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Synthesis and antioxidant evaluation of isochroman-derivatives of hydroxytyrosol: structure-activity relationship

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Running title: Synthesis and antioxidant evaluation of isochromans.

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ABSTRACT

Isochroman-derivatives of the natural olive oil phenol hydroxytyrosol (HT) have been synthesized via Oxa-Pictet-Spengler reaction in high yields. Lipophilicity and antioxidant activity were determined to establish the structure-activity relationship of isochromans compared to HT, BHT and α-tocopherol. Antioxidant capacity was tested in two different media: bulk oils, using the Rancimat test, and brain homogenates, by measuring malondialdehyde (MDA) levels as a lipoperoxidation biomarker. In addition, other antioxidant assays (FRAP, ABTS and ORAC) were carried out. Rancimat and MDA results show that antioxidant activity was related with lipophilicity, directly in brain homogenates and inversely in the oils, in agreement with the polar paradox. Free *o*-diphenolic groups positively determined the activity in the oils, whereas reducing and radical-scavenging activities were related to the number of free hydroxyl moieties. BHT and α-tocopherol showed lower antioxidant activity than isochromans and HT. We conclude that HT-isochromans present significant potential as bioactive compounds.

Keywords: Olive oil isochromans, hydroxytyrosol, antioxidant activity, lipophilic phenolic antioxidants, polar paradox.

1. INTRODUCTION

The beneficial effects on health of mono and polyunsaturated fatty acids are well known, particularly their effects on cardiovascular function (Schwingshackl & Hoffmann, 2012; Kromhout & De Goede, 2014). During food processing, transport or storage, oxidative degradation of unsaturated fatty acids takes place having negative repercussions on food quality. Therefore, avoiding such oxidation represents a challenge for the food industry as well as the cosmetic industry (Sanches-Silva et al., 2014; Liu, Jin & Zhang, 2014). Oxidation may be counteracted to a certain extent by adding antioxidants that may prevent or delay oxidative reactions. Nowadays, most antioxidants used to control lipid oxidation are synthetic, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butyl hydroquinone (TBHQ), and propyl gallate (EEC/2006/52, 2006). However, certain synthetic antioxidants have shown potential carcinogenic effects; besides, consumers prefer natural ingredients over synthetic compounds. The use of natural antioxidants, such as phenolic compounds, is a safer and healthier alternative to synthetic compounds. Most natural phenols are hydrophilic polyhydroxy derivatives, which reduces their application, particularly under hydrophobic conditions (Liu et al., 2014). Other lipophilic phenolic compounds such as phenolic acid esters or phenolic acid lactones, among others, are also natural antioxidants although present in foods in small amounts, requiring time-consuming and not cost-profitable procedures for their isolation and purification, which limits their potential industrial application. Therefore, the synthesis of lipophilic phenolic compounds is an ongoing challenge.

Among the phenolic compounds naturally present in our diet, hydroxytyrosol HT (**1**), as secoiridoid derivative, in free form or as an acetate ester (Mateos et al. 2001), is one of the most abundant and representative olive oil phenols. The phenolic fraction in virgin and/or extra virgin olive oil has well established beneficial health effects. In fact, EFSA has recently issued a positive opinion on HT and other olive oil phenols' capacity to protect low-density lipoproteins (LDL) against oxidation (EFSA, 2011). Another minor group of phenolic compounds related to HT that have been identified in olive oil are 6,7-dihydroxyisochromans, in particular 1-phenyl-6,7-dihydroxyisochroman and 1-(4'-hydroxy-3'-

methoxyphenyl)-6,7-dihydroxyisochroman (**4**) (Bianco, Coccioli, Guiso, & Marra, 2001). These compounds have shown antiplatelet activity (Togna, Togna, Franconi, Marra, & Guiso, 2003), cytoprotective properties (Zeh, Lorenz, Kreutzmann, & Schonfeld, 2008), in addition to antioxidant activity, reducing oxygen/nitrogen reactive species (Schönfeld, Kruska, & Reiser, 2009), and anti-inflammatory activity (Trefiletti, Togna, Latina, Marra, Guiso, & Togna, 2011, Togna, Latina, Trefiletti, Guiso, Moschini, & Togna, 2013). All these effects confirm dihydroxyisochromans' contribution to the beneficial health effects of the phenolic fraction of olive oil.

Taking into account the bioactivity already attributed to isochromans and the increasing interest in obtaining new lipophilic phenolic compounds with lower hydrophilic/lipophilic balance (HLB) than HT in order to enhance its solubility in more lipophilic media, the aim of the present study was to synthesize a batch of isochromans from HT, via oxa-Pictet-Spengler reaction. Lipophilicity and antioxidant activity of isochromans were determined by complementary tests (Rancimat, FRAP, ABTS, ORAC and MDA assays). Comparison with natural (HT, α-tocopherol) and synthetic (BHT) antioxidants was also carried out.

2. MATERIALS AND METHODS

2.1. Materials

All solvents and reagents were of analytical grade unless otherwise stated. Cumene hydroperoxide (CH), *p*-toluenesulphonic acid, hexadeuterated methylsulfoxide (DMSO-d_6), sucrose, trichloroacetic acid, chloroform, sodium chloride, *n*-octanol, iron (III) chloride hexahydrate, potassium persulphate, phenylmethylsulphonyl fluoride, ethylenediaminetetraacetic acid tetrasodium salt hydrate (EDTA), chymostatin, leupeptin, microbial pepstatin A, α-tocopherol, 2,6-di-*tert*-butyl-4-methylphenol (BHT), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), fluorescein, randomized methylated β-cyclodextrin (RMCD), 2,2'-azobis(2-amidinopropane) dihydrocloride (AAPH), 2,2'-azino bis-(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (98%), 2,4,6-tri-(2-pyridyl)-1,3,5-triazine (TPTZ) and 2-thiobarbituric acid (TBA) were from Sigma-Aldrich (Madrid, Spain). Sodium hydroxide, methanol,

sodium hydrogen phosphate and potassium dihydrogen phosphate were from Panreac (Madrid, Spain). Neutral alumina, type 507C, grade I, was acquired from Fluka AG (Buchs, Switzerland). Acetone, 3,4-dihydroxybenzaldehyde, 3,4-dimethoxybenzaldehyde, 3,4-dioctyloxybenzaldehyde, 3-hydroxy-4-methoxybenzaldehyde, 4-hydroxy-3-methoxybenzaldehyde were purchased from Acros Organics (Geel, Belgium). HT was recovered with 95% purity from olive oil wastewaters following a patented industrial procedure (Fernández-Bolaños, Heredia, Rodriguez, Rodriguez, Jimenez, & Guillen, 2005).

