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Cocoa bean (*Theobroma cacao* L.) phenolic extracts as PTP1B inhibitors, hepatic HepG2 and pancreatic β-TC3 cell cytoprotective agents and their influence on oxidative stress in rats

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ABSTRACT

Recently attention has been focused on cocoa beans that are the primary raw material used for the preparation of cocoa powder or chocolate as valuable source of bioactive substances with high antioxidant potential and well documented beneficial health properties, including prevention and treatment of type 2 diabetes. The ability of phenolic compounds to inhibit the activity of enzyme hydrolyzing carbohydrates is already quite well studied, however the anti-obesity and antidiabetic activity of cocoa extracts obtained from roasted beans as cytoprotective agents or insulin signaling regulators is not known. In the present study for the first time compounds of raw and roasted cocoa bean of *Forastero* variety phenolic extracts were separated and purificated via centrifugal partition chromatography (CPC) technique. Obtained preparations were *in vitro* investigated in terms of the PTP1B inhibition and cytoprotective activity against oxidative stress using human hepatoma HepG2 and mouse pancreatic β-TC3 cell lines. Additionally the influence of preparations on fat tissue and antioxidant properties *in vivo* on rat animal model was studied. Taking into account obtained results it can be concluded that cocoa phytochemicals, including pigment's fraction of roasted beans with melanoidins, are potential modulators of insulin signaling, protect beta and hepatic cells against cellular damage induced by excessive oxidative stress. This study for the first time reports potential anti-obesity properties of roasted cocoa bean extract rich in MRP, which makes this extract as promising candidate for diabetes prevention and associated metabolic disorder.

1. Introduction

One of the richest known natural sources of antioxidants is cocoa bean, which contains more phenolic compounds and has higher antioxidant activity than tea or red wine (Lee, Kim, Lee, & Lee, 2003). Cocoa beans are the seeds of the tropical cocoa tree *Theobroma cacao* L. and are one of the most valuable ingredients of noble confectionery and pharmaceutical or cosmetic industries (Ali, Ranneh, & Ismail, 2015; Farahany, Selamat, Man, & Idris, 2008; Liendo, Padilla, & Quintana, 1998). Among three main varieties of cocoa bean: *Forastero*, *Criollo*, and *Trinitario*, the first one is the most common and widely cultivated for mass production. Cocoa beans of *Forastero* variety are dark brown and their aroma is less pleasant and aromatic comparing to the *Criollo* variety. They have more robust, sour–bitter flavor with moderate acidity, are richer in valuable cocoa butter and have higher content of phenolic compounds (Motamayor, Risterucci, Heath, & Lanaud, 2003; Oracz, Żyżelewicz & Nebesny, 2015; Rusconi & Conti, 2010). Phenolic compounds of cocoa beans represent on average around 10% of the dry weight of the whole bean (Ali et al., 2015; Gallo, Vinci, Graziani, De Simone, & Ferranti, 2013). Most of them, due to the nature of their construction and the presence of hydroxyl groups, have a high biological *in vitro* and *in vivo* activity and play an important role in the prevention of many diseases. They exhibit a wide range of physiological properties, such as antioxidant, anti-free radical, anti-allergic, antibacterial, antiviral, anti-inflamatory, antiatherogenic, antithrombotic, antihypertensive, anticarcinogenic, cardioprotective and vasodilatory effects (Arlorio et al., 2005; Aron & Kennedy, 2008; Faisal, Yazan, Amin, & Mohd Esa, 2015; Field & Newton, 2013; Keen, Holt, Polagruto, Wang, & Schmitz, 2002; Kirch & Ellinger, 2014; Kumar Pandurangan, Saadatdous, Mohd Esa, Hazilawati, & Ismail, 2015; Maskarinec, 2009; Quinones, Miguel, Muguerza, & Aleixandre, 2011; Rein et al., 2000; Rodriguez-Ramiro et al., 2011; Sies, Schewe, Heiss, & Kelm, 2005; Zanotti et al., 2015). It is known that the consumption of modest amounts of cocoa products is associated with ~40% reduction in cardiometabolic risk, decrease of blood glucose levels, improvement of glucose tolerance in diabetic patients, improvement of sensitivity to insulin and

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modulation of oxidative stress markers in skeletal muscle (Ramirez-Sanchez et al., 2013). However, the antioxidant defense mechanism for the preventive activities of cocoa during obesity and diabetes is not fully understood (Ali et al., 2015; Arlorio et al., 2005; Helal, Desobry, Banon, & Shamsia, 2015; Oracz, Żyżelewicz, et al. 2015; Ramiro et al., 2005). More recently it has been shown, that some phenolic compounds possessing antidiabetic effect can inhibit activity of PTP1B protein and protect pancreatic beta cells against oxidative stress (Zakłos-Szyda, Majewska, Redzynia, & Koziołkiewicz, 2015). Due to the high concentration of phenolic compounds in cocoa bean this variety will constitute research material in these studies.

Cocoa bean is the main raw material used for the preparation of chocolate and cocoa powder. One of the most important processes of the cocoa processing is roasting. As a result of roasting among others Maillard reaction products (MRP) are formed in the beans. Maillard reactions play very important role among multiple conversions taking place during the thermal processing of cocoa beans. This process is very complex and in the advanced and final steps of the Maillard reactions it leads to formation of high molecular weight polymers or co-polymers with molecular masses up to 100 kDa that are named melanoidins (Oliviero, Capuano, Cammerer, & Fogliano, 2009). These compounds are responsible for the taste, color, aroma and texture of roasted food products such as cocoa beans, coffee and malt (Wang, Qian, & Yao, 2011). Melanoidins have been shown to possess the strong antioxidant activity, antimicrobial ability and antihypertensive properties (Liu, Kong, Han, Sun, & Li, 2014). MRP isolated from roasted coffee and biscuits protect human hepatoma HepG2 cells against oxidative stress (Goya, Delgado-Andrade, Rufián-Henares, Bravo, & Morales, 2007; Martin et al., 2009; Wang et al., 2011).

The aim of the study was to determine the biological activity of two freeze-dried aqueous extracts obtained from cocoa bean of *Forastero* variety (CBEs), i.e. raw and roasted beans and fractions of the compounds isolated from these extracts as potential anti-obesity agents using the hepatoma HepG2 and pancreatic β-TC3 cell lines. In addition to the *in vitro* study, the above mentioned extracts were used as dietary supplements in laboratory rats fed high-fat diets for 4 weeks. In *in vivo* study it was hypothesized that: *i*) polyphenols along with other cocoa bean compounds possess antioxidant properties irrespective if they are extracted from raw or roasted beans, *ii*) the antioxidant activity should be ascribed to the main polyphenolic fraction, i.e. monomeric flavan-3-ols, and *iii*) the dietary cocoa extracts may mitigate negative consequences of high-fat diet consumption.

