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Targeting excessive free radicals with peels and juices of citrus fruits: Grapefruit, lemon, lime and orange

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ABSTRACT

A comparative study between the antioxidant properties of peel (flavedo and albedo) and juice of some commercially grown citrus fruit (Rutaceae), grapefruit (*Citrus paradisi*), lemon (*Citrus limon*), lime (*Citrus* × *aurantiifolia*) and sweet orange (*Citrus sinensis*) was performed. Different in vitro assays were applied to the volatile and polar fractions of peels and to crude and polar fraction of juices: 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity, reducing power and inhibition of lipid peroxidation using β-carotene–linoleate model system in liposomes and thiobarbituric acid reactive substances (TBARS) assay in brain homogenates. Reducing sugars and phenolics were the main antioxidant compounds found in all the extracts. Peels polar fractions revealed the highest contents in phenolics, flavonoids, ascorbic acid, carotenoids and reducing sugars, which certainly contribute to the highest antioxidant potential found in these fractions. Peels volatile fractions were clearly separated using discriminant analysis, which is in agreement with their lowest antioxidant potential.

1. Introduction

During the past years, reactive oxygen species (ROS) and reactive nitrogen species (RNS) have been implicated in the oxidative deterioration of food products as well as in the pathogenesis of several human diseases such as atherosclerosis, diabetes mellitus, chronic inflammation, neurodegenerative disorders and certain types of cancer (Halliwell, 1996). Recently, interest has increased considerably in finding naturally occurring antioxidants for use in food or pharmaceutical applications, which can protect the human body from free radicals and retard the progress of many chronical diseases as well as retard lipid oxidative rancidity in food (Prior, 2003). In fact, many antioxidant compounds extracted from plant sources (phytochemicals) have been identified as free radical or active oxygen scavengers (Ramarathnam et al., 1995).

Citrus (*Citrus* L. from Rutaceae) is one of the most important world fruit crops and is consumed mostly as fresh produce or juice because of its nutritional value and special flavour. Most popular within European and North American consumers are grapefruits (*Citrus paradisi*), lemons (*Citrus limon*), limes (*Citrus* × *aurantiifolia*) and sweet oranges (*Citrus sinensis*) (Mabberley, 1997; Citrus Pages, 2009). Consumption of citrus fruit or juice is found to be inversely associated with several diseases (Joshipura et al., 2001). The health benefits of citrus fruit have mainly been attributed to the presence of bioactive compounds, such as phenolics (e.g., flavanone glycosides, hydroxycinnamic acids) (Marchand, 2002), vitamin C (Halliwell, 1996), and carotenoids (Rao and Rao, 2007). Although, the fruits are mainly used for dessert, they are also sources of essential oils due to their aromatic compounds (Minh Tu et al., 2002; Chutia et al., 2009). For instance, lime flavours are used in beverage, confectionary, cookies and desserts (Dharmawan et al., 2007; Chutia et al., 2009). Many authors have reported antioxidant and radical-scavenging properties of essential oils (Sacchetti et al., 2005) and in some cases, a direct food-related application also (Madsen and Bertelsen, 1995).

So far, studies on bioactive compounds and antioxidant activity of citrus have mainly focused on the fruits (peels, pulps and juices) polar fractions (Abeysinghe et al., 2007; Gorinstein et al., 2001). Herein we developed a comparative study between four citrus fruits (peels and juices) in order to understand which of them are preferable for dietary prevention of cardiovascular and other diseases related to oxidative stress. Volatile and polar fractions of grapefruits, lemons, limes and oranges studied and compared considering free radical-scavenging properties, reducing power, and inhibition of lipid peroxidation capacity (in liposomes and in brain homogenates). Antioxidant molecules such as phenolics, sugars, ascorbic acid and carotenoids were also quantified in order to understand their contribution to the overall bioactive properties.