NMR spectra were recorded on a Bruker Avance 500 spectrophotometer operating at 500.13 MHz (^{1}H) and 125.75 MHz (^{13}C). Samples were dissolved (0.1 mmol/mL) in DMSO-d_6 and spectra were recorded at 303 K. Chemical shifts are given in ppm with the residual solvent signals (2.49 ppm for ^{1}H and 39.5 ppm for ^{13}C) as references. Elemental analyses were carried out on a Leco CHNS-932 apparatus. High-resolution EI mass spectra were obtained on a Micromass AUTOSPECQ spectrometer.

2.2. Isochromans synthesis

Isochromans (**2**-**7**) were synthesized by means of the oxa-Pictet-Spengler reaction from HT (**1**) and the corresponding carbonylic compounds, using an acid catalyst (Guiso, Marra, & Cavarischia, 2001, Guiso, Bianco, Marra, & Cavarischia, 2003) (Figure 1). Briefly, 1 mmol of HT (**1**, 154 mg) was dissolved in 4 mL of anhydrous methyl alcohol and mixed with 2 mmol of carbonyl compound (100% molar excess) in the presence of 10 mg of *p*-toluenesulphonic acid as acid catalyst. The mixture was heated under reflux for 2 hours. After reaction completion (TLC plate), the mixture was cooled and the solvent evaporated under low pressure. Finally, the products were purified using silica gel column chromatography.

Data for 1,1-dimethyl-6,7-dihydroxyisochroman (**2**): using acetone as carbonyl compound and purification through silica gel column chromatography with ether/hexane (2:3, v/v) as eluent, a white solid was obtained with a yield of 98%. M.p. 130-2ºC [130-2ºC] (Guiso et al., 2003). NMR data (see Tables 1 and 2). Elem. Anal. Calcd. for $C_{11}H_{14}O_3$: C, 68.02%; H, 7.27%; found: C, 67.84%; H, 7.41%. HRMS (EI) m/z found 194.093926 (1.9 ppm).

Data for 1-(3',4'-dihydroxyphenyl)-6,7-dihydroxyisochroman (**3**): using 3,4-dihydroxybenzaldehyde as carbonyl compound and purification through silica gel column chromatography with dichloromethane/methanol (20:1, v/v) as eluent, a pink solid was obtained with a yield of 84.3%. M.p. 204-6ºC [177-9ºC] (Lorenz, Zeh, Martens-Lobenhoffer, Schmidt, Wolf, & Horn, 2005). NMR data (see Tables 1 and 2). Elem. Anal. Calcd. for $C_{15}H_{14}O_5$: C, 65.69%; H, 5.15%; found: C, 65.34%; H, 5.34%. HRMS (El) m/z found 274,083643 (1.8 ppm).

Data for 1-(4'-hydroxy-3'-methoxyphenyl)-6,7-dihydroxyisochroman (**4**): using 4-hydroxy-3-methoxybenzaldehyde as carbonyl compound and purification through silica gel column chromatography with dichloromethane/methanol (30:1, v/v) as eluent, a slightly pink solid was obtained with a yield of 72.6%. M.p.: 165-7ºC [154-6ºC] (Lorenz et al., 2005). NMR data (see Tables 1 and 2). Elem. Anal. Calcd. for $C_{16}H_{16}O_5$ x ¾ H_2O: C, 63.67%; H, 5.84%, found: C, 63.72%; H, 5.52%. HRMS (El) m/z found 288,100202 (1.5 ppm).

Data for 1-(3'-hydroxy-4'-methoxyphenyl)-6,7-dihydroxyisochroman (**5**): using 3-hydroxy-4-methoxybenzaldehyde as carbonyl compound and purification through silica gel column chromatography with dichloromethane/methanol (30:1, v/v) as eluent, an orange solid was obtained with a yield of 82.3%. M.p.: 180-2ºC. NMR data (see Tables 1 and 2). Elem. Anal. Calcd. for $C_{16}H_{16}O_5$ x H_2O: C, 62.74%; H, 5.92%. Found: C, 62.03%; H, 5.84%. HRMS (El) m/z found 288,100311 (1.9 ppm).

Data for 1-(3',4'-dimethoxyphenyl)-6,7-dihydroxyisocroman (**6**): using 3,4-dimethoxybenzaldehyde as carbonyl compound and purification through silica gel column chromatography with dichloromethane/methanol (30:1, v/v) as eluent, a white solid was obtained with a yield of 77.5%. M.p.: 169-71ºC [156-8ºC] (Lorenz et al., 2005). NMR data (see Tables 1 and 2). Elem. Anal. Calcd. for $C_{17}H_{18}O_5$: C, 67.54%; H, 6.00%; found: C, 67.35%; H, 6.18%. HRMS (El) m/z found 302,115349 (0.2 ppm).

Data for 1-(3',4'-dioctiloxiphenyl)-6,7-dihydroxyisocroman (**7**): using 3,4-dioctyloxybenzaldehyde as carbonyl compound and purification through silica gel column chromatography with ether/hexane (1:4, v/v) as eluent, a white solid was obtained with a yield of 88.1%. M.p.: 93-5ºC. NMR data (see Tables 1 and

2). Elem. Anal. Calcd. for $C_{31}H_{46}O_5$: C, 74.66%; H, 9.30%; found: C, 74.61%; H, 9.98%. HRMS (EI) m/z found 498,334416 (0.2 ppm).

2.3. Determination of compound polarity

Two procedures were used to determine the polarity of the synthesized isochromans (**2**-**7**) in comparison with their precursor HT (**1**) and antioxidants, α-tocopherol (**8**) and BHT (**9**): the retention factor (Rf) and partition coefficient (logP). In addition, theoretical values of logP were determined by ChemBioDraw Ultra (version 13.0).

