Food Chemistry 129 (2011) 1045–1053

Contents lists available at ScienceDirect

Food Chemistry

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

Antioxidant profile of strawberry tree honey and its marker homogentisic acid in several models of oxidative stress

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ARTICLE INFO

Article history:
Received 7 January 2011
Received in revised form 18 March 2011
Accepted 18 May 2011
Available online 25 May 2011

Keywords:
Arbutus unedo L. honey
Homogentisic acid
Antioxidant activity
Antiradical activity
Caco-2

ABSTRACT

The antioxidant activity of several honeys was evaluated considering the different contribution of entire samples. The strawberry tree honey emerged as the richest in total phenols and the most active honey in the DPPH and FRAP tests, and could protect cholesterol against oxidative degradation (140 °C). Homogentisic acid (2,5-dihydroxyphenylacetic acid, HGA), the main phenolic compound from strawberry tree honey, showed interesting antioxidant and antiradical activities, and protective effect against thermal-cholesterol degradation, comparable to those of well known antioxidants. Moreover, the pre-treatment with HGA significantly preserved liposomes and LDL from Cu^{2+}-induced oxidative damage at 37 °C for 2 h, inhibiting the reduction of polyunsaturated fatty acids and cholesterol and the increase of their oxidative products. This phenol had no toxic effect in human intestinal epithelial Caco-2 cells within the concentration range tested (5–1000 μM). HGA was able to pass through the Caco-2 monolayers, the apparent permeability coefficients (P_{app}) in the apical-to-basolateral and basolateral-to-apical direction were $3.48 \pm 1.22 \times 10^{-6}$ and $2.18 \pm 0.34 \times 10^{-6}$ cm/s, respectively, suggesting a passive diffusion pathway as the dominating process. The results of the work qualify HGA as natural antioxidant, able to exert a significant *in vitro* protective effect and to contribute to the strawberry tree honey antioxidant activity.

1. Introduction

Honey is a supersaturated solution of fructose and glucose and contains a wide range of minor constituents such as minerals, proteins, vitamins, organic acids, enzymes, and phenolic compounds (Al et al., 2009; Ferreira, Aires, Barreira, & Estevinho, 2009; Gheldof, Wang, & Engeseth, 2002). This natural product is considered a part of traditional medicine (treatment of burns, gastrointestinal disorders, asthma, skin ulcers, and cataracts) and is used as a food preservative and sweetening agent (Al et al., 2009; Ferreira et al., 2009). The beneficial role of honey is partially attributed to its antibacterial and antioxidant activities. The antioxidants present in honey include both enzymatic (catalase, glucose oxidase, peroxidase) and non-enzymatic substances (ascorbic acid, α-tocopherol, carotenoids, amino acids, proteins, Maillard reaction products, flavonoids, and phenolic acids) (Al et al., 2009; Ferreira et al., 2009; Gheldof et al., 2002). The amount and type of these antioxidants depends largely upon the floral source/variety of the honey and a correlation between its antioxidant activity and total phenolic content has been demonstrated (Al et al., 2009; Gheldof et al., 2002).

Strawberry tree (*Arbutus unedo* L., Ericaceae) unifloral honey is a typical product of some Mediterranean regions, Sardinia in particular (Spano et al., 2006). Due to the characteristic taste, this honey is known as "bitter honey" and it has been traditionally employed mainly for curative aims (Tuberoso et al., 2010). Chemical investigation of the phenolic fraction highlighted homogentisic acid (2,5-dihydroxyphenylacetic acid; HGA) as a useful marker to assess the botanical origin of strawberry tree honey (Tuberoso et al., 2010). It was reported that HGA is the most abundant phenolic compound in this honey, with an average amount of 414.1 ± 69.8 mg/kg (Tuberoso et al., 2010). In vegetables, HGA is involved in the pathway outlined for the biosynthesis of plastoquinones and tocopherols in higher plants (Whistance & Threlfall, 1968) and is a catabolite of phenolic metabolism in a wide variety of higher organisms (mammals, fish, birds, amphibians, and plants) (Frases, Salazar, Dadachova, & Casadevall, 2007). In humans, HGA is an intermediate in the metabolism of tyrosine, normally metabolised by the enzyme homogentisate 1,2-dioxygenase (Hegedus & Nayak, 1994). It was found to scavenge DPPH radicals (Kang et al., 2005), like other hydroxyphenylacetic acid derivatives (Nahar, Russel, Middleton, Shoeb, & Sarker, 2005; Ordoudi, Tsimidou, Vafiadis, & Bakalbassis, 2006; Sroka & Cisowski, 2003), and to prevent H_2O_2-induced lipid peroxidation in human fibroblasts (Kang et al., 2005). HGA has been reported to induce cytotoxic and mutagenic