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2. Materials and methods

2.1. Samples

Commercially grown grapefruit (*C. paradisi* 'Star Ruby'), lime (*Citrus* × *aurantiifolia* (Christm.) Swingle) were purchased from a local supermarket, and lemon (*C. limon* (L.) Burm.f.) and sweet orange (*C. sinensis* (L.) Osbeck, 'Valencia' group) from a rural market, in February 2009. The citrus *taxa* studied were botanically classified using the synthetic proposal of Mabberley (1997) and the information published in Citrus Pages (http://users.kymp.net/citruspages/introduction.html, last update April 2009). Morphological characterization of the samples (eight fruits analysed per sample and species) was performed (Table 1) for botanical description and comparison in future research. Size, shape, form of the basal (stem) and apical (stylar) ends, and other distinctive general characters (Fig. 1), such as peel (flavedo and albedo) thickness and the smoothness of the surface, number of segments of the endocarp, central axis or medulla, some special structures that are or may be present in the apex (areole, mammilla, navel) and seed presence were described according to horticultural criteria defined by Hodgson (1986). Fruits range in size is expressed by the average *D*/*H* index (Table 1). The *D*/*H* index is obtained by dividing the diameter of each fruit measured by its height (distance from stem to apex).

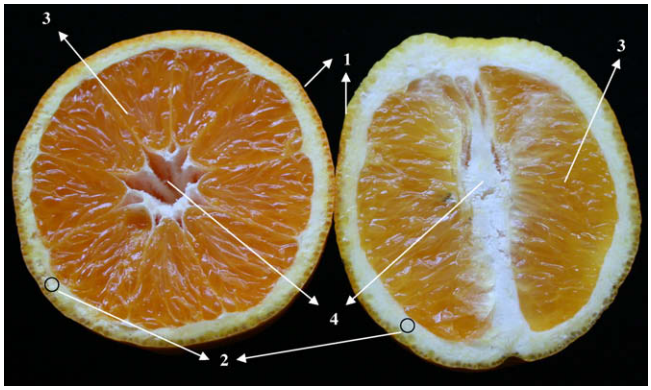

Fig. 1. 1 – Flavedo (the orange peripheral surface of the peel or epicarp); 2 – albedo (the white soft fiber middle layer of the peel or mesocarp); 3 – the inside layer of the fruit or endocarp, divided in segments or carpels with juicy vesicles; 4 – central column or medulla.

2.2. Standards and reagents

All the solvents were of analytical grade purity; methanol was supplied by Lab-Scan (Lisbon, Portugal). The standards used in the antioxidant activity assays: BHA (2-*tert*-butyl-4-methoxyphenol), TBHQ (tert-butylhydroquinone), L-ascorbic acid, α-tocopherol, gallic acid and (+)–catechin were purchased from Sigma (St. Louis, MO, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). The standard butylated hydroxytoluene (BHT) was purchased from Merck (Darmstadt, Germany). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.3. Volatile fraction

The essential oils were isolated from the fresh material (~150 g peels plus 350 mL of distilled ultra pure water) by hydro-distillation for 3 h, using a Clevenger-type apparatus. The extracts were dried with anhydrous sodium sulphate and concentrated under reduced pressure by rotary evaporator (Büchi R-210). The extraction yield was calculated in g of oil/100 g of fresh material. The collected oil was weighed, dissolved in methanol at a concentration of 500 mg/mL, and stored in sealed vials at −20 °C for further use.

2.4. Polar fraction

Lyophilized (Ly-8-FM-ULE, Snijders, HOLLAND) powdered samples (peels and juice; ~3 g) were extracted by stirring with 50 mL of methanol at 25 °C at 150 rpm for 12 h and filtered through Whatman No. 4 paper. The residue was then extracted with one additional 50 mL portion of the methanol. The extracts were evaporated to dryness and redissolved in methanol at a concentration of 20 mg/mL, and stored at 4 °C for further use. Also, the lyophilized juices were directly dissolved in water at a concentration of 20 mg/mL (Crude juices), and stored at 4 °C for further use.

Total phenolics were estimated by a colorimetric assay, based on procedures described by (Wolfe et al., 2003) with some modifications. An aliquot of the extract solution was mixed with *Folin–Ciocalteu* reagent (5 ml, previously diluted with water 1:10 v/v) and sodium carbonate (75 g/l, 4 ml). The tubes were vortexed for 15 s and allowed to stand for 30 min at 40 °C for colour development. Absorbance was then measured at 765 nm (Analytikijena 200-2004 spectrophotometer). Gallic acid was used to calculate the standard curve (0.05–0.8 mM; $y = 1.9799x + 0.0299$; $R^2 = 0.9997$), and the results were expressed as mg of gallic acid equivalents (GAEs) per g of extract.