Retention factor (Rf) values were determined by silica gel thin layer chromatography (TLC) using chloroform/methanol (20:1, v/v) as eluent, as described by Porter et al (Porter, Black, & Drolet, 1989). Partition coefficient (logP) was determined by the 'shake-flask method' based on the quantification of substance distribution between *n*-octanol and water (EEC/92/69, 1992). Briefly, 0.01 M stock solution of each compound was diluted with *n*-octanol to yield different concentrations and absorbances were measured at their corresponding maxima wavelengths using a UV-vis spectrophotometer (HT (**1**) at 282 nm, isochromans (**2**-**6**) at 286 nm and isochroman (**7**) at 284 nm). 50 mL of each compound dissolved in *n*-octanol were placed in a separatory funnel and extracted three times with 50 mL of water by shaking 10 min and resting 30 min to equilibrate phases. Subsequently, phases were separated and analysed spectrophotometrically. Partition coefficient was determined by the following equation: log P = [X]o/[X]w where [X]o represents the analyte concentration in the organic phase (*n*-octanol) and [X]w the analyte concentration in the aqueous phase (water). All measurements were carried out in triplicate.

2.4. Evaluation of lipid oxidative stability by the Rancimat method

Lipid oxidative stability was evaluated by an accelerated automated test using the Rancimat equipment (Model 743, Metrohm Co. Basel, Switzerland). The lipid matrix was obtained from commercial sunflower oil by purification through alumina (Yoshida, Kondo, & Kajimoto, 1992) following the 'free solvent' procedure. The purified matrix, free of antioxidants, was stored at -18°C under nitrogen atmosphere. Absence of polyphenols and tocopherols was checked by solid phase extraction and HPLC analysis with UV detector at

280 nm and C18 Column (Tecknokroma, 5 µm, 25 cm x 4.6 mm i.d.) (Mateos et al., 2001) and by HPLC analysis on a silica gel column (Lichrorb SI, 5 µm 25 cm x 4.0 cm i.d.) using a UV-vis detector at 292 nm (Paquot & Hautfenne, 1992), respectively. The fatty acid composition of the matrix was C16:0 (5.7%), C16:1 (0.1%), C18:0 (3.6%), C18:1 (27.4%), C18:2 (61.4%), C18:3 (0.1%), others (1.7%). Aliquots of the glyceridic matrix were spiked with increasing amounts of compounds, ranging from 0.2 mmol/kg to 2.0 mmol/kg and then subjected to accelerated oxidation in the Rancimat apparatus at 90°C. Results are expressed as induction time (IT) in hours corresponding to the stability of the lipid matrix evaluated. All determinations were carried out in duplicate.

2.5. Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay was carried out according to the procedure described by Pulido, Bravo, and Saura-Calixto (2000). The antioxidant potential of the synthesized compounds was estimated from their ability to reduce the ferric tripyridyltriazine (TPTZ-Fe^{+3}) complex to its stable ferrous form (TPTZ-Fe^{+2} complex). Briefly, the FRAP reagent contained 2.5 mL of a 10 mM TPTZ solution in 40 mM HCl, plus 2.5 mL of 20 mM $FeCl_3x6H_2O$ and 25 mL of 0.3 M acetate buffer was added to obtain a final pH of 3.6. This reagent was freshly prepared and warmed to 37 ºC prior to its use. 900 µL of FRAP reagent were mixed with 90 µL of distilled water and 30 µL of either test sample, standard (Trolox), or methanol, which was the reagent blank since standards were dissolved in methanol, and shaken. Readings at 595 nm were taken every 20 s, and the reaction was monitored up to 30 min at 37°C using a UV-Visible Varian (Cary 50 BIO, Holland) spectrophotometer, equipped with a thermostated auto-cell-holder. The reading at 30 min was selected in each case for the calculation of FRAP values. Methanolic solutions of Trolox were used for calibration. FRAP values are expressed as mM TE (Trolox Equivalent). All analyses were run in triplicate.

2.6. ABTS assay

Free-radical scavenging capacity was measured using the ABTS decoloration method (Re, Pellegrini, Proteggente, Pannala, Yang, & Rice-Evans, 1999) with some modifications. The method is based on the capacity of different components to scavenge the ABTS radical cation ($ABTS^{\bullet+}$) compared to a standard

antioxidant (Trolox). Briefly, ABTS was dissolved in a 2.45 mM $K_2S_2O_8$ solution in the dark at room temperature for 12-16 hours to obtain a 7 mM concentration of ABTS radical cation ($ABTS^{\bullet+}$) stock solution. The $ABTS^{\bullet+}$ stock solution was diluted with methanol to obtain an absorbance of 0.70 ± 0.02 at 730 nm. After the addition of 0.1 mL of sample (dissolved in methanol), methanol (blank), or Trolox (standard) to 3.9 mL of diluted $ABTS^{\bullet+}$ solution, absorbance readings were taken every 20 s at 30°C over 6 min, using a UV-Visible Varian (Cary 50 BIO, Holland) spectrophotometer equipped with a thermostatted auto-cell-holder. The percentage of inhibition of absorbance versus time was plotted, and the area under the curve (0-360 s) was calculated. Methanolic solutions of known concentrations of Trolox were used for calibration. Results are expressed as mM TE (Trolox Equivalent). Each value is the average of three determinations.

2.7. ORAC assay

The oxygen radical scavenging capacity was measured using the lipophilic ORAC assay according to the method developed by Huang, Ou, Hampsch-Woodill, Flanagan, and Deemer (2002) with some modifications. This assay is based on the fluorescence decay of a reference substance (fluorescein) after the addition of a peroxyl radical (AAPH), which acts as an initiator of the oxidative reaction. Briefly, HT (**1**), isochromans (**2**-**7**) and Trolox standards were dissolved in 7% methylated β-cyclodextrin (RMCD) in acetone/water (1:1, v/v) solution. Then, 25 µL of either test sample, solvent (blank), or Trolox were added to a 96-well microplate followed by the addition of 150 µL of fluorescein work solution (8.5×10^{-5} mM) prepared in 75 mM phosphate buffer (pH 7.4). After the addition of 30 µL of AAPH (153 mM) as peroxyl radical generator, also prepared in 75 mM phosphate buffer (pH 7.4), fluorescence was recorded every 2 min on a microplate reader (Bio-Tek, Winooski, VT, USA) for 120 min at 485 and 528 nm excitation and emission wavelengths, respectively. Each value is the average of four determinations. Results are expressed as mM TE (Trolox Equivalent).

2.8. Oxidative stress model in brain homogenates

Three months old Wistar rats were housed in a pathogen-free room under controlled conditions (19–23°C, 50–60% humidity and 12 h light/darkness cycles) with access to food and water ad libitum.

