Food Chemistry 125 (2011) 841–849

Contents lists available at ScienceDirect

Food Chemistry

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

Antioxidative effects of phenolic extracts from chestnut leaves, catkins and spiny burs in streptozotocin-treated rat pancreatic β-cells

Aida Mujić [a,1], Nevena Grdović [b,1], Ibrahim Mujić [c], Mirjana Mihailović [b], Jelena Živković [d], Goran Poznanović [b], Melita Vidaković [b,*]

[a] Clinical Hospital Center Rijeka, Department of Transfusion Medicine, Tome Stizica 3, 51000 Rijeka, Croatia
[b] Institute for Biological Research, University of Belgrade, Bulevar Despota Stefana 142, 11060 Belgrade, Serbia
[c] Biotechnical Faculty, University of Bihac, Kulina Bana 2, 77000 Bihac, Bosnia and Herzegovina
[d] Faculty of Medical Sciences in Nis, Department of Pharmacy, Bulevar Zorana Djindjica 81, 18000 Nis, Serbia

ARTICLE INFO

Article history:
Received 4 December 2009
Received in revised form 12 July 2010
Accepted 25 August 2010

Keywords:
Oxidative stress
Phenolic-rich chestnut extracts
Antioxidative activity
DNA damage
Cell death

ABSTRACT

Oxidative stress plays a central role in the onset of diabetes which is characterised by progressive pancreatic β-cell failure. We studied the ability of phenolic extracts prepared from chestnut leaf, catkin and spiny burs to prevent oxidative stress-induced β-cell (Rin-5F) death. The Rin-5F cell death was induced by treatment with the diabetogenic agent streptozotocin (STZ) and the protective effects of the chestnut extracts were assessed after cells treatment with a STZ/extracts mixture. The chestnut extracts increased cell viability by protecting DNA from oxidative damage and by enhancing the natural antioxidant system. Moreover, the chestnut extracts had a significant impact on the cell's redox-status which was observed as an increase in the levels of reduced glutathione. Chestnut extracts were capable of inhibiting lipid peroxidation, which was manifested as a lowering of malondialdehyde levels. We concluded that chestnut extracts increased cell viability after STZ treatment as a result of their antioxidative properties.

1. Introduction

Oxidative stress results when highly reactive oxygen species (ROS) that possess the ability to extensively damage cell structures increase to a level that overloads an organism's endogenous enzymatic and non-enzymatic antioxidant defences. While diabetic conditions produce oxidative stress, which is responsible for the development of serious long-term complications arising from damage to the vascular and nervous systems, oxidative stress plays a particularly important role in the aetiology and progression of diabetes. The susceptibility of specific tissues to the deleterious effect of oxidative stress is critically influenced by endogenous tissue levels of antioxidant defence mechanisms (Maritim, Sanders, & Watkins, 2003). This is very relevant to pancreatic β-cells that are more vulnerable than other cell types to oxidative stress as a result of possessing low levels of enzymatic antioxidant defences (Moussa, 2008). Progressive β-cell failure characterises diabetes types 1 and 2 (according to the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus). It is estimated that about 30 million people, world-wide, suffer from some type of *diabetes mellitus*, one of the most common non-communicable diseases.

Important data concerning the cellular mechanisms that lead to diabetes have been obtained from studies of animal models of diabetes induced by the administration of the alkylating agent streptozotocin [2-deoxy-2-(3-methyl-3-nitrosourea)-1-D-glucopyranose] (STZ) to rodents. It is generally accepted that STZ brings about insulin-producing β-cell death by directly causing DNA damage, thereby effectively replicating one of the central deleterious effects of oxidative stress, as well as through STZ-induced secondary effects manifested as bona fide oxidative stress. After entering β-cells *via* the GLUT2 glucose transporter, STZ causes DNA damage by DNA alkylation, which in turn rapidly triggers the induction of the DNA repair process. Immediately prior to activation of DNA repair, the nuclear enzyme poly(ADP-ribose) polymerase-1 (PARP-1), that regulates the molecular events responsible for DNA repair, is activated. The level of PARP-1 activation that is correlated with the amount of DNA damage is manifested as an increase in poly(ADP-ribosyl)ation. Thus, intensive poly(ADP-ribosyl)ation leads to a rapid depletion of cellular NAD^+ and ATP levels and the process of cell death becomes activated. Some of the secondary effects of STZ are enhanced dephosphorylation of ATP that provides a substrate for xanthine oxidase which generates more ROS, as well as the liberation of toxic amounts of nitric oxide

