Food Chemistry 170 (2015) 16–21

Contents lists available at ScienceDirect

Food Chemistry

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

Beverages of lemon juice and exotic noni and papaya with potential for anticholinergic effects

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ARTICLE INFO

Article history:
Received 13 May 2013
Received in revised form 11 June 2014
Accepted 7 August 2014
Available online 17 August 2014

Keywords:
Exotic fruits
Bioactive
Antioxidant
Anticholinergic
Noni
Papaya
Lemon juice

ABSTRACT

Lemon (*Citrus limon* (L.) Burm. f.) juice beverages enriched either with noni (*Morinda citrifolia* L.) (LN) or papaya (*Carica papaya* L.) (LP), were characterized by HPLC-DAD-ESI/MS^n, the antioxidant capacity was evaluated by (DPPH$^•$), superoxide ($O_2^{•-}$), hydroxyl radicals ($^•OH$) and hypochlorous acid (HOCl) assays, and their potential as acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) inhibitors was also assessed. The fruits are rich in a wide range of bioactive phenolics. Regarding DPPH$^•$, $^•OH$ and HOCl assays, the LP displayed strong activity, and LN was the most active against $O_2^{•-}$. Concerning cholinesterases, LP was the most active, mainly due to lemon juice contribution. The effect on the cholinesterases was not as strong as in previous reports on purified extracts, but the bioactive-rich beverages offer the possibility of dietary coadjutants for daily consumption of health-promoting substances by adults with aging-related cognitive or physical disorders.

1. Introduction

Nowadays it is widely accepted the fact that diets rich in fruits and vegetables have a positive impact on health and wellbeing. Therefore, the current trend is to demonstrate the relationship between food consumption and reduction of diseases risk or prevention of different health conditions. The growing interest in new added-value foods and beverages with health-promoting properties has prompted to develop new beverages based on different type of waters, juices, and non-alcoholic drinks enriched with fruits, as natural sources of nutrients, colours, and bioactive phytochemicals, being the evaluation of their bioactivity necessary.

In the regulation of cognitive functions, the central cholinergic system is considered to be the most important neurotransmitter involved (Mukherjee, Kumar, Mal, & Houghton, 2007). Furthermore, cholinesterases, such as acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE), are key enzymes that play significant roles in cholinergic transmission, hydrolysing the neurotransmitter acetylcholine (Brühlmann, Marston, Hostettmann, Carrupt, & Testa, 2004). The loss of basal forebrain cholinergic cells results in an important reduction of acetylcholine, which is believed to play a determinant role in the cognitive impairment associated with Alzheimer's disease (AD), senile dementia, ataxia, myasthenia gravis and Parkinson's disease (Mukherjee et al., 2007). The drugs used as cholinesterase inhibitors have adverse side-effects, like gastrointestinal disturbances, nausea, vomiting, and diarrhoea, as well as problems of bioavailability. For this reason, scientific research is seeking natural alternatives of AChE and BuChE inhibitors with a safer profile. A range of plant compounds with this inhibitory activity have been found, including alkaloids (Mukherjee et al., 2007), or xanthones (Brühlmann et al., 2004), but natural foods or juices for this purpose may be a better option in treatment of aging adults under nutrition because of mastication, improper deglutition, or swallowing, usual problems in age-related cognitive decline disorders.

Noni (*Morinda citrifolia* L.) and papaya (*Carica papaya* L.) fruits are grown in tropical and sub-tropical regions, with demonstrated positive implications in the cholinergic system (Essa et al., 2012; Pachauri et al., 2012). However no previous works about noni or papaya fruits used as inhibitors of cholinesterases has been performed to the best of our knowledge. Other positive effects against cancer (Brown, 2012; Nguyen, Shaw, Parat, & Hewavitharana, 2013), diabetes (Juárez-Rojop et al., 2012; Sabitha, Adhikari Prabha, Shetty Rukmini, Anupama, & Asha, 2009), and obesity (Athesh, Karthiga, & Brindha, 2012; Lin et al., 2012) have been

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http://dx.doi.org/10.1016/j.foodchem.2014.08.021
0308-8146/

ascribed to these exotic fruits. In this sense, previous research designing beverages by combining lemon juice (*Citrus limon* (L.) Burm. f.) with some exotic fruits or berries (Gironés-Vilaplana, Valentão, Andrade, et al., 2012; Gironés-Vilaplana, Valentão, Moreno, et al., 2012) revealed an effective increase of the antioxidant properties of the lemon juice and great anticholinesterases activity in the blends, complementary to the neurodegenerative, cardiovascular and metabolic reported for lemon fruits (González-Molina, Domínguez-Perles, Moreno, & García-Viguera, 2010).

With all this in mind, the aim of this work was to design new blends of lemon juice enriched with noni and papaya fruits looking for potential benefits on cognitive function as cholinesterases' inhibitors (acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE)) for food product developments in the area of nutrition for aging adults. First, the phytochemical characterization of polyphenols from beverages by HPLC-DAD-ESI-MS^n, and the determination of their antioxidant capacity by different assays (2, 2-diphenyl-1-picrylhydrazyl (DPPH$^{\cdot}$), superoxide ($O_2^{\cdot-}$) and hydroxyl ($^{\cdot}OH$) radicals and hypochlorous acid (HOCl)) was carried out.