The study was approved by the Animal Ethics Committee of the University of Seville (Seville, Spain). Protocols for animal handling and experimentation were in strict conformation with the Spanish law for the protection of experimental animals (RD 53/2013, BOE nº 34 Sec I page 11370, February 8th, 2013).

Rats were sacrificed by decapitation and brains were isolated and homogenized in three volumes of 50 mM Tris/HCl buffer, pH 7.5, containing 1 mM sodium chloride and 0.25 M sucrose in addition to a mixture of protease inhibitors (0.2 mM phenylmethylsulphonyl fluoride, 50 mM EDTA and 1 µg/mL of chymostatin, leupeptin and pepstatin A) to prevent proteolysis. After centrifugation (1000 g, 5 min, 4°C), supernatants were collected for later experiments. Protein content was determined using the Bradford assay.

A volume of brain homogenates containing 4 mg protein/mL was incubated in the absence and presence of three doses of antioxidant (0.2, 1 and 5 mM) for 30 min at 37°C. Oxidation was initiated by adding CH to obtain a final concentration of 2 mM and the mixture was maintained for 30 min at 37°C. Proteins were precipitated by adding two volumes of 10% (v/v) ice-cold trichloroacetic acid to the samples and centrifuging at 8000 g for 5 min. MDA quantification was carried out in supernatants.

2.9. Determination of malondialdehyde (MDA)

In order to quantify lipid peroxidation in brain homogenates (Esterbauer & Cheeseman, 1990), malondialdehyde was determined using the thiobarbituric acid reactive substances (TBARS) assay. Samples were treated with TBA reagent (20 mM TBA in 50% (v/v) glacial acetic acid) and then heated at 100°C for 1 h. TBARS were quantified at 532 nm on a spectrophotometer (Perkin-Elmer C-532001, Waltham, MA, USA). Results are expressed as percentage referred to MDA controls. Brain homogenates treated with 2 mM CH in the absence of any antioxidant were used as positive control.

2.10. Statistical Analysis

Prior to statistical analysis, data were tested for homogeneity of variance using the Levene test. Multiple comparisons were carried out using one-way ANOVA followed by Bonferroni tests when variances were homogeneous and Tamhane test when not. The level of significance was established at $p < 0.05$. The statistical package SPSS (version 19.0) was used.

3. RESULTS

3.1. Preparation and characterization of isochromans from HT

Oxa-Pictet-Spengler reaction allowed to easily synthetize isochroman compounds (**2**-**7**) from HT (**1**) after the reaction with a carbonyl compound and acid catalyst (Guiso et al., 2001; Guiso et al., 2003). Isochromans (**2**-**7**) were synthetized in good to excellent yields (represented in **Figure 1**). Compounds were characterized by NMR and HR-MS spectrometry. ^{1}H and ^{13}C NMR chemical shifts (Tables 1 and 2, respectively) were unequivocally assigned to each compound by 2D homo- and heteronuclear correlation experiments (COSY, NOESY, HSQC and HMBC spectra) and are completely in agreement with the proposed structures.

3.2. Polarity determination of compounds

Values of partition coefficient (LogP) and retention factor (Rf) (summarized in Table 3) allowed to characterize isochromans' (**2**-**7**) polarity in comparison with their precursor, HT (**1**), and antioxidants α-tocopherol (**8**) and BHT (**9**). The relative order of Rf was as follows: **1** ≈ **3** < **4** ≈ **5** < **6** ≈ **2** < **7** < **8** < **9**. Regarding experimental partition coefficient ($LogP_{exp}$), the compounds showed the following behavior: **1** < **2** ≈ **3** < **4** ≈ **5** < **6** < **7** < **9** < **8**. Theoretical partition coefficient ($LogP_{theor}$) values were almost coincident with $LogP_{exp}$ values, showing the following order: **1** < **2** < **3** < **4** ≈ **5** < **6** < **9** < **7** < **8**.

3.3. Antioxidant activity in lipid matrices

The efficacy of isochromans (**2**-**7**) as antioxidants in sunflower oil in comparison with HT (**1**), α-tocopherol (**8**) and BHT (**9**) was evaluated using the Rancimat method at 90°C in air saturation conditions. Results were expressed as the induction time (IT) corresponding to the stability of the lipid matrix. Considering that minimal differences in fatty acid composition of lipid matrixes critically determine stability and consequently induction time, the antioxidant activity of the references (**1**, **8** and **9**) was analysed in order to carry out a more accurate comparison with that of isochromans (**2**-**7**), although the former data had been published previously (Pereira-Caro et al., 2009). Figure 2 shows the IT values of purified matrices of sunflower oil spiked with increasing concentrations of the studied compounds (**1**-**9**). Attending to the

present results, HT (**1**) and isochromans (**2**-**7**) showed better activity against induced oxidation in lipid matrices than α-tocopherol (**8**) and BHT (**9**). Regarding isochromans (**2**-**7**) and their precursor HT (**1**), a direct relationship between their polar nature and antioxidant capacity in lipid matrices was observed, except for compound **3**, which showed the highest antioxidant power of all the evaluated compounds in spite of being less polar than HT (**1**) and compound (**2**).

3.4. Ferric-reducing antioxidant power (FRAP)

Results of the reducing power of isochromans (**2**-**7**) evaluated by the FRAP assay (expressed as mM TE Trolox equivalent) in comparison with HT (**1**), α-tocopherol (**8**) and BHT (**9**) are summarized in Table 4. The antioxidant activity of isochromans (**2**-**7**) varied depending on the number of free hydroxyl groups contained in the molecule, observing a direct relationship with the antioxidant power. Thus, HT (**1**) showed lower activity than isochromans (**2**-**5**), similar to that of compound (**6**) but slightly higher than that of compound (**7**). Regarding the two commercial food antioxidants, α-tocopherol (**8**) and BHT (**9**), both showed lower antioxidant potency than isochromans (**2**-**7**), except compound (**7**), which was less active than α-tocopherol (**8**).

3.5. The radical-scavenging activity by ABTS and ORAC assays

The radical-scavenging activity of isochromans (**2-7**) in comparison with HT (**1**), α-tocopherol (**8**) and BHT (**9**) determined by ABTS and ORAC assays are shown in Table 4. Results regarding the free radical scavenging capacity evaluated by ORAC assay were similar to those of the ferric reducing activity, as isochromans (**2**-**6**) showed higher antioxidant capacity than HT (**1**) except for the most lipophilic compound (**7**), which showed similar activity as HT (**1**). According to both assays, α-tocopherol (**8**) and BHT (**9**) had lower activity than isochromans (**2**-**7**), except for compounds **6** and **7,** with similar values to those of α-tocopherol (**8**) according to the ABTS assay.