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doi:10.1016/j.foodchem.2011.05.072

effects and it appears to occur through an oxidative mechanism based on the ability of this compound to undergo autoxidation, leading to quinone formation with the production of reactive oxygen species (Hiraku, Yamasaki, & Kawanishi, 1998; Urios et al., 2003). The accumulation of HGA in connective tissues, the formation of its oxidised/polymerised products and oxygen radical generation have been implicated in aetiology of ochronosis, inflammation and tissue degeneration observed in patients with alkaptonuria, a rare genetic disease associated with deficient homogentisate 1,2-dioxygenase activity in the liver (Braconi et al., 2010; Phornphutkul et al., 2002).

In this study the antiradical and total antioxidant activities (by DPPH and FRAP tests, respectively) of honeys from different floral sources were preliminarily investigated. The strawberry tree honey emerged as the most active and the richest honey in total phenols, and its biological profile was further investigated in *in vitro* model of lipid oxidation, namely the thermal (140 °C), solvent-free oxidation of cholesterol. HGA was then screened in the same chemical and biochemical assays used for strawberry tree honey, in order to assess its contribution to the antioxidant properties of this honey, and its activity compared to that of other well-known antioxidants. The misleading literature data, coupled to the HGA interesting protective effect in simplified assay systems, prompted us to study in deep the ability of this attractive antioxidant molecule in preventing lipid component degradation in oxidative stress systems of biological relevance, like the oxidative damage to liposome membranes and low density lipoproteins (LDL). The effect of HGA on the copper-induced consumption of specific liposome and LDL lipid targets (fatty acids and cholesterol), and the formation of oxidative products: conjugated diene fatty acid hydroperoxides HP, 7β-hydroxycholesterol, and 7-ketocholesterol for LDL and malondialdehyde for liposomes were investigated. The cytotoxicity of HGA and the rate by which this compound passes through a biological membrane (by calculating the apparent permeability coefficient, P_{app}) was also assessed by using Caco-2 cell monolayers as an intestinal epithelial cell model.

2. Material and methods

2.1. Chemicals

Human low density lipoproteins (LDL), fatty acids, triolein, trilinolein, vitamin E (α-tocopherol), cholesterol, 5-cholesten-3β-ol-7-one (7-keto), 5-cholestene-3β,7β-diol (7β-OH), Bradford protein reagent, desferal (deferoxamine mesylate salt), the mixture of phospholipids (bovine brain extract, Type VII, purity >99%), 1,1,3,3,-tetraethoxypropane (TEP), homogentisic acid (HGA), gallic acid, resveratrol, butylated hydroxyanisole (BHA), ascorbic acid, trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA), ferrous sulphate, 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), (±)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,4,6-tris(2-pyridyl)-1,3,5-triazine (TPTZ), Folin–Ciocalteau's reactive were obtained from Sigma–Aldrich, Fluka (Milan, Italy). All solvents used, of the highest available purity, were also purchased from Sigma–Aldrich. Sodium carbonate, ferric chloride and $CuSO_4{\cdot}5H_2O$ were supplied by Carlo Erba (Milan, Italy). Spectra Por float-a-lyzer dialysis membrane was purchased from Prodotti Gianni (Milan, Italy). *Cis*, *trans*-13-hydroperoxy-octadeca-dienoic acid (*c*,*t*-13-HPODE) and *cis*, *trans*-9-hydroperoxy-octadeca-dienoic acid (*c*,*t*-9-HPODE) were purchased from Cascade (Cascade Biochem. Ltd., London). Alamarblue was purchased from Biosource Europe (Nivelles, Belgium). Cell culture materials were purchased from Invitrogen (Milan, Italy). All the chemicals used in this study were of analytical grade. Ultrapure water (18 mΩ) was obtained with a Elix/Milli-Q Advantage A10 System apparatus (Millipore, Milan, Italy).