Total flavonoids contents were determined spectrophotometrically using the method of Jia et al. (1999) based on the formation of a complex flavonoid-aluminum, with some modifications. An aliquot (0.5 ml) of the extract solution was mixed with distilled water (2 ml) and subsequently with $NaNO_2$ solution (5%, 0.15 ml). After 6 min, $AlCl_3$ solution (10%, 0.15 ml) was added and allowed to stand further 6 min, thereafter, NaOH solution (4%, 2 ml) was added to the mixture. Immediately, distilled water was added to bring the final volume to 5 mL. Then the mixture was properly mixed and allowed to stand for 15 min. The intensity of pink colour was measured at 510 nm. (+)–Catechin was used to calculate the standard curve (0.0156–1.0 mM; $y = 0.9186x - 0.0003$; $R^2 = 0.9999$) and the results were expressed as mg of (+)–chatequin equivalents (CEs) per g of extract.

Ascorbic acid was determined according to the method of Klein and Perry (1982). A fine powder (20 mesh) of sample (150 mg) was extracted with metaphosphoric acid (1%, 10 ml) for 45 min at room temperature and filtered through Whatman No. 4 filter paper. The filtrate (1 ml) was mixed with 2,6-dichloroindophenol (9 ml) and the absorbance was measured within 30 min at 515 nm against a blank. Content of ascorbic acid was calculated on the basis of the calibration curve of authentic L-ascorbic acid (0.006–0.1 mg/ml; $y = 3.0062x + 0.007$; $R^2 = 0.9999$), and the results were expressed as μg of ascorbic acid per g of extract.

For β-carotene and lycopene determination a fine dried powder (150 mg) was vigorously shaken with 10 mL of acetone–hexane mixture (4:6) for 1 min and filtered through Whatman No. 4 filter paper. The absorbance of the filtrate was measured at 453, 505, 645 and 663 nm (Barros et al., 2008). Contents of β-carotene and lycopene were calculated according to the following equations: lycopene (mg/100 mL) = $-0.0458 \times A_{663} + 0.204 \times A_{645} + 0.372 \times A_{505} - 0.0806 \times A_{453}$; β-carotene (mg/100 mL) = $0.216 \times A_{663} - 1.220 \times A_{645} - 0.304 \times A_{505} + 0.452 \times A_{453}$. The results were expressed as μg of carotenoid per g of extract.

Reducing sugars were determined by the DNS (dinitrosalicylic acid) method and glucose was used to calculate the standard curve (250–1500 μg/mL; $Y = 0.0007X - 0.0567$; $R^2 = 0.9997$); the results were expressed as g of reducing sugars per g of extract.

2.5. Radical scavenging activity

This methodology was performed using an ELX800 Microplate Reader (Bio-Tek Instruments, Inc). The reaction mixture in each one of the 96-wells consisted of extract solution (30 μL) and aqueous methanolic solution (80:20 v/v, 270 μL) containing DPPH radicals (6×10^{-5} mol/L). The mixture was left to stand for 60 min in the

Table 1
Morphological characterization of citrus fruits samples purchased in local markets. Grapefruit and lime collection date unknown; lemon and orange collected in January 2009. Average values and patrons.

Samples	Origin	Weight (g)	*H*/*D*	Shape	Skin	Flavedo (mm)	Albedo (mm)	Segments number	Medulla (core)	Apex	Seed
Grapefruit	Spain	296.4	0.80	Subglobose	Smooth dotted	2.0	4.0	12.8	Hollow	Flattened slightly depressed	Vestigial
Lemon	Portugal (Trás-os-Montes)	102.3	1.27	Elliptical	Rough	0.9	4.4	7.4	Solid	Nipple	Seedless
Lime	Spain	76.6	1.22	Ovate	Smooth	1.0	1.3	9.5	Solid	Small papilla	Seedless
Orange	Portugal (Trás-os-Montes	168.7	1.21	Spherical	Smooth	1.3	2.8	10.3	Semi solid	Navel	Seedless