3.5. Antioxidant activity evaluation in tissue homogenates

The oxidative damage induced by cumene hydroperoxide (CH) was reverted after treatment with 0.2, 1 and 5 mM of HT (**1**) and isochromans (**2**-**7**) in a dose-dependent manner (Figure 2). An association between the

compounds' lipophilic nature and their antioxidant capacity was observed. Surprisingly, although α-tocopherol (**8**) is highly lipophilic, it showed little protection against oxidation whereas BHT (**9**) was as active as the most lipophilic isochroman derivatives (**6** and **7**).

4. DISCUSSION

The fact that HT is easily recovered from alperujo, an abundant by-product obtained during olive oil production (Fernandez-Bolanos et al., 2005), together with the high bioactivity and antioxidant power it presents, turns HT into an interesting substrate to obtain phenolic derivatives with a modified hydrophilic/lipophilic balance (HLB) and enhanced solubility in lipophilic media. Moreover, the HT derivatives may represent an alternative that answers the food industry' demand for this type of antioxidants. Isochromans are HT derivatives naturally present in the phenolic fraction of virgin and/or extra-virgin olive oil (Bianco et al., 2001); in addition, they may be easily obtained from HT by means of the Oxa-Pictet-Spengler reaction in high to excellent yields (Guiso et al., 2001; Guiso et al., 2003). In the present work a wide range of derivatives have been studied: compound (**4**), naturally occurring in olive oil (Bianco et al., 2001), in addition to other isochromans which have been previously synthesized, (**2**) (Guiso et al., 2003), (**3**) and (**6**) (Lorenz et al., 2005), together with two new derivatives, namely 1-(3'-hydroxy-4'-methoxyphenyl)-6,7-dihydroxyisochroman (**5**) and 1-(3',4'-dioctyloxyphenyl)-6,7-dihydroxyisochroman (**7**), which to our knowledge have been synthesized for the first time, in order to establish a structure-antioxidant activity association. ^{1}H and ^{13}C NMR chemical shifts, as indicated in Tables 1 and 2, in addition to COSY, NOESY, HSQC and HMBC spectra, allowed us to unequivocally identify the chemical structure of isochromans (**2**-**7**). It is noteworthy that ^{13}C NMR data of compounds (**3**) and (**6**), in particular C10 and C1', are exchanged in the literature (Lorenz et al., 2005).

Lipophilicity of isochromans (**2**-**7**) was evaluated by means of estimating their retention factor (Rf) and both experimental ($LogP_{exp}$) and theoretical ($LogP_{theor}$) partition coefficients (Table 3). Rf results provided only a guidance of lipophilicity as these values are not constant and can vary by using different eluents in TLC.

Bearing this in mind, after observing the correspondence between theoretical and experimental partition coefficients, $LogP_{exp}$ values were taken as a basis to discuss the results observed in the present work.

HT (**1**) showed the highest polar nature among all the evaluated compounds, presenting a LogP value in agreement with data previously published (Grasso, Siracusa, Spatafora, Renis, & Tringali, 2007). Compound (**2**) was the second most hydrophilic compound. Among isochromans (**3**-**7**), compound (**3**) showed the most hydrophilic nature due to the presence of two free *o*-catechol groups in its chemical structure in contrast to the other compounds. The higher degree of hydroxylation of compound (**3**) compared to HT (**1**) did not result in increased polarity, probably due to its higher carbon skeleton density in comparison with HT (**1**). The succeeding compounds in polarity ranking were the regioisomers (**4**) and (**5**), showing similar lipophilicity since the only difference between these compounds is the methyl group in position 3' (compound **4**) or 4' (**5**). The double substitution of 3' and 4' positions in the phenyl moiety by two methyl groups in compound (**6**) or two octyl groups in compound (**7**) enhanced their lipophilic character, particularly that of compound (**7**). Finally, standards BHT (**8**) and α-tocopherol (**9**) showed the highest lipophilic values of all the evaluated compounds in agreement with their chemical structure.

Regarding the in vitro antioxidant activity of isochromans (**2**-**7**), which was evaluated by complementary methods (Rancimat, FRAP, ABTS and ORAC assays) and compared with HT (**1**) and α-tocopherol (**8**) and BHT (**9**), isochromans (**2**-**7**) and HT (**1**) showed higher antioxidant capacity than α-tocoferol (**8**) and BHT (**9**) with minor changes depending on their chemical structure and the medium in which they were dissolved.

Lipid matrix stability after spiking with isochromans (**2**-**7**) and references (**1**, **8** and **9**) was evaluated using the Rancimat test (Figure 2). Results partially agreed with the polar paradox (Porter et al., 1989), which indicates that polar antioxidants are more efficient in bulk oil than lipophilic antioxidants, whereas non-polar antioxidants are more efficient in emulsions or cell membrane systems. From this point of view, a direct association was observed between hydrophilicity and antioxidant activity, with some exceptions related to the chemical structure of the compounds. Compound (**3**), which was more lipophilic than HT (**1**) and compound (**2**), showed the highest antioxidant capacity among all the evaluated products due to the

double free *o*-diphenolic moieties present in its chemical structure. However, isochromans **4**, **5**, **6** and **7**, which contain two *o*-diphenolic groups partially substituted by one (**4** and **5**) and two methyl groups (**6**) or two octyl groups (**7**), showed an antioxidant activity similar to molecules containing one *o*-diphenolic group [(**1**) and (**2**)], which is modulated by their lipophilic nature. Similarly, α-tocopherol (**8**) was more effective than BHT (**9**) in spite of having a more lipophilic nature than (**9**) due to its polyunsaturated chemical structure, which favours radical stabilization.