2. Material and methods

2.1. Chemicals and reagents

Standards of (+)-catechin (≥99%), (−)-epicatechin (≥98%), epigallocatechin (≥90%), procyanidin B2 (≥90%), procyanidin C1 (≥75%), quercetin (≥95%), quercetin-3-O-glucoside (≥98%), quercetin-3-O-galactoside (≥97%), quercetin-3-O-arabinoside (≥95%), acetonitrile of HPLC grade (≥99.9%), formic acid for LC–MS (~98%), DCFH-DA dye, DPPH and ABTS radicals were all obtained from Sigma-Aldrich (St. Louis, MO, USA) and dichloromethane of HPLC grade from CHEMPUR (Piekary Śląskie, Poland). All other reagents used were of analytical grade and purchased from POCH (Gliwice, Poland). Ultrapure water was obtained from a Millipore Milli-Q Plus purification system (Bedford, MA, USA).

2.2. Material

Raw and fermented cocoa bean (*T. cacao* L.) of *Forastero* variety harvested in Peru in 2014 with 5.8% water content was used in the study. It was purchased from Barry Callebaut Polska Sp. z o.o. Raw and roasted beans were used to prepare two aqueous extracts. Roasting parameters and the procedure of extract obtaining is described in Section 2.3.

2.3. Cocoa bean processing

2.3.1. Roasting

A part of cocoa bean was convectively roasted in the tunnel roaster described by Żyżelewicz, Krysiak, Budryn, Oracz, and Nebesny (2014). The process was conducted for 35 min at 135 °C and with 1 m/s air velocity and 0.3% relative humidity of roasting air. The water content in the roasted beans was 2.05%.

2.3.2. Preparation of cocoa bean extracts (CBEs)

Raw and roasted cocoa beans were dehulled, ground and sieved to a particle size ranging from 0.200 to 1.0 mm to achieve satisfactory extractability of phenolic compounds in water and good effectiveness of filtration. The extracts were obtained using ground and sieved cocoa beans (raw or roasted) and water in 1:3 (*w*/*w*) ratio, respectively. Water was used as a solvent for the preparation of extracts because presented in the article researches are part of the work focused on the application of CBEs and isolated from them fractions in the food. The suspensions in Erlenmeyer flasks were placed in a SV 1422 Memmert water baths with heating and shaker (Schwabach, Germany). Extraction of biological compounds, primarily phenolic compounds, was carried out at 60 °C for 30 min. After this process, suspensions were filtered under vacuum using filter paper from POCH (Gliwice, Poland) and a vacuum pump KNF 18 035.3 N (Neuberger, NJ, USA). The extracts were then evaporated (approx. 50%) using a Heidolph evaporator (Schwabach, Germany), frozen, freeze-dried in a BETTA2-8LSC plus Christ freeze drier (Osterode am Harz, Germany) and stored at −24 °C until separation, purification and analysis.

Then, one part of each extract was fractionated into three fractions (three groups of compounds — listed in Section 2.4) and purified using the technique of centrifugal partition chromatography (CPC), which uses a two-phase liquid–liquid system without solid phase. For this purpose, two phases should be prepared, upper – more hydrophobic and lower – less hydrophobic. These phases consist of three–four solvents, in most cases. Solvents used to prepare phases for the separation and purification of CBEs are listed in Section 2.4.

2.4. Separation and purification of cocoa bioactive compounds

In case of rotor with 1000 mL capacity, separation and purification of CBE was carried out using CPC technique with a chromatograph SPOT Prep II 50 from Armen Instrument (Saint-Avé, France) integrated with a 2-channel UV/VIS detector, a fraction collector and additional external pump. CPC was equipped in Armen Glider CPC v5.0b.11 software. The two-phase solvent system was prepared from four solvents: hexane, ethyl acetate, ethanol and water in the ratio of 1:8:2:7 (*v*/*v*/*v*/*v*) (two-phase solvent system), in accordance with Delaunay, Castagnino, Chèze, and Vercauteren (2002). Complete cycle of CPC resulting in a full separation of bioactive compounds into three groups, i.e. 1. monomeric flavan-3-ols, 2. procyanidins, and 3. colored compounds, takes 5 h and 17 min, consumes more than 5.5 L of eluents and comprises the following steps:

I. filling and balancing of the rotor (with capacity 1000 mL)

- filling the rotor with a hydrophilic stationary lower phase (B) in 32 min at a flow rate of 50 mL/min and at a rotor speed of 500 rpm,
- injecting 5 g of freeze-dried CBE dissolved in 50 mL of a two-phase solvent system (up to 10% of rotor volume), purified on a nylon syringe filter 0.45 μm via an external pump onto a rotor,

– balancing with a hydrophobic mobile upper phase (A) in 18 min in an ascending mode at a flow rate of 25 mL/min and at a rotor speed of 1500 rpm.

II. separation of bioactive compounds

– elution with upper phase (A — 100%) in 192 min at a flow rate of 10 mL/min and 1500 rpm rotor speed,
– elution with lower phase (B — 100%) in 60 min at a flow rate of 30 mL/min and 500 rpm rotor speed.

During this period 50 mL volume fractions were collected.

III. washing the motionless rotor (parking) at a flow rate of 30 mL/min for 15 min with phase C (distilled water) and phase D (methanol) (1:1 ratio).

After 1 cycle of chromatograph followed by washing the external pump with warm distilled water and subsequently with methanol and isopropanol.

Detection of phenolic compounds was provided by UV at the wavelength of 280 and 365 nm. Fractions collected during these time slots were combined and concentrated by the evaporation of hexane, ethanol and ethyl acetate using a ScanMaxiVac Labogene (Lynge, Denmark) concentrator. Then, fractions were purified from methylxanthines with dichloromethane in the following way. Individual evaporated from organic solvent fractions were placed in the separatory funnels and then, 300 mL of dichloromethane for each 100 mL of fraction was added. The whole was mixed. After 24 h, the upper phase from the separator back was treated with dichloromethane, adding it in an amount of 150 mL, followed by vigorous shaking. This was repeated twice. In the next step, dichloromethane layer was discarded and remained organic solvent was evaporated under a stream of nitrogen. Then obtained aqueous solutions of monomeric flavan-3-ols, procyanidins and colored compounds were subjected to freeze-drying process in the freeze drier CHRIST BETA 2-8 LSC plus (Osterode am Harz, Germany) and forwarded for further study.

2.5. UHPLC–DAD–ESI–MS/MS analysis of phenolic compounds

Phenolic compounds were analyzed using UHPLC–DAD–ESI–MS/MS technique described by Oracz, Nebesny, and Żyżelewicz (2015). Extract or fraction isolated with a CPC technique (1 g) was first twice defatted with 20 mL of hexane for 15 min in a multirotator Biosan Multi Bio RS-24 (Riga, Latvia) and centrifuged at 4000 ×*g* for 15 min at 4 °C using Centurion Scientific K3 series model K2015R (Stoughton, UK). Then, the hexane layer was discarded and remained solvent was evaporated under a steam of nitrogen. The sample was then dissolved in 10 mL of distilled water and filtered through a 0.2 μm nylon syringe filter to a glass vial. A standard stock solution of phenolic compounds at a concentration of 0.5 mg/mL was initially prepared in acetonitrile/water (1:1, *v*/*v*) from which solutions in ultrapure water with different dilution were obtained.