* Corresponding author. Tel.: +381 11 2078 326; fax: +381 11 2761 433.
E-mail address: melita@ibiss.bg.ac.rs (M. Vidaković).
[1] These two authors contributed equally to this work.

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doi:10.1016/j.foodchem.2010.08.068

that participate in further DNA damage. As a result of an overload of primary and secondary STZ actions, the cell is unable to restore homoeostasis and β-cells undergo destruction by necrosis (Lenzen, 2007).

Endogenous antioxidant mechanisms inhibit or delay specific substrate oxidation by ROS and are important in preventing many diseases (Halliwell, Gutteridge, & Cross, 1992). Their effectiveness can be improved by exogenous antioxidants of natural and synthetic origin that are present in the diet. Phenolics, also known as high and low molecular weight polyphenols, represent the main class of natural antioxidants. They are synthesised by plants as secondary products and represent one of the most numerous and widely-distributed groups of substances in the plant kingdom, with more than 8000 phenolic structures currently known (Urquiaga & Leighton, 2000). These bioactive compounds participate in defence processes against deleterious oxidative damage through the antioxidant effects associated with their scavenging of harmful reactive species (Fresco, Borges, Diniz, & Marques, 2006). Furthermore, certain phenolic compounds were shown to inhibit ROS-generating transcription factors, such as NF-κB, which is closely linked to inflammation (Le Corre, Chalabi, Delort, Bignon, & Bernard-Gallon, 2005).

As the use of synthetic drugs in diabetes-related oxidative stress management, aside from its high cost, is associated with a host of side-effects, interest in antioxidants of natural origin has increased. The relationship between total phenolic contents and antioxidant capacity has recently been shown for different nuts and their by-products: in *Gevuilla avellana* hull (Moure et al., 2000), almond hull (Pinelo, Rubilar, Sineiro, & Núñez, 2004), hazelnuts (Contini, Baccelloni, Massantini, & Anelli, 2008), pecans, chestnut flowers, fruits, leaves and skins (Barreira, Ferreira, Beatriz, Oliveira, & Pereira, 2008). Since purified phenolic compounds are difficult to obtain and because extracts sometimes have better antioxidant activities than have pure molecules, there is growing interest in studying complex plant extracts (Calliste, Trouillas, Allais, & Duroux, 2005) that could control diabetes through hypoglycaemic and antioxidant effects. In the present work we focused our interest on the antioxidative effects of three different chestnut extracts prepared from chestnut catkins, leaves, and spiny burs from *Castanea sativa* Mill. The protective effect of phenolic-rich chestnut extracts was examined in STZ-treated rat pancreatic β-cells (Rin-5F) in an *in vitro* experimental model that simulates the molecular events which lead to cell death and would bring about diabetes *in vivo*.