On the other hand, the reducing and radical-scavenging capacities of compounds (**2**-**7**), depicted in Table 4, showed an association between the number of free hydroxyl groups and the antioxidant activity of the compounds, regardless the *o*-diphenolic unit was partially substituted or not. Thus, isochromans (**4** and **5**) with two *o*-diphenolic groups and four hydroxyl groups (three free and one substituted by a methyl group) presented higher antioxidant activity than HT (**1**) and isochromans (**2**) in contrast to results obtained by the Rancimat analysis. The present outcome is in agreement with previous studies in which FRAP and ABTS activities of the homovanillic alcohol (1.44 and 1.29 mM Trolox Equivalent, respectively), with an hydroxyl group substituted with a methyl group in position 3, was significantly higher than that of HT (1.39 and 0.84 mM Trolox Equivalent, respectively) (Madrona, Pereira-Caro, Bravo, Mateos, & Espartero, 2011). Furthermore, these results are in line with those reported by Lorenz et al., (2005) who studied the radical scavenging activity for 1,1-diphenyl-2-picrylhydrazyl (DPPH), superoxide $O_2^{\cdot-}$ and peroxinitrite ($ONOO^-$) of isochromans (**3**), (**4**) and (**6**), observing an association between the degree of hydroxylation and the antioxidant activity, being molecule (**3**) the most active of the tested compounds, followed by (**4**) and (**6**).

To complete the characterization of the antioxidant activity of isochromans (**2**-**7**), the protective effects against oxidative stress induced by cumene hydroperoxide (CH) was assessed in homogenate brain using malondialdehyde (MDA) as an index of lipoperoxidation. Brain homogenate is a substrate widely used as an oxidative stress model because of the high oxygen demand of this organ, the abundance of redox-active metals (iron and copper), the high levels of polyunsaturated fatty acids susceptible to oxidation, and also because of the scarcity of endogenous antioxidant defences (Wang & Michaelis, 2010). Non physiological

doses of HT and isochromans were used to compare the results obtained with this model with those observed in lipid matrices in order to further investigate the polar paradox. In the present study, the oxidative damage induced by CH was reverted in a dose and lipophilic dependent manner. This outcome contrasts with that observed in bulk oils, but was in agreement with the polar paradox, where lipophilic antioxidants are more efficient in emulsions or cell membrane systems, as brain homogenates.

It may be concluded that isochromans are antioxidants with significant bioactive potential that can be easily obtained from HT (**1**), being more efficient than α-tocopherol (**8**) and BHT (**9**). Isochromans (**2**-**7**) are excellent antioxidants in bulk oils as well as in biological systems, in agreement with the polar paradox, also showing high reducing and radical-scavenging activities.

5. ACKNOWLEDGEMENTS

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Figure Legends

Figure 1. Chemical reactions for the synthesis of isochromans (**2**-**7**) from hydroxytyrosol (**1**) and chemical structures of synthesized compounds: 1,1-dimethyl-6,7-dihydroxyisochroman (**2**); 1-(3',4'-dihydroxyphenyl)-6,7-dihydroxyisochroman (**3**); 1-(4'-hydroxy-3'-methoxyphenyl)-6,7-dihydroxyisochroman (**4**); 1-(3'-hydroxy-4'-methoxyphenyl)-6,7-dihydroxyisochroman (**5**); 1-(3',4'-dimethoxyphenyl)-6,7-dihydroxyisochroman (**6**); 1-(3',4'-dioctyloxyphenyl)-6,7-dihydroxyisochroman (**7**).

Figure 2. Panel A: Induction time (IT) of sunflower oil lipid matrices spiked with antioxidants; Panel B: Inhibitory capacity of the test compounds at three concentrations (0.2, 1 and 5 mM) against lipid peroxidation in brain homogenates. The compounds evaluated were hydroxytyrosol (**1**), 1,1-dimethyl-6,7-dihydroxyisochroman (**2**); 1-(3',4'-dihydroxyphenyl)-6,7-dihydroxyisocroman (**3**); 1-(4'-hydroxy-3'-methoxyphenyl)-6,7-dihydroxyisocroman (**4**); 1-(3'-hydroxy-4'-methoxyphenyl)-6,7-dihydroxyisocroman (**5**); 1-(3',4'-dimethoxyphenyl)-6,7-dihydroxyisocroman (**6**); 1-(3',4'-dioctyloxyphenyl)-6,7-dihydroxyisocroman (**7)**, α-tocopherol (**8**) and BHT (**9**). Values represent means ± SD. Different letters indicate statistically significant differences ($p < 0.05$) among groups.

Figure 1.

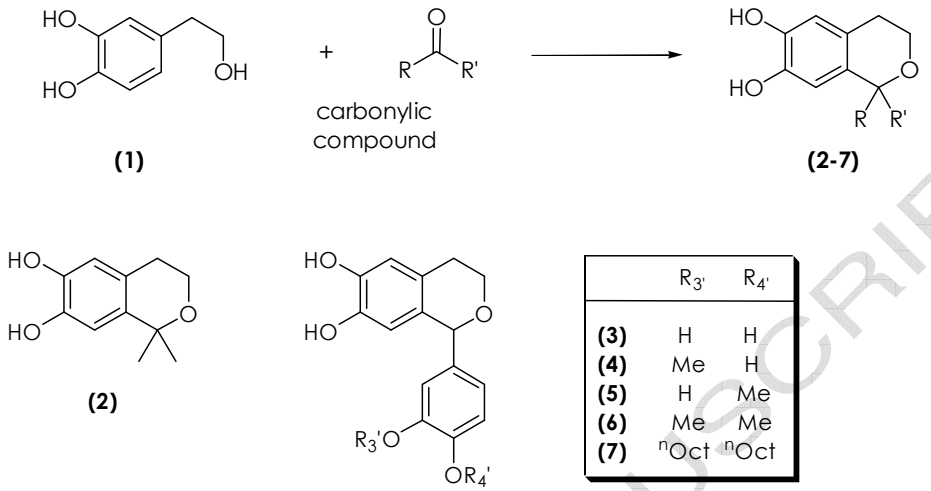
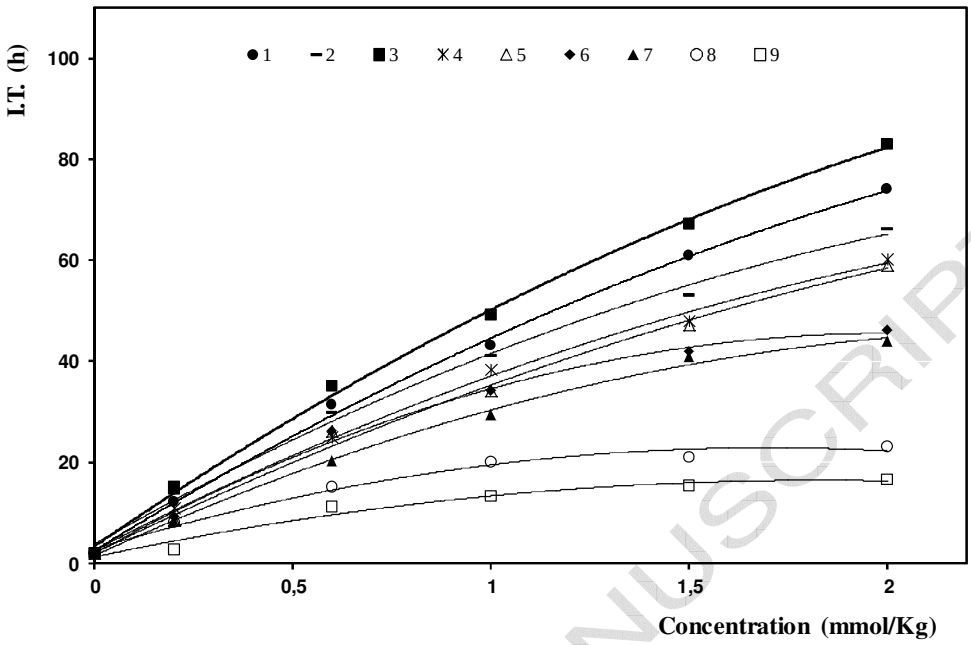