2.2. Cell cultures

Caco-2 cell line, obtained from ECACC (Salisbury, Wiltshire UK), has been obtained from human colon adenocarcinoma; after confluence, these cells spontaneously differentiate into enterocytes, retaining many of the morphological and functional characteristics of normal small intestine polarised enterocytes (Travelin, Gråsjö, Taipalensuu, Ocklind, & Artursson, 2002). Sub-cultures of the Caco-2 cells were grown in T-75 culture flasks and passaged with a trypsin–EDTA solution. Cells were cultured in minimum essential medium (MEM) supplemented with 10% FCS, 1% non essential amino acids, 2 mM L-glutamine, penicillin (100 U/ml)-streptomycin (100 μg/ml), at 37 °C in 5% CO_2.

2.3. Honey samples

Seven unifloral honeys types, Strawberry tree (St), Honeydew (Ho), Heather (He), Eucalyptus (Eu), Asphodel (As), Citrus spp (Ct), Acacia (Ac), produced in Italy in 2009 were analysed. Samples were obtained from professional beekeepers and selected according to their sensorial and melissopalynological properties. All samples were stored at 4 °C in the dark until the analysis was carried out. Quantification of HGA in strawberry tree honey was performed as previously described (Tuberoso et al., 2010), and the amount of HGA ranged between 377.6 ± 20.1 and 520.3 ± 17.2 mg/kg. Amount of HGA in the strawberry tree honey used for the biological tests was 489.1 ± 23.5 mg/kg.

2.4. Total polyphenols

The total phenol content (TP) was measured through spectrophotometric determination with a modified Folin–Ciocalteau method (Tuberoso et al., 2009). One hundred μl of honey diluted with water (1:5–1:10, w/v) were added to 0.5 ml of Folin–Ciocalteau's reactive. After 5 min, 3 ml of 10% Na_2CO_3 (w/v) were added, the mixture was shaken, and then diluted with water to a final volume of 10 ml. After a 90 min incubation period at room temperature, the absorbance was read at 725 nm on a 10 mm quartz cuvette using a Varian Cary 50 Scan spectrophotometer (Varian, Leinì, TO, Italy) against a blank. The TP results, expressed as mg/kg of gallic acid equivalent (GAE), were obtained using a calibration curve of a freshly prepared gallic acid standard solution (10–200 mg/kg).

2.5. DPPH assay

A spectrophotometric analysis using DPPH and a comparison with the Trolox calibration curve was performed (Tuberoso et al., 2009). Fifty microlitres of diluted honey (1:5–1:10, w/v, with ultra pure water) was dissolved in 2 ml of DPPH 0.04 mmol/l in methanol. Spectrophotometric readings were carried out with a Cary 50 spectrophotometer at 517 nm using a 10 mm plastic cuvette. A calibration curve in the range 0.02–0.8 mmol/l was used for the Trolox, and data were expressed as Trolox equivalent antioxidant capacity (mmol TEAC/kg). Pure compounds (HGA, resveratrol, gallic acid, ascorbic acid, BHA) were analysed at 10, 20, 50 and 100 mg/kg in triplicate following the previously described procedure. From calibration equation, activity for 1 g of pure standard was calculated.

2.6. FRAP assay

The ferric reducing-antioxidant assay (FRAP) is based on the reduction at low pH of ferric 2,4,6-tris(2-pyridyl)-1,3,5-triazine [Fe(III)-TPTZ] to the ferrous complex followed by a spectrophotometric analysis (Tuberoso et al., 2009). The reagent was prepared