LWT - Food Science and Technology 42 (2009) 458–463

Contents lists available at ScienceDirect

LWT - Food Science and Technology

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

Cyphomandra betacea Sendt. phenolics protect LDL from oxidation and PC12 cells from oxidative stress

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ARTICLE INFO

Article history:
Received 30 May 2008
Received in revised form 17 August 2008
Accepted 9 September 2008

Keywords:
C. betacea
Antioxidant effect
LDL oxidation
PC12 cells
ROS

ABSTRACT

Cyphomandra betacea Sendt. (tamarillo) is a subtropical fruit containing rich contents of anthocyanins and carotenoids. The antioxidant activity was investigated using a crude ethanol extract of *C. betacea* fruit and its partitioned fractions, i.e. the ethyl acetate, butanol and water fractions. The ethyl acetate fraction exhibited the highest DPPH scavenging activity, Trolox equivalent antioxidant capacity (TEAC) and total phenol content. *C. betacea* phenolics in ethyl acetate fraction inhibited copper-induced LDL oxidation equally to or more effectively than DL-α-tocopherol, as measured by decreased formation of thiobarbituric acid-reactive substances (TBARS), conjugated diene and relative electrophoretic mobility (REM). Furthermore, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) reduction assay showed that *C. betacea* phenolics in ethyl acetate fraction prevent oxidative stress-induced cell death in neuronal PC12 cells in a dose-dependent manner via attenuation of ROS production. In conclusion, *C. betacea* phenolics are potent antioxidants which can inhibit LDL oxidation *in vitro* and ROS production in PC12 cells.

1. Introduction

Hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), superoxide radicals ($O_2^{\bullet-}$), and hydroxyl radicals ($OH^{\bullet}$), collectively known as the reactive oxygen species (ROS), are the most reactive species derived from the metabolism of oxygen in aerobic systems (Gutteridge, 1994). Classically oxidative stress is described as an imbalance between generation and elimination of ROS. ROS provide a wide potential for causing damages of cellular components such as DNA, protein and lipid. It is widely acknowledged that the accumulation of oxidative damages in intracellular macromolecules is an essential element in the pathogenesis of many diseases such as respiratory distress syndrome, ischemia/reperfusion injury, renal failure, rheumatoid arthritis, local or systemic inflammatory disorders, diabetes, atherosclerosis, cancer and neurodegenerative diseases (Aruoma, Kaur, & Halliwell, 1991; Emerit, Edeas, & Bricaire, 2004). As the major component of ROS, H_2O_2 induces neuronal apoptosis thereby perturbing cellular natural antioxidant defense system and this effect can be blocked by addition of antioxidants (Jiang, Liu, Bao, & An, 2003; Tang et al., 2005).

There is extensive evidence indicated that atherogenesis is initiated and promoted by lipid oxidation of low-density lipoprotein (LDL), ultimately leading to modification of apolipoprotein B such that the LDL particle becomes recognized by the macrophage scavenger receptor(s) and produces massive cholesterol loading and foam cell formation (Aviram, 1999; de Winther, van Dijk, Havekes, & Hofker, 2000). Increased fruit and vegetable consumption is associated with a decreased incidence of cardiovascular diseases, cancer, and other chronic diseases. Their beneficial health effects have been attributed, in part, to antioxidant polyphenols present in these foods. These observations led to the hypothesis that dietary antioxidants might reduce the risk of degenerative diseases by reduction of oxidative stress and inhibition of macromolecule oxidation. Lots of researches have been devoted to investigate the antioxidant activities of natural antioxidants and their roles in neural protection or prevention of atherosclerosis (Colognato et al., 2006; Enkhmaa et al., 2005; Heo & Lee, 2005; Koyama et al., 2006; Yamakoshi, Kataoka, Koga, & Ariga, 1999).

Cyphomandra betacea Sendt., also known as tree tomato or tamarillo, is a commercial crop for international export in New Zealand and Portugal. Delphinidin 3-rutinoside and β-cryptoxanthin are its major anthocyanin and carotenoid, respectively (de Rosso & Mercadante, 2007). Anthocyanins and carotenoids are not only natural pigments

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0023-6438/$34.00
doi:10.1016/j.lwt.2008.09.010

responsible for the color of vegetables and fruits, but are also bioactive compounds, both demonstrating important biological, therapeutic, and preventative properties (Bast, Haenen, van den Berg, & van den Berg, 1998; Kong, Chia, Goh, Chia, & Brouillard, 2003). To better understand whether *C. betacea* fruit could prevent oxidative stress, we analyzed the total phenol contents and investigated the biochemical properties of the ethanol crude extract and its partitioned fractions on DPPH and ABTS cation radical scavenging activity. The polyphenol-rich fractions were further used to study their protective effect against LDL oxidation and oxidative stress-induced cytotoxicity on neural PC12 cells. Results in the present study will facilitate our understanding of *C. betacea* fruit on its antioxidant activity as well as its effect in preventing atherosclerosis and neurodegenerative diseases.

2. Materials and methods

2.1. Sample

Ripen *C. betacea* fruits were purchased from the orchard in Fencihu, Alishan National Scenic Area, Chiayi, Taiwan in July 2007.

2.2. Proximate composition analysis of C. betacea fruit

The moisture, carbohydrate, crude protein, crude fat, fiber, ash, vitamins and calcium of the edible portion of the fresh fruit were determined according to the CNS (Chinese National Standard) method stipulated by the Bureau of Standards, Metrology and Inspection, Taiwan, ROC.