2. Materials and methods

2.1. Plant material and extraction procedure

2.1.1. General

Samples of catkins (ext 1), leaves (ext 2) and spiny burrs (ext 3) of the sweet chestnut (*C. sativa* Mill.) were collected in four chestnut-producing orchards (Bužim, Velika Kladuša, Cazin and Bosanska Krupa) located in the area of The Una-Sana Canton in the northwest of Bosnia and Herzegovina. The chestnut samples were harvested in the chestnut-ripening season, from the middle of September to the end of October, 2006. The collected samples were kept at −20 °C and protected from light prior to further use. Samples were milled for further analysis with a homogenizer. For antioxidant compound extraction (performed at room temperature), a fine dried powder of the sample (50 g of all samples) was soaked in 250 ml of 50% ethanol (ratio of sample:solvent was 1:5; w/v). Extraction was performed by ultrasound (using an Ultrasonic bath, Branson model b-220, the Smith-Kline Company) for 30 min at 50 Hz/125 W. The liquid extracts were obtained by filtration through Whatman No. 4 paper and dried by evaporation under vacuum at 40 °C. Dry extracts were kept at −80 °C.

2.1.2. Analyses of total phenolics and flavonoids contents

The contents of total phenolic compounds in dry chestnut extracts were determined by the Folin–Ciocalteu procedure at 765 nm. The values are expressed as g of gallic acid equivalents (GAE) per 100 g of the dry chestnut extract sample. The total flavonoids content was analysed by aluminium-chloride colorimetric assay at 510 nm. The values are expressed as g of catechin equivalents (CE) per 100 g of the dry extract sample.

Identification of the constituents of the chestnut extracts was performed by LC/UV/MS and HPLC/DAD. In this study, the extracts were dissolved in cell medium at a concentration of 10 mg/ml and analysed for their DPPH radical-scavenging activity, cell viability, comet assay, GSH content and inhibition of lipid peroxidation.

2.2. Cell culture and treatment

The Rin-5F, rat insulinoma pancreatic β-cells, were cultivated at 37 °C under 5% CO_2 in a humidified atmosphere in RPMI (Roswell Park Memorial Institute medium), supplemented with 10% foetal calf serum, 100 U of penicillin/ml, and 100 μg of streptomycin/ml. Medium was changed every 72 h. Treatment procedure was as follows: cells were incubated for 24 h with 5 mM STZ (citrate buffer, pH 4.5) and processed immediately; chestnut extracts 1, 2 and 3 were dissolved in RPMI medium and, depending on experimental design, were combined with 5 mM STZ before applying to cells.

2.3. MTT viability test

Rin-5F cell viability was estimated using the MTT (Sigma, Cat. No. M5655-1G) viability assay. The MTT assay estimates cell viability, based on the quantification of mitochondrial activity by measuring the formation of a dark-purple formazan formed by the reduction of the tetrazolium ring of MTT. Rin-5F cells were grown in 96 well plates, treated with 5 mM STZ in combination with different chestnut extracts and, after 24 h, 200 μl of MTT (0.5 mg/ml RPMI medium) were added to each well. After incubation for 2 h in the dark the insoluble purple formazan products formed in living cells were dissolved in dimethyl sulfoxide. Formazan product formation was quantified by measuring the absorbance at 570 nm. Cell viability was expressed as a percentage after comparison to the control cells that were assumed to be 100%.

2.4. DPPH radical-scavenging activity

Various concentrations (5, 10, 20 and 50 μg/ml) of chestnut extracts (in total volume of 100 μl) were mixed with 500 μl of a freshly prepared DPPH (1,1-diphenyl-2-picrylhydrazyl) (250 μM) working solution and 400 μl of 100 mM Tris–HCl buffer, pH 7.4. The mixture was shaken vigorously and left to stand for 20 min in the dark. Reduction of the DPPH radical was determined by reading the absorbance at 517 nm. Ascorbic acid was used as a standard at 50, 100, 300 and 500 μM concentrations. All tests were performed in triplicate. The inhibitory percentage of DPPH· was calculated according to the following formula: % Inhibition = $[(A_{blanc} - A_{test})/A_{blanc}] \times 100$ (A_{blanc} is the absorbance of the DPPH· in solution without the test sample; A_{test} is the absorbance of DPPH· in the solution with the test sample – different chestnut extracts). IC50 values were calculated as concentration of extract required to decrease the absorbance at 517 nm by 50%.