0.035
Ferulic acid	**3**	48.124	294; 322	50.986 ± 1.835
Flavones				
Eriocitrin	**4**	52.586	284; 334	1,012.407 ± 22.764
Neoeriocitrin	**5**	53.764	284; 338	4.037 ± 0.082
Hesperidin	**9**	60.149	284; 338	600.377 ± 6.225
Diosmin	**10**	63.065	252; 266; 346	22.800 ± 0.840
Roifolin	**12**	64.276	266; 338	2.583 ± 0.025
Neodiosmin	**13**	65.477	254; 266; 347	3.329 ± 0.018
Tangeretin	**14**	68.413	258; 318	23.375 ± 0.422
Apigenin	**15**	77.394	266; 338	9.669 ± 0.124
Flavonols				
Quercetin-3-*O*-galactoside (Hyperoside)	**6**	58.974	256; 355	14.913 ± 0.284
Quercetin-3-*O*-glucoside (Isoquercetin)	**7**	59.673	262; 354	4.303 ± 0.088
Quercetin-3-*O*-rutinoside (Rutin)	**8**	59.703	256; 356	10.923 ± 0.358
Kaempferol 7-*O*-neohesperidoside	**11**	63.497	264; 320; 368	4.178 ± 0.122

[§] FW= Fresh Weight

Highlights

- The ancient Mediterranean species *Citrus lumia* Risso is a rich source of bioactive compounds;
- Albedo extract showed a high polyphenol content with flavonoids as the most abundant fraction;
- A peculiar flavonoid profile, with high content of eriocitrin and hesperidin, was found;
- The presence of hesperidin was confirmed by microscopic detection of crystal aggregates;
- *C. lumia* albedo extract showed strong antioxidant and cytoprotective activities.