Figure 2.

Panel A



Panel B

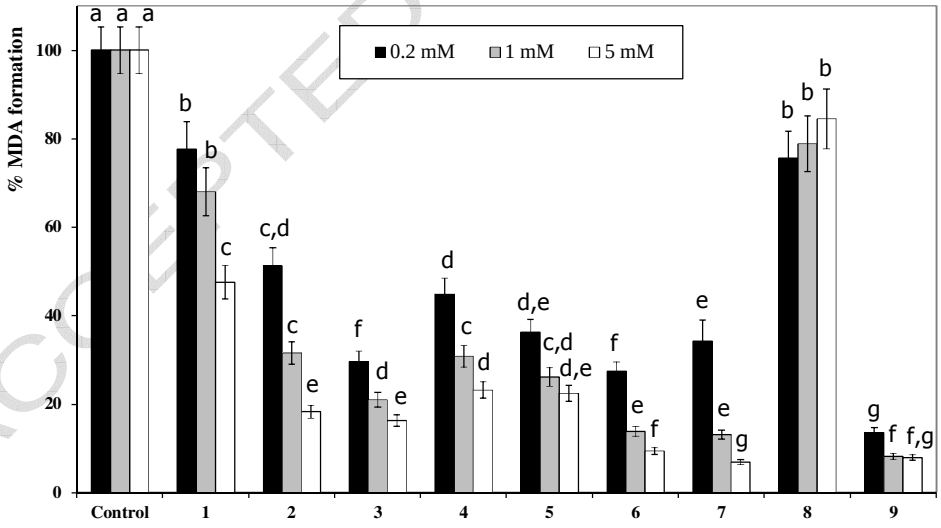


Table 1. ^{1}H NMR (500.13 MHz, DMSO-d_6, 303k) for isochromans (**2**-**7**). Chemical shifts (δ) are expressed in ppm and coupling constants *(J)* in Hz.

	2	**3**	**4**	**5**	**6**	**7**
• *Isochroman unit*						
1		5.32 *(s)*	5.40 *(s)*	5.36 *(s)*	5.45 *(s)*	5.42 *(s)*
3_{eq}	3.74 *(t)* $^{3}J_{3,4}$ = 5.5	3.94 *(ddd)* $^{2}J_{3a,3e}$ = 11.4 $^{3}J_{3e,4a}$ = 5.4 $^{3}J_{3e,4e}$ = 4.0	3.98 *(ddd)* $^{2}J_{3a,3e}$ = 11.1 $^{3}J_{3e,4a}$ = 5.4 $^{3}J_{3e,4e}$ = 3.8	3.95 *(ddd)* $^{2}J_{3a,3e}$ = 11.1 $^{3}J_{3e,4a}$ = 5.5 $^{3}J_{3e,4e}$ = 3.9	3.99 *(ddd)* $^{2}J_{3a,3e}$ = 11.1 $^{3}J_{3e,4a}$ = 5.4 $^{3}J_{3e,4e}$ = 3.7	3.97 *(ddd)* $^{2}J_{3a,3e}$ = 11.2 $^{3}J_{3e,4a}$ = 5.5 $^{3}J_{3e,4e}$ = 3.7
3_{ax}		3.67 *(ddd)* $^{3}J_{3a,4a}$ = 9.2 $^{3}J_{3a,4e}$ = 4.0	3.69 *(ddd)* $^{3}J_{3a,4a}$ = 9.7 $^{3}J_{3a,4e}$ =3.8	3.68 *(ddd)* $^{3}J_{3a,4a}$ = 9.5 $^{3}J_{3a,4e}$ = 4.0	3.71 *(m)*	3.69 (ddd) $^{3}J_{3a,4a}$ = 9.6 $^{3}J_{3a,4e}$ = 4.0
4_{eq}	2.51 *(t)*	2.51 *(dt)* $^{2}J_{4a,4e}$ = 15.9	2.52 *(dt)* $^{2}J_{4a,4e}$ = 15.6	2.52 *(dt)* $^{2}J_{4a,4e}$ = 15.8	2.53 *(dt)* $^{2}J_{4a,4e}$ = 15.9	2.52 *(dt)* $^{2}J_{4a,4e}$ = 16.0
4_{ax}		2.77 *(ddd)*	2.82 *(ddd)*	2.78 *(ddd)*	2.83 *(ddd)*	2.81 *(ddd)*
5	6.40 *(s)*	6.48 *(s)*	6.49 *(s)*	6.49 *(s)*	6.50 *(s)*	6.49 *(s)*
8	6.48 *(s)*	6.05 *(s)*	6.05 *(s)*	6.04 *(s)*	6.05 *(s)*	6.03 *(s)*
• *1-Substituent*						
1'	1.34 *(s)*					
2'		6.57 *(d)* $^{4}J_{2',6'}$ = 2.0	6.78 *(d)* $^{4}J_{2',6'}$ = 1.9	6.61 *(d)* $^{4}J_{2',6'}$ = 2.0	6.82 *(d)* $^{4}J_{2',6'}$ = 1.9	6.79 *(d)* $^{4}J_{2',6'}$ = 1.9
5'		6.66 *(d)* $^{3}J_{5',6'}$ = 8.0	6.71 *(d)* $^{3}J_{5',6'}$ = 8.0	6.84 *(d)* $^{3}J_{5',6'}$ = 8.2	6.89 *(d)* $^{3}J_{5',6'}$ = 8.1	6.87 *(d)* $^{3}J_{5',6'}$ = 8.2
6'		6.53 *(dd)*	6.62 *(dd)*	6.66 *(dd)*	6.75 *(dd)*	6.72 *(dd)*
• *3'-Substituent*						
1''			3.70 *(s)*		3.69 *(s)*	3.86 *(ddt)* *(sist. ABX_2)* ^{2}J = 9.5 ^{3}J = 6.3
2''						1.66 *(m)*
3'' – 7''						1.25 *(m)*
8''						0.85 *(t)*
• *4'-Substituent*						
1'''				3.74 *(s)*	3.74 *(s)*	3.91 *(t)* ^{3}J = 6,3
2'''						1.66 *(m)*
3''' – 7'''						1.25 *(m)*
8'''						0.85 *(t)*