UHPLC analyses were performed using an UHPLC + Dionex UltiMate 3000 liquid chromatographic system consisted of a UHPLC pump, an autosampler, a column oven, a diode array detector with multiple wavelength (Thermo Fisher Scientific Inc., Waltham, MA, USA), and an UHR-Q-TOF–MS/MS (Bruker Daltonics GmbH, Bremen, Germany) using an electrospray ionization (ESI) source. Instrument control, data acquisition, and evaluation were done with the OTOFControl 3.2, HyStar 3.2, and Chromeleon 6.8.1 Chromatography Data System softwares, respectively. Separation was carried out using a Accucore™ C18 2.6 μm, 150 mm × 3.0 mm i.d. column (Thermo Scientific, PA, USA) with two-phase gradient system of formic acid/water (0.1/99.1, *v*/*v*) as mobile phase A, and acetonitrile/water/formic acid (80/19.98/0.02, *v*/*v*/*v*) as mobile phase B. The mobile-phase gradient used was: 0–5 min, 5% B; 5–6 min, 5–8% B; 6–25 min, 8–15% B; 25–30 min, 15–20% B; 30–35 min, 20–25% B; 35–38 min, 25–30% B; 38–45 min, 30–85% B; 45–52 min, 85–5% B; 52–62 min, 5% B. The flow rate of the mobile phase was 0.300 mL/min, and the column temperature was 30 °C. The injection volume was 10 μL. Monomeric flavan-3-ols, procyanidins and gallic acid were monitored and quantified at 280 nm, while flavonols were monitored and quantified at 365 nm. The mass spectrometric conditions were as follows: negative ion mode, capillary voltage, 4500 V; drying gas temperature, 200 °C; drying gas flow, 8.0 L/min; and nebulizing gas pressure, 1 bar. Full scan mass spectra were acquired over a mass range from m/z 50 to 1500 in the negative ion mode for flavan-3-ols and flavonols and positive ion mode for anthocyanins. The MS/MS spectra were obtained in collision-induced dissociation (CID) mode using nitrogen as the collision gas.

Identification and peak assignment of phenolic compounds was based on the comparison of their retention times, UV–vis spectra characteristics, full scan mass spectra, and MS/MS fragmentation patterns with those of authentic standards analyzed under identical conditions, as well as the bibliographic references used in the characterization process. Quantification of individual phenolic compounds was carried out using external standard method. The calibration curves were constructed for each compound using six different concentration levels (0.01–0.1 mg/mL). All analyses were repeated three times. The chromatograms of examined cocoa bean preparations are presented in Fig. 1. Mass spectra on $[M–H]^-$ ion of gallic acid (peak 1) at m/z 171, (+)-catechin (peak 2) at m/z 289, (−)-epicatechin (peak 3) at m/z 289, procyanidin B2 (peak 4) at m/z 577, procyanidin C1 (peak 5) at m/z 865, quercetin 3-*O*-galactoside (peak 6) at m/z 463, quercetin 3-*O*-glucoside (peak 7) at m/z 463, quercetin 3-*O*-arabinoside (peak 8) at m/z 433, and quercetin (peak 9) at m/z 301 are presented in Fig. 2.

2.6. Spectrophotometric and spectrofluorimetric analysis

The absorbance of the freeze-dried CBEs and fractions of colored compounds solutions was measured using a UV-1800 Shimadzu UV Spectrophotometer (Kyoto, Japan) at 294 and 420 nm, as markers at the early and late high molecular weight Maillard reaction products, respectively (Stanic-Vucinic, Prodic, Apostolovic, Nikolic, & Cirkovic Velickovic, 2013). The specific extinction coefficients at 294 and 420 nm, respectively $K_{mix294\ nm}$, and $K_{mix420\ nm}$, were determined using the methodology described by Bellesia and Tagliazucchi (2014).

The fluorescence of the freeze-dried CBEs and obtained from them fractions of colored compounds solutions was measured at an excitation wavelength of 350 nm and an emission wavelength 360–600 nm using a RF-5301PC Shimadzu Spectrofluorophotometer (Kyoto, Japan).

A 1 g/L sample of each freeze-dried CBEs and obtained from them fractions was prepared in water.

2.7. PTP1B inhibition assay

The antiphosphatase activity of extracts was measured with CycLex Protein Tyrosine Phosphatase PTP1B Fluorometric Assay Kit according to manufacturer procedure. Briefly, the mix of 40 μL of reaction buffer and 5 μL of tested extract (different concentrations were performed by serial dilutions) was added to each well of 96-well microplate. Next 5 μL of human recombinant PTP1B enzyme solution was added and reaction mixture was incubated for 15 min at room temperature. After incubation, 20 μL of development buffer and 5 μL of development reagent were added. After 15 min of incubation at room temperature, 25 μL of stop solution was added to each well. The fluorescence was measured at 530 nm emission and 490 nm excitation (Synergy™ 2, BioTek Instruments Inc.). Experiments were performed in triplicate. As internal inhibitor of PTP1B sodium, orthovanadate was used. Analysis of inhibitory effect was calculated as:

$$\text{inhibition}(\%) = A_A/A_B \times 100;$$

where A_A and A_B were fluorescence intensities of tested sample and solvent control, respectively. The concentration of the inhibitor (expressed