2. Methods and materials

2.1. Chemicals

The reagents used were commercially available: DPPH$^{\cdot}$, β-nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), nitrotetrazolium blue chloride (NBT), trizina hydrochloride, bovine albumin, sodium chloride, AChE from electric eel, BuChE from equine serum, acetylthiocholine iodide, S-butyrylthiocholine chloride, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), sodium borohydride, sodium hypochlorite solution, ferrium chloride (45% solution), 2-deoxy-D-ribose, and 2-thiobarbituric acid were obtained from Sigma–Aldrich (Steinheim, Germany); potassium dihydrogen phosphate, ethylenediaminetetraacetic acid (EDTA), hydrogen peroxide (30%), and trichloroacetic acid were purchased from Merck (Darmstadt, Germany); magnesium chloride hexahydrate and ascorbic acid were bought from Fluka Chemika (Neu-Ulm, Switzerland). Ultrapure water was produced using a Millipore water purification system.

2.2. Samples

Papaya and noni lyophilized fruits were provided by Ecuadorian Rainforest, LLC. (USA). Lemon juice was obtained, from 'Fino' lemons freshly collected from the CEBAS-CSIC experimental farm ('La Matanza', Santomera, Murcia, SE Spain; 38°6′14″N, 1°1′59″W), using a domestic squeezer ('Citromatic', Braun Española S.A., Barcelona, Spain). The juice was stored frozen (−20 °C) until use.

2.3. Experimental design

Lyophilized and powdered fruits were added to lemon juice separately to obtain final concentrations of 5% w/v (5 g of powdered fruit in 100 ml of lemon juice). In addition, a control solution using the same proportion in 0.18 M citric acid buffer (pH 2.46) was prepared, to study the activities of the fruits without lemon. Lemon juice alone was also assayed (pH 2.14). The homogenized mixtures and control solutions were centrifuged (7 min at 4000 rpm), and the supernatant was separated from the solid. The juices were stored frozen (−20 °C) until use. The samples were labelled as follows: L (lemon juice control), LP (5 g of papaya fruit in 100 ml of lemon juice), AP (5 g of papaya fruit in 100 ml of citric acid buffer), LN (5 g of noni fruit in 100 ml of lemon juice), and AN (5 g of noni fruit in 100 ml of citric acid buffer).

2.4. HPLC-DAD-ESI/MS^n

Chromatographic analyses were carried out on a Luna C18 column (250 × 4.6 mm, 5 mm particle size; Phenomenex, Macclesfield, UK). Water:formic acid (99:1, v/v) and acetonitrile were used as the mobile phases A and B, respectively, with a flow rate of 1 mL/min. The linear gradient started with 8% of solvent B, reaching 15% at 25 min, 22% at 55 min, and 40% at 60 min, which was maintained up to 70 min. The injection volume was 30 μL. Chromatograms were recorded at 280 and 360 nm for all flavonoids. The HPLC-DAD-ESI/MS^n analyses were carried out in an Agilent HPLC 1100 series machine equipped with a photodiode array detector and a mass detector in series (Agilent Technologies, Waldbronn, Germany). The equipment consisted of a binary pump (model G1312A), an autosampler (model G1313A), a degasser (model G1322A), and a photodiode array detector (model G1315B). The HPLC system was controlled by ChemStation software (Agilent, version 08.03). The mass detector was an ion trap spectrometer (model G2445A), equipped with an electrospray ionization interface and controlled by LCMSD software (Agilent, version 4.1). The ionization conditions were 350 °C and 4 kV, for capillary temperature and voltage, respectively. The nebulizer pressure and nitrogen flow rate were 65.0 psi and 11 L/min, respectively. The full-scan mass covered the range from m/z 100 to m/z 1200. Collision induced fragmentation experiments were performed in the ion trap using helium as the collision gas, with voltage ramping cycles from 0.3 up to 2 V. The mass spectrometry data were acquired in the positive ionization mode for anthocyanins and in the negative ionization mode for other flavonoids. The MS^n was carried out in the automatic mode on the more abundant fragment ion in MS$^{(n-1)}$. Prior to injection, the samples were centrifuged (12000 rpm, 5 min) and filtered through a PVDF Filter (0.22 μm). Flavonols and flavones were quantified as quercetin 3-*O*-rutinoside (rutin) at 360 nm, and flavanones as hesperidin at 280 nm.

2.5. DPPH$^{\cdot}$ scavenging activity

The antiradical capacity was estimated spectrophotometrically in a Multiskan Ascent plate reader (Thermo Electron Corporation), by monitoring the disappearance of DPPH$^{\cdot}$ at 515 nm, according to Ferreres et al. (2009). Three experiments were performed in triplicate.

2.6. Superoxide radical ($O_2^{\cdot-}$) scavenging activity

This antiradical activity was determined spectrophotometrically, in a 96-well plate reader, by monitoring the effect of the controls and the new drink on the $O_2^{\cdot-}$ induced reduction of NBT at 560 nm. Superoxide radicals were generated by the NADH/PMS system according to a described procedure (Ferreres et al., 2009). Three experiments were performed in triplicate.

2.7. Hypochlorous acid (HOCl) scavenging activity

The inhibition of the hypochlorous acid-induced oxidation of 5-thio-2-nitrobenzoic acid (TNB) to 5,5′-dithiobis(2-nitrobenzoic acid) was studied according to a described procedure (Valentão et al., 2002), in a double beam spectrophotometer (Heλios α, Unicam, Leeds, UK) at 412 nm. Three experiments were performed in triplicate.

2.8. Hydroxyl radical ($^{\cdot}OH$) scavenging activity

The deoxyribose method for determining the scavenging effect of samples on hydroxyl radicals was performed as described before