Table 2. ^{13}C NMR (125.75 MHz, DMSO-d_6, 303k) for isochromans (**2**-**7**). Chemical shifts (δ) are expressed in ppm.

	2	**3**	**4**	**5**	**6**	**7**
• *Isochroman unit*						
1	73.3	78.1	78.3	77.9	78.2	78.1
3	58.8	62.8	63.1	62.9	63.2	63.1
4	28.2	27.5	27.5	27.5	27.5	27.5
5	114.9	114.9	115.0	114.9	115.0	114.9
6	143.4	143.9	143.9	143.9	143.9	143.9
7	143.4	143.1	143.1	143.2	143.2	143.2
8	112.3	113.4	113.3	113.3	113.3	113.3
9	133.3	128.3	128.3	128.1	128.0	128.0
10	123.0	123.9 [a]	123.9	123.9	123.9 [a]	123.9
• *1-Substituent*						
1'	29.7	133.9 [a]	133.9	135.6	135.4 [a]	135.5
2'		115.8	112.6	115.7	112.1	114.4
3'		144.8	147.2	146.2	148.5	148.2
4'		144.8	146.1	147.2	148.3	148.3
5'		114.8	114.8	111.6	111.2	113.3
6'		119.7	121.2	119.5	120.8	121.1
• *3'-Substituent*						
1''			55.6		55.5	68.5
2'' – 6''						25.5-31.1
7''						22.0
8''						13.8
• *4'-Substituent*						
1'''				55.6	55.4	68.5
2''' – 6'''						25.5-31.1
7'''						22.0
8'''						13.8

[a] Data interchanged in literature (Lorenz et al., 2005).

Table 3. Retention factor (Rf) and partition coefficient ($LogP_{exp}$) values obtained for hydroxytyrosol (**1**), isochromans (**2**-**7**), α-tocopherol (**8**) and BHT (**9**). Theoretical partition coefficient ($LogP_{theor}$) values were calculated using ChemBioDraw Ultra Software.

Compound	Rf	$LogP_{exp}$	$LogP_{theor}$
1	0.12 ± 0.01[a]	0.14 ± 0.02[a]	0.96
2	0.36 ± 0.02[c]	1.26 ± 0.07[b]	1.56
3	0.10 ± 0.01[a]	1.34 ± 0.04[b]	1.95
4	0.24 ± 0.02[b]	1.46 ± 0.03[c]	2.22
5	0.28 ± 0.02[b]	1.47 ± 0.02[c]	2.22
6	0.32 ± 0.02[c]	2.62 ± 0.06[d]	2.48
7	0.69 ± 0.02[d]	3.64 ± 0.05[e]	8.30
8	0.93 ± 0.02[e]	10.0 ± 0.06[g*]	9.98
9	0.97 ± 0.02[f]	5.10 ± 0.06[f*]	5.54

* For comparative purposes, LogP of α-tocopherol (**8**) and BHT (**9**) taken from The Drugbank 4.0 (http://www.drugbank.ca) and The Human Metabolome Database (HMDB, http://www.hmdb.ca), respectively, were included for comparative purposes.

Table 4. Antioxidant capacity of the studied compounds (**1**-**9**)*.

Compound	FRAP (mM TE)	ABTS (mM TE)	ORAC (mM TE)
1†	1.39 ± 0.05 ^{d,e}	0.84 ± 0.02 ^{f}	1.92 ± 0.04 ^{e}
2	1.46 ± 0.02 ^{d}	1.12 ± 0.01 ^{c}	2.26 ± 0.04 ^{c}
3	4.20 ± 0.05 ^{a}	2.06 ± 0.02 ^{a}	4.86 ± 0.11 ^{a}
4	2.18 ± 0.02 ^{b}	1.37 ± 0.02 ^{b}	3.51 ± 0.14 ^{b}
5	2.22 ± 0.02 ^{b}	1.38 ± 0.01 ^{b}	3.70 ± 0.06 ^{b}
6	1.53 ± 0.02 ^{c}	1.03 ± 0.01 ^{d}	2.15 ± 0.12 ^{c,d}
7	1.18 ± 0.02 ^{f}	0.94 ± 0.01 ^{e}	1.99 ± 0.06 ^{d,e}
8†	0.80 ± 0.04 ^{g}	1.01 ± 0.02 ^{d}	0.63 ± 0.06 ^{f}
9†	1.32 ± 0.05 ^{e}	0.27 ± 0.01 ^{g}	0.12 ± 0.01 ^{g}

* Reducing antioxidant power was determined by the FRAP assay, and free radical-scavenging capacity by the ABTS and ORAC methods. FRAP and ABTS data is the mean of triplicate measurements ± standard deviation; ORAC data represents the mean of quadruplicate measurements ± standard deviations. Results are expressed as mM TE (Trolox Equivalents). Values within a column with different superscripts are significantly different, $p < 0.05$.

†FRAP, ABTS and ORAC values of HT (**1**), α-tocopherol (**8**) and BHT (**9**) have been published by Madrona (Madrona et al., 2011) and are included for comparative purposes.

HIGHLIGHTS

- Isochromans are obtained from hydroxytyrosol (HT) via Oxa-Pictet Spengler reaction
- Most isochromans are better antioxidants than HT, α-tocopherol and BHT
- Antioxidant activity of isochromans agrees with the polar paradox
- Isochromans' *o*-diphenolic groups in oils positively determine the antioxidant power
- Hydroxyl groups are responsible for isochromans' reducing and radical-scavenging power