2.3. Extraction and partition of C. betacea phenolics

The fresh *C. betacea* fruits were washed and the pedicel was removed. They were diced into 5-cm pieces and weighed (~500 g) followed by freezing dry. The lyophilized fruit pieces were crushed into powder (~70 g) and were extracted with ethanol (~1.5 L) at room temperature over night for three times followed by filtration. The flow-through was concentrated in a rotary evaporator under reduced pressure at 50 °C to yield dark-brown syrup. A small portion of the syrup was redissolved in DMSO and denoted as the crude ethanol extract.

The rest of the dark-brown syrup was partitioned between ethyl acetate and H_2O. The ethyl acetate layer was concentrated in a rotary evaporator at 50 °C and the resulting residue was dissolved in DMSO and denoted as the EA fraction.

The aqueous layer was again partitioned with equal volume of *n*-butanol to give *n*-butanol and aqueous layers. The *n*-butanol layer was concentrated in a rotary evaporator at 60 °C and the resulting brown syrup was dissolved in DMSO and denoted as the *n*-butanol fraction. The aqueous layer was lyophilized and the resulting dark-brown residue was dissolved in DMSO and denoted as water fraction.

2.4. Total phenol content

Total phenol content was determined by the Folin–Denis method (Waterman & Mole, 1994). 50 μL of Folin–Denis reagent (Sigma Chemical, St. Louis, MO, USA) and 100 μL of saturated sodium carbonate were added to 850 μL of the diluted extract. After incubating the mixture for 30 min at 50 °C and cooling down to room temperature, the absorbance of mixture was measured at 760 nm. D-catechin (5–50 μg/mL) was used to construct the reference curve. The contents of phenolic compounds in crude ethanol extract and various partitioned fractions were expressed as mg catechin equivalents (CE)/g dry weight.

2.5. DPPH scavenging capacities

The crude extract and different fractions of *C. betacea* fruit were evaluated for their activities to scavenge the stable DPPH radical (0.1 mM, Sigma) according to the method described previously (Dinis, Maderia, & Almeida, 1994). The activity of the test material to quench the DPPH free radical was evaluated according to the equation: scavenging $\% = (A_c - A_s)/A_c \times 100\%$. A_s and A_c are absorbance at 517 nm of the reaction mixture with sample and control, respectively. DMSO was used as a control (blank). The IC_{50} values were obtained through interpolation from linear regression analysis and denoted the concentration of sample required to scavenge 50% of DPPH radicals. All experiments were repeated at least three times.

2.6. Trolox equivalent antioxidant capacity (TEAC) analysis

The TEAC value is based on the ability of samples to scavenge the blue–green 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical cation relative to the ability of the water-soluble vitamin E analogue, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox). The ABTS radical cation was prepared by mixing an ABTS stock solution (Sigma, 7 mM in water) with 2.45 mM potassium persulfate. This mixture was maintained for 12–24 h until the reaction is completed and the absorbance is stable. For measurement of the $ABTS^{\bullet+}$, the solution was diluted to an absorbance of 0.700 ± 0.020 at 734 nm. 1 mL of the $ABTS^{\bullet+}$ solution and 100 μL of tested sample (0–1 mg/mL) were mixed for 45 s and the absorbance at 734 nm was recorded after 1 min of incubation (Re et al., 1999). Trolox (0–50 μg/mL) was used for calibration and results expressed as mg Trolox equivalents (TE) per gram of dry matter.

2.7. Preparation and oxidation of LDL

LDL ($d = 1.019$–1.063) was prepared from the plasma of anonymous donors from Tainan Blood Center (Tainan, Taiwan) by sequential ultracentrifugation. Lipoprotein was desalted and concentrated by filtration (Amicon Ultra-4, Millipore, Beverly, MA) against phosphate-buffered saline (PBS) at $450 \times g$ for 120 min at 4 °C. The protein concentration was measured according to the method of Bradford (Bradford, 1976), using bovine serum albumin as a standard. Oxidation of LDL was carried out by incubating EDTA-free LDL (0.1 mg/mL) with 10 μM Cu^{2+} in PBS in the presence of vehicle or different concentrations of test reagents at 37 °C.

2.8. Analysis of LDL oxidation

Peroxidation of LDL was measured by determination of thiobarbituric acid-reactive substances (TBARS) and expressed as malondialdehyde (MDA) equivalents (Hermann & Gmeiner, 1993). The percentage of inhibition was measured for different concentrations of each tested sample. IC_{50} values were determined by constructing a dose–response curve and calculated by determining the concentration needed to inhibit 50% MDA formation.

The quantity of conjugated dienes in LDL was assessed by monitoring the change at A_{234} (ΔA_{234}) (Esterbauer, Striegl, Puhl, & Rotheneder, 1989). To measure the electrophoretic mobility, the oxLDL was concentrated by filtration (Microcon YM-3, Millipore) at $8000 \times g$ for 120 min at 4 °C. About 1–2 μL of each concentrated sample was loaded onto Titan lipoprotein gel (Helena Laboratories, Beaumont, TX) and run at 80 V for 45 min. The electrophoretic mobility of LDL was determined by Fat Red 7B staining according to the manufacturer's instructions. Relative electrophoretic mobility (REM) was calculated as the mobility of oxLDL relative to that of native LDL (n-LDL).