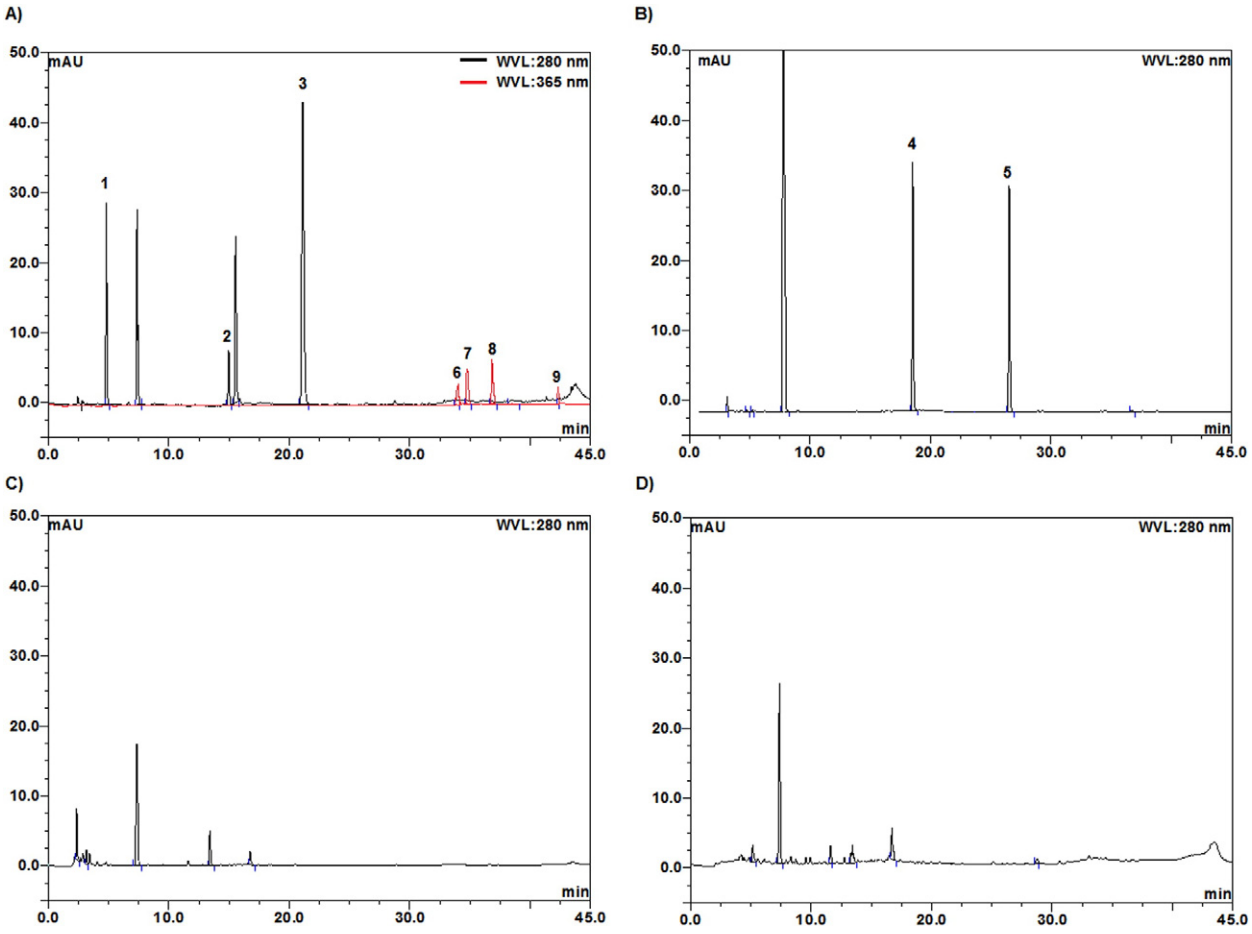

Fig. 1. Typical UHPLC-DAD chromatograms of monomeric flavan-3-ol fraction (A), procyanidins fraction (B), fraction of colored compounds from raw cocoa bean (C) and fraction of colored compounds from roasted cocoa bean (D), recorded using UV detection at 280 nm for flavan-3-ols and gallic acid and 365 nm for flavonols. Peak designations: 1 — Gallic acid, 2 — (+)-catechin, 3 — (−)-epicatechin, 4 — procyanidin B2, 5 — procyanidin C1, 6 — quercetin 3-*O*-galactoside, 7 — quercetin 3-*O*-glucoside, 8 — quercetin 3-*O*-arabinoside, and 9 — quercetin.

as mg of dry weight of extract per mL of reaction mixture under assay conditions) required to inhibit 50% of the PTP1B activity is defined as the IC_{50} value. If the compounds at analyzed dilution achieved higher than 50% level of inhibition their inhibitory activities were further measured at different concentrations to obtain the IC_{50} values by regression analyses.

2.8. Cell culture

All cell culture reagents were obtained from the Life Technologies (Carlsbad, USA). Tissue culture plastics were supplied by Greiner Bio-One GmbH (Frickenhausen, Austria). Mouse insulinoma β-TC3 and human hepatoma HepG2 cells were purchased from Leibniz Institute DSMZ — German Collection of Microorganisms and Cell Cultures (Leibniz, Germany). Cells were maintained at 37 °C in a humidified incubator containing 5% CO_2 and 95% air. β-TC3 cell were grown in DMEM medium with 25 mM glucose, supplemented with 15% horse serum and 3% fetal bovine serum, 1% Hepes, 1 mM sodium pyruvate, 100 μg/mL ampicillin, 100 μg/mL streptomycin. HepG2 cells were grown in RPMI medium with 11 mM glucose, supplemented with 10% fetal bovine serum, 1% Hepes, 100 μg/mL ampicillin, and 100 μg/mL streptomycin.

2.9. Cell viability and proliferation assay

Cells were seeded into 96-well plates in the number of 10^4 per well in complete medium and grown for 20 h. Cells were incubated in the presence of investigated extracts diluted in DMEM or RPMI culture medium respectively for either 24 h. Following incubation 10 μL of PrestoBlue cell viability reagent (Life Technologies, Van Allen Way, CA, USA), a resazurin-based solution, was added into each well and incubated further for 40 min at 37 °C and 5% CO_2. Cell viability was determined by measuring the fluorescent signal F530/590 on a Synergy 2 Microplate Reader (Bio-Rad, CA, USA). The obtained fluorescence magnitudes were used to calculate cell viability expressed as a percent of the viability of the untreated control cells. Cell proliferation was evaluated with the CyQUANT Direct Cell Proliferation Assay (Life Technologies). After addition of 2× Detection Reagent to the wells the assay was incubated 60 min at 37 °C and 5% CO_2 and fluorescent signal F485/528 (directly proportional to proliferation) was measured. To evaluate the protective effect of preparations against oxidative stress cells were preincubated with different concentrations of extracts for 20 h. To induce the oxidative stress condition 500 μM t-BOOH was added for 2 h, then the cell viability and proliferation were measured.

2.10. Detection of reactive oxygen species

To determine the effect of cocoa bean extracts on the intracellular generation of ROS the DCFH assay was performed. Cells were treated with different doses of extracts for 20 h and after washing with PBS were loaded with the DCFH dye at a final concentration of 1 μM in serum-free medium for 40 min. Then cells were washed twice with PBS, fresh medium was added and fluorescent signal F485/528 was measured; intracellular ROS production was evaluated and expressed

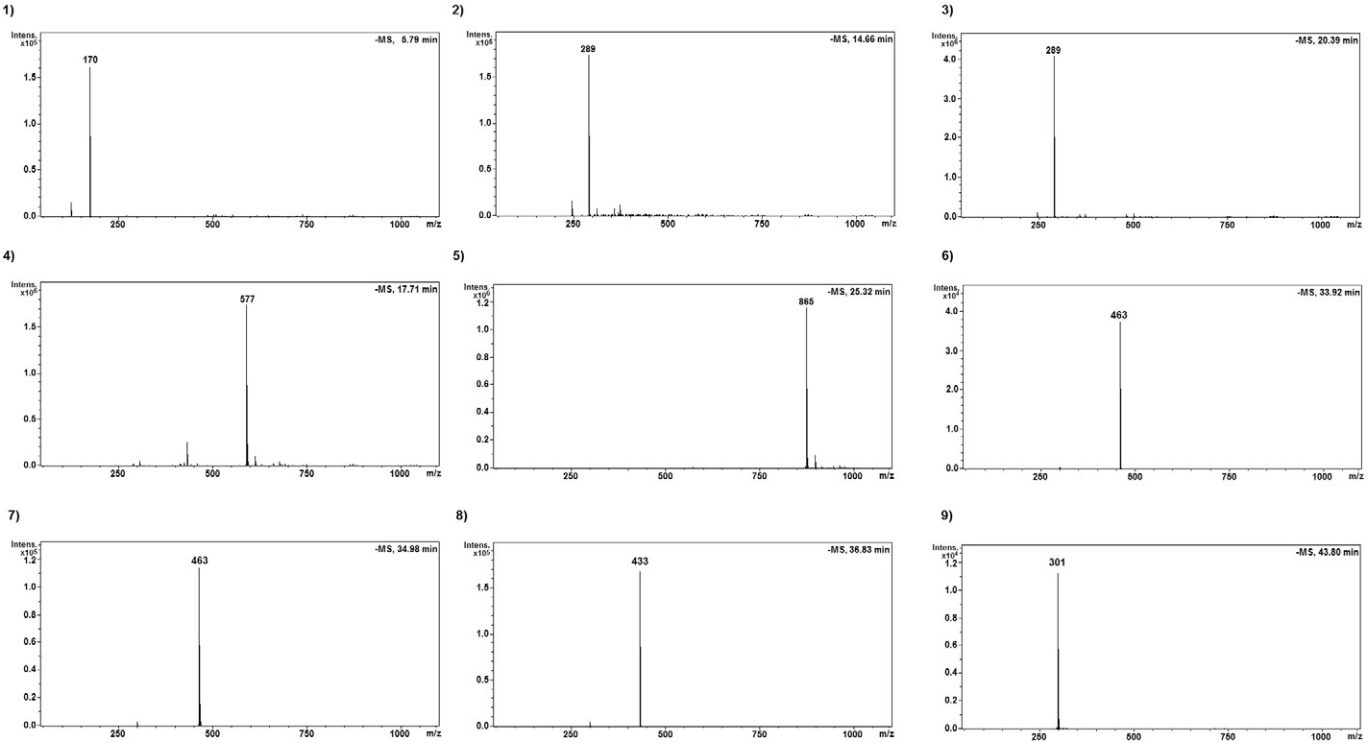

Fig. 2. Mass spectra on $[M–H]^-$ ion of gallic acid (peak 1) at m/z 171, (+)-catechin (peak 2) at m/z 289, (−)-epicatechin (peak 3) at m/z 289, procyanidin B2 (peak 4) at m/z 577, procyanidin C1 (peak 5) at m/z 865, quercetin 3-*O*-galactoside (peak 6) at m/z 463, quercetin 3-*O*-glucoside (peak 7) at m/z 463, quercetin 3-*O*-arabinoside (peak 8) at m/z 433, and quercetin (peak 9) at m/z 301.

as fluorescence % of control (cells without preparations). As a positive oxidative stress inducer 500 μM t-BOOH was used.

2.11. Nile red staining

To determine the influence of preparations on lipid droplet formation in HepG2 cells were cultured in RMPI medium supplemented with 30 mM glucose for 48 h. Then cells were incubated in FBS-free medium for 24 h with extracts in the presence of 400 μM oleic acid (Cui, Chen, & Hu, 2010). After treatment cells were washed with cold PBS and fixed in 10% paraformaldehyde for 10 min and stained with Nile red solution (1 μg/mL) for 30 min at 37 °C and 5% CO_2. Lipid-bound Nile red fluorescence was measured (F485/530) (Hwang et al., 2011).

2.12. Antioxidant activity

2.12.1. DPPH radical scavenging assay

The antioxidant capacity was determined using the DPPH radical scavenging assay according to Scherer and Godoy (2009). The test using aqueous suspension of CBEs and obtained from them fractions at concentrations of 0.06–0.3 g·100 g^{-1} was performed. Reaction between 0.2 mL of a suspension and 7.8 mL of methanolic radical solution prepared by dissolving 5 mg of DPPH radical in 100 mL of methanol was carried out. Methanol was used as a blank sample and 0.2 mL of water with 7.8 mL of methanolic radical solution as a control and absorbance of tested batches at 517 nm after a 30 min reaction in the darkness was measured. Each test was repeated three times and the IC_{50} value was calculated.

2.12.2. ABTS radical scavenging assay

The scavenging activity was assayed according to the method of Re et al. (1999) with modifications, for 0.1% methanol cocoa preparation (extract or fraction). ABTS was dissolved in distilled water to 7 mM a concentration. Then, this ABTS stock solution was mixed with 2.45 mM potassium persulfate (Solution I, prepared earlier) and left to stand in the dark at room temperature for 14 h before further use (Solution II). The reaction mixture contained 10 mL of Solution II and 0.2 mL of the sample. After incubation for 6 min at 30 °C, the absorbance was measured at 734 nm. The scavenging activity was measured according to the formula: $[(A_0 - A_1) / A_0] \times 100\%$, where A_0 is absorbance of the control (without cocoa preparation) and A_1 is the absorbance in the presence of the cocoa preparation. Test results are given as IC_{50} value.

2.12.3. Hydroxyl radical scavenging assay

The scavenging activity was assayed according to the method of Sudha, Priya, Shree, and Vadivukkarasi (2011), for different concentrations of cocoa extracts. The reaction mixture contained 3 mL of 1.5 mM $FeSO_4$, 2.1 mL of 6 mM hydrogen peroxide, 0.9 mL of 20 mM sodium salicylate and 3 mL of cocoa preparation (extract or fraction). After incubation for 1 h at 37 °C, the absorbance of the complex was measured at 562 nm. The scavenging activity was measured according to the formula: $[1 - (A_1 - A_2) / A_0] \times 100\%$, where A_0 is absorbance of the control (without examined cocoa preparation), A_1 is the absorbance in the presence of the cocoa preparation and A_2 is the absorbance without sodium salicylate. Test results are given as IC_{50} value.

2.13. Animal study

The experiment was conducted on 40 male Wistar rats randomly assigned to one of 5 groups of 8 rats each. The animals were maintained individually in metal cages under a stable temperature (21–22 °C), a 12-hour light:12-hour dark cycle and a ventilation rate of 15 air changes per hour. For 4 weeks, the rats had free access to water and semipurified casein diets composed on the basis of the AIN-93 diet (Reeves, 1997). The control (C) group was fed a diet containing among others 53% corn starch, 7% rapeseed oil and 5% cellulose, as the source of carbohydrates, fat and fiber, respectively. In the high-fat (HF) group, the diet was modified by the addition of palm oil (14% of the diet) at the expense of corn starch and cellulose. In the other three groups, the high-fat and low-fiber diet was additionally supplemented with the CBE from raw

cocoa bean (group D5, 2.25% of the diet), the CBE from roasted cocoa bean (group D6, 2.45% of the diet) or the monomeric flavan-3-ols fraction of CBE (group D2, 0.114% of the diet). The extracts were added at the expense of corn starch and the detailed composition of each diet is given in Table 1. The dietary polyphenol content in the D5, D6 and D2 groups was similar and equaled 73.1, 70.1 and 78.1 mg/100 g diet, respectively. Energetic values for experimental control and high-fat diets were estimated according to *Research Diets*, *Inc.* (New Brunswick, NJ, USA). The rats were used in compliance with the European guidelines for the care and use of laboratory animals, and the animal protocol was approved by the Local Institutional Animal Care and Use Committee (Olsztyn, Poland).

2.13.1. Sample collection and analysis

After 4 weeks of experimental feeding, the rats were weighed and anesthetized with sodium pentobarbital (50 mg/kg body weight). The body fat and lean mass was then determined by time-domain nuclear magnetic resonance using a minispec LF 90II analyzer (Bruker, Karlsruhe, Germany). Afterwards, the blood was collected from the caudal vein, whereas the liver was removed, weighed, immersed in liquid nitrogen and stored at −20 °C until analyses. The blood was then centrifuged for 15 min at 380 ×*g* and the obtained serum was stored at −70 °C until analyses.

In the blood serum, the antioxidant capacity of water-soluble and lipid-soluble substances (ACW and ACL, respectively) was determined by a photochemiluminescence detection method using a Photochem and respective kits (ACW-Kit and ACL-Kit, Analytik Jena AG, Germany). In the photochemiluminescence assay, the generation of free radicals was partially eliminated through reactions with antioxidants present in the plasma samples, and the remaining radicals were quantified by luminescence generation. Ascorbate and Trolox calibration curves were used to evaluate ACW and ACL, respectively. The serum concentration of glucose (GL), total cholesterol (TC), HDL cholesterol fraction (HDL-C) and uric acid were estimated using a biochemical analyzer (Horiba, Pentra C200, Kyoto, Japan). Based on the serum lipid profile, the atherogenic index (AI) was calculated using the following formula: [(TC − HDL) / HDL]. Non-HDL fraction was calculated by subtracting HDL-C from total cholesterol.

In the liver and kidneys, thiobarbituric acid-reactive substances (TBARS) were determined as an indicator of lipid peroxidation in this organ. A procedure developed by Botsoglou et al. (1994) was used in the assay, and the TBARS concentrations were determined spectrophotometrically at 532 nm and expressed in ng malondialdehyde per g of tissue. The liver concentration of reduced and oxidized glutathione (GSH and GSSG, respectively) were determined by using an enzymatic recycling method described by Rahman, Kode, and Biswas (2006).

Table 1
Composition of the diets fed to rats (%).

	Group[a]				
	C	HF	HF_{D5}	HF_{D6}	HF_{D2}
Casein	20	20	20	20	20
DL-methionine	0.3	0.3	0.3	0.3	0.3
Rapeseed oil	7	7	7	7	7
Palm oil	–	14	14	14	14
Cellulose	5	2	2	2	2
Sucrose	10	10	10	10	10
Mineral mix[3]	3.5	3.5	3.5	3.5	3.5
Vitamin mix[3]	1	1	1	1	1
Choline chloride	0.2	0.2	0.2	0.2	0.2
CBE from raw cocoa bean	–	–	2.25	–	–
CBE from roasted cocoa bean	–	–	–	2.45	–
Monomeric flavan-3-ols fraction of CBE	–	–	–	–	0.114
Corn starch	53.0	42.0	39.75	39.55	41.886
Gross Energy, MJ/kg	16.7	19.6	19.6	19.6	19.6

[a] C, control; HF, high-fat; HF_{D5}, high-fat supplemented with CBE from raw cocoa bean; HF_{D6}, high-fat supplemented with CBE from roasted cocoa bean; HF_{D2}, high-fat supplemented with monomeric flavan-3-ols fraction of CBE.

2.14. Statistical analysis

Analyses were carried out in triplicate, starting from cocoa bean roasting and preparing of CBEs. All obtained results were subjected to statistical analysis. The determination comprised of both, average values and one-way analysis of variance ANOVA using STATISTICA 10 software (StatSoft Inc., Tulsa, USA) at the significance level of $p \leq 0.05$.

3. Results and discussion

3.1. Characteristic of CBEs and their fractions

Freeze-dried CBE obtained from raw cocoa bean, CBE prepared from roasted cocoa bean and isolated from them fractions of following bioactive compound groups obtained by CPC: 1. monomeric flavan-3-ols, 2. procyanidins, 3. colored compounds from raw cocoa bean and 4. colored compounds from roasted cocoa bean were analyzed for the content of phenolic compounds and Maillard reaction products. The results of these determinations are summarized in Table 2.

Preserved by freeze drying CBEs before separation into fractions are characterized by a relatively low total concentration of phenolic compounds, among which the dominant group was group of flavan-3-ols. CBE from roasted cocoa bean contained more HMW Maillard reaction products than CBE from raw beans. As a result of the CBEs separation into fractions and their purification from contaminants via CPC technique followed by purification of the obtained fractions from methylxanthines four preparations (fractions) were obtained, which were further subjected to freeze drying: 1. monomeric flavan-3-ols fraction, 2. procyanidins fraction, 3. fraction of colored compounds from raw cocoa bean, and 4. fraction of colored compounds from roasted cocoa bean. All of obtained fractions possessed higher levels of bioactive compounds than the initial CBEs.

Antioxidant capacities of cocoa bean preparations, which *in vivo* may contribute to inhibition of lipid oxidation and reduction of hydroperoxide formation, have been assessed with DPPH•, ABTS• and OH^- assay (Table 3). The highest DPPH radical scavenging activity showed both monomeric flavan-3-ols, (1.50 mg of dw/mL) and procyanidins (1.64 mg of dw/mL) fractions. In contrast, colored compounds from roasted cocoa bean revealed as the weakest antioxidant agents (4.29 mg of dw/mL). Similar dependencies were obtained in ABTS tests. The highest ABTS radical scavenging activity showed both procyanidins and color compounds from raw cocoa bean fractions and the weakest the fraction of colored compounds from roasted cocoa bean. The highest hydroxyl radical scavenging activity showed CBE from roasted cocoa bean and fraction of color compounds from roasted cocoa beans (with MRP). CBE from raw cocoa bean exhibited a similar ability to scavenge hydroxyl radicals as fraction of color compounds from roasted cocoa beans. The hydroxyl radical is the most reactive of the reactive oxygen species (ROS), and it can be formed by a Fenton-type reaction in the presence of reduced transition metals and H_2O_2 (Galano, Macias-Ruvalcaba, Medina Campos, & Pedraza-Chaverri, 2010). Scavenging of hydroxyl radical is an important antioxidant activity because •OH radicals are very reactive species that can damage molecules of high biological importance like proteins and DNA. This is particularly important because the dysfunctions of cells caused by free radicals and reactive oxygen species is one of the factors contributing to aging process and cancer development, thus removing hydroxyl radical is very important for the protection of living systems (Sowndhararajan & Kang, 2013).

3.2. Cytoprotective activity against t–BOOH induced cytotoxicity and influence on the intracellular ROS generation

Liver plays crucial role in maintaining lipid and glucose homeostasis and its altered metabolism is strongly correlated with obesity and insulin resistance, which leads to type 2 diabetes (Chen, Cai, Huang, Meng, & Li, 2015). In the regulation of plasma glucose concentration also insulin

Table 2
Characteristic of CBEs and obtained from them fractions.

Chemical composition	CBE from raw cocoa bean (D5)	CBE from roasted cocoa bean (D6)	Monomeric flavan-3-ols fraction (D2)	Procyanidins fraction (D4)	Fraction of colored compounds from raw cocoa bean (D3)	Fraction of colored compounds from roasted cocoa bean (D1)
Phenolic compounds (mg/g dw)						
Flavan-3-ols						
(+)-Catechin	0.24 ± 0.01^{b}	0.47 ± 0.02^{a}	27.08 ± 0.03^{c}	–	–	–
(−)-Epicatechin	8.68 ± 0.04^{b}	6.35 ± 0.03^{a}	575.57 ± 0.11^{c}	–	–	–
Epigallocatechin	1.36 ± 0.01^{b}	0.34 ± 0.01^{a}	–	–	–	–
Procyanidin B2	7.59 ± 0.02^{b}	7.21 ± 0.02^{a}	–	242.25 ± 0.09^{c}	–	–
Procyanidin C1	2.44 ± 0.02^{b}	1.36 ± 0.01^{a}	–	149.14 ± 0.07^{c}	–	–
Other procyanidins	11.40 ± 0.05^{b}	10.41 ± 0.04^{a}	–	–	–	–
Flavonols						
Quercetin	0.01 ± 0.00^{a}	0.00 ± 0.00^{a}	0.33 ± 0.02^{b}	–	–	–
Quercetin 3-*O*-glucoside	0.30 ± 0.01^{a}	0.26 ± 0.01^{a}	4.07 ± 0.02^{b}	–	–	–
Quercetin 3-*O*-arabinoside	0.31 ± 0.01^{b}	0.27 ± 0.01^{a}	4.60 ± 0.01^{c}	–	–	–
Quercetin 3-*O*-galactoside	0.09 ± 0.01^{a}	0.08 ± 0.00^{a}	1.49 ± 0.01^{b}	–	–	–
Phenolic acid						
Gallic acid	–	1.85 ± 0.02^{a}	14.63 ± 0.04^{b}	–	–	–
Total phenolics	32.48	28.62	685.12	391.39	–	–
Colored compounds						
$K_{mix294\ nm}$ ($L \times g^{-1} \times cm^{-1}$)	1.38 ± 0.01^{b}	1.95 ± 0.02^{d}	–	–	1.45 ± 0.01^{c}	1.20 ± 0.02^{a}
$K_{mix420\ nm}$ ($L \times g^{-1} \times cm^{-1}$)	0.10 ± 0.01^{a}	0.10 ± 0.01^{a}	–	–	0.13 ± 0.01^{b}	0.16 ± 0.01^{c}
HMW Maillard reaction products (%)	56.41 ± 0.31^{b}	83.18 ± 0.41^{c}	–	–	42.49 ± 0.12^{a}	88.80 ± 0.52^{d}
Other compounds (%)	43.59 ± 0.14^{c}	16.82 ± 0.09^{b}	–	–	57.50 ± 0.16^{d}	11.19 ± 0.06^{a}

CBE — cocoa bean's extract; HMW — high molecular weight; dw — dry weight. [abcd] Mean values not sharing the same superscript letters within a row are significantly different at $p \leq 0.05$.

secreting pancreatic beta cells are highly involved (Zakłos-Szyda et al., 2015). They lose their function or undergo cell death due to chronic hyperglycemic conditions and excessive oxidative stress resulting from high levels of reactive oxygen species (ROS) or dysfunction of intracellular antioxidant defense (Martin, Ramos, Cordero-Herrera, Bravo, & Goya, 2013; Martin et al., 2008; Ortega et al., 2008).

In the current study we investigate the effects of cocoa bean preparations on human hepatoma HepG2 and mouse β-TC3 cell function as cytoprotective agents against oxidative stress. As *in vitro* oxidative stress inducer tert-butylhydroperoxide (t-BOOH) was used, which is metabolized via cytochrome p-450 or GSH oxidation with reactive oxygen species generation.

The effect of different preparation concentrations on cell viability was studied with Presto Blue® reagent. The results indicated that 24 h incubation with preparations caused a concentration-dependent reduction of cell metabolic activity. Via comparison of IC_{50} parameters the most active against beta cells is procyanidins fraction (0.91 ± 0.09 mg of dw/mL); the lowest cytotoxic potential possess the CBEs from raw and roasted cocoa bean (Table 4). In the case of HepG2 cells the preparation activity profile is maintained. Dosages obtained for both cell types are comparable. The highest nontoxic concentrations (IC_0) selected for further studies are summarized in Table 4. The IC_0 values ranged from 0.125 to 0.50 mg of dw/mL for full raw and roasted CBEs and monomeric flavan-3-ols fractions, and raw cocoa bean colored compounds respectively. In order to examine the preparations protection effects against t-BOOH oxidative damage cells were preincubated with the IC_0 dosages of extracts and the control samples were untreated cells. Adding 500 μM t-BOOH for 2 h decreased studied cell viability almost by 30%. All studied compounds reduced t-BOOH induced oxidative stress in both cell lines, although their higher cytoprotective activity was observed in case of HepG2 cell line. The lowest inhibition of metabolic activity by toxin was observed for cells previously preincubated with procyanidins. Additionally at the same time the effect of the extracts on beta cell proliferation was tested using CyQuant Proliferation Assay, which allows sample's DNA content measurement. The results showed that t-BOOH inhibits β-TC3 proliferation only by 15% comparing to untreated cells and cell preincubation with preparations practically did not affect DNA rate synthesis.

To evaluate whether cocoa preparations prevent the generation of oxidative stress the intracellular ROS production in living cells was quantified by measurement of DCF fluorescence. Cells treated with 500 μM t-BOOH showed a significant increase in ROS generation after 40 min (positive control) as compared to untreated cells (Fig. 3). The 20 h pretreatment of cells with the IC_0 dosages of preparations significantly reduced ROS generation in the presence of stress inducer to values that were intermediate between those of control and t-BOOH treated cells. In HepG2 all preparations reduced oxidative stress to the

Table 3
CBEs and their fractions as PTP1B inhibitors and their antioxidant capacities ($n \geq 3$).

Name of tested cocoa preparation	PTP-1B inhibition IC_{50} (mg dw of preparation/mL)	Antioxidant activity		
		IC_{50} (mg/1 μmol DPPH•)	IC_{50} (mg/1 μmol ABTS•)	IC_{50} (mg/1 μmol OH^{-})
CBE from raw cocoa bean (D5)	$1.12 \pm 0.01^{*}$	3.05 ± 0.11^{c}	0.31 ± 0.02^{c}	1.32 ± 0.02^{b}
CBE from roasted cocoa bean (D6)	$0.83 \pm 0.07^{*}$	3.48 ± 0.23^{d}	0.22 ± 0.01^{b}	1.02 ± 0.02^{a}
Monomeric flavan-3-ols fraction (D2)	$1.21 \pm 0.04^{*}$	1.50 ± 0.04^{a}	0.25 ± 0.01^{b}	1.97 ± 0.02^{e}
Procyanidins fraction (D4)	$1.05 \pm 0.34^{*}$	1.64 ± 0.05^{a}	0.11 ± 0.0^{a}	1.79 ± 0.03^{d}
Fraction of colored compounds from raw cocoa bean (D3)	$0.91 \pm 0.19^{*}$	2.78 ± 0.07^{b}	0.10 ± 0.01^{a}	1.68 ± 0.03^{c}
Fraction of colored compounds from roasted cocoa bean (D1)	$0.61 \pm 0.10^{*}$	$4.29 \pm 0,14^{e}$	0.59 ± 0.03^{d}	1.29 ± 0.01^{b}

CBE — cocoa bean's extract; dw — dry weight; Data are expressed as the mean of triplicates ± standard deviation. [abcde] Mean values not sharing the same superscript letters within a column are significantly different at $p \leq 0.05$. * Indicates $p < 0.05$ compared with the control cells.

Table 4
Biological activity of CBEs and their fractions against β-TC3 and HepG2 cells (n ≥ 9).

Name of tested cocoa preparation	β-TC3				HepG2		
	IC_0 (mg dw of preparation/mL)[a,b]	IC_{50} (mg dw of preparation/mL)[a,b]	% of cell viability[c]	% of cell proliferation[d]	IC_0 (mg dw of preparation/mL)[a]	IC_{50} (mg dw of preparation/mL)[a,b]	% of cell viability[c]
500 μM t-BOOH[e]			68.26 ± 6.02*	82.68 ± 2.35*			67.44 ± 2.56*
CBE from raw cocoa bean (D5)	0.125	>7.50	80.25 ± 1.15*	88.50 ± 4.27*	0.25	>7.50	97.13 ± 7.31
CBE from roasted cocoa bean (D6)	0.125	>7.50	77.96 ± 2.41*	87.16 ± 2.02*	0.25	>7.50	100.47 ± 6.41
Monomeric flavan-3-ols fraction (D2)	0.125	1.88 ± 0.09*	79.21 ± 3.02*	89.18 ± 1.20*	0.25	1.92 ± 0.12*	102.29 ± 7.45
Procyanidins fraction (D4)	0.25	0.91 ± 0.19	83.81 ± 3.41*	88.91 ± 3.22*	0.50	1.55 ± 0.32	88.27 ± 5.33*
Fraction of colored compounds from raw cocoa bean (D3)	0.25	3.01 ± 0.25*	80.13 ± 1.14*	83.11 ± 0.91*	0.25	2.10 ± 0.07	91.74 ± 2.11*
Fraction of colored compounds from roasted cocoa bean (D1)	0.50	1.86 ± 0.14*	76.34 ± 1.05*	84.10 ± 0.09*	0.50	1.93 ± 0.08*	91.28 ± 8.10

[a] Values were determined by regression analyses and expressed as mean ± SD, n ≥ 6.
[b] The influence of tested compounds was measured with PrestoBlue assay.
[c] The influence of cells preincubation with maximal nontoxic extract concentrations (IC_0) for 20 h against oxidative stress induced by 500 μM t-BOOH on metabolic activity measured with PrestoBlue assay.
[d] The influence of cells preincubation with maximal nontoxic extract concentrations (IC_0) for 20 h against oxidative stress induced by 500 μM t-BOOH on proliferation measured with CyQuant assay.
[e] The influence of 2 h incubation of cells with 500 μM t-BOOH on metabolic activity or proliferation.
* Indicates $p < 0.05$ compared with the control cells.

level comparable to untreated cells, which shows that studied preparations prevent conditions favoring intracellular oxidative stress. In the case of beta cells CBEs were the strongest in prevention of the enhanced ROS generation, although even activities of colored compounds fractions were at similar level. These observations are consistent with the data reported by Martin et al. (2013), in which chemoprotective effect on Ins-1E pancreatic beta cells of a cocoa phenolic extract against oxidative stress induced by t-BOOH was observed. Furthermore, these experiments revealed that with progressive oxidative stress the level of GSH glutathione decreases remarkably and cellular pretreatment with cocoa phenolic compounds evoked significant recovery of GSH from oxidized glutathione. Comparable results were observed during studies performed with hepatic HepG2 cells (Martin et al., 2008). In particular, the cocoa polyphenolic extract increased the activity of GPx and glutathione reductase (GR) via extracellular regulated kinase (ERK) activation.

3.3. PTP1B inhibition activity and lipid droplet accumulation

It is known that insulin enhances glucose uptake in muscle and adipose tissue and reduces gluconeogenesis and lipolysis (Elchebly et al., 1999). Protein tyrosine phosphatase 1B is a cytosolic enzyme which modulates insulin sensitivity and carbohydrate metabolism. In the insulin signaling pathway PTP1B dephosphorylates the insulin receptor and the insulin receptor substrate IRS-1 disturbing signal transition from insulin, thereby PTP1B suppressors are established as potential therapeutic agents in the treatment of type 2 diabetes mellitus. Although many synthetic inhibitors of PTP1B are known among phenolic compound extracts so far there have been only few identified (Bower, Hernandez, Berhow, & Gonzalez de Mejia, 2014; Zakłos-Szyda et al., 2015). The activity of PTP1B was determined with the use of assay based on the detection of fluorescence emitted from fluoro-phospho-substrate due to its dephosphorylation catalyzed by the enzyme. All of the cocoa bean preparations studied (Table 3) had the ability of PTP1B inhibition with the IC_{50} values between 0.61–1.21 mg of dw/mL, where the lowest IC_{50} indicates the strongest inhibition potential. Protein tyrosine phosphatase 1B inhibition by total polyphenolic extract (CBE) activity is comparable with the results obtained for other plants rich in flavanols (e.g. Japanese quince, Guelder rose) (Zakłos-Szyda et al., 2015). Taking into account obtained preparation the order of the most potent PTP1B inhibitors was as follows: fraction of colored compounds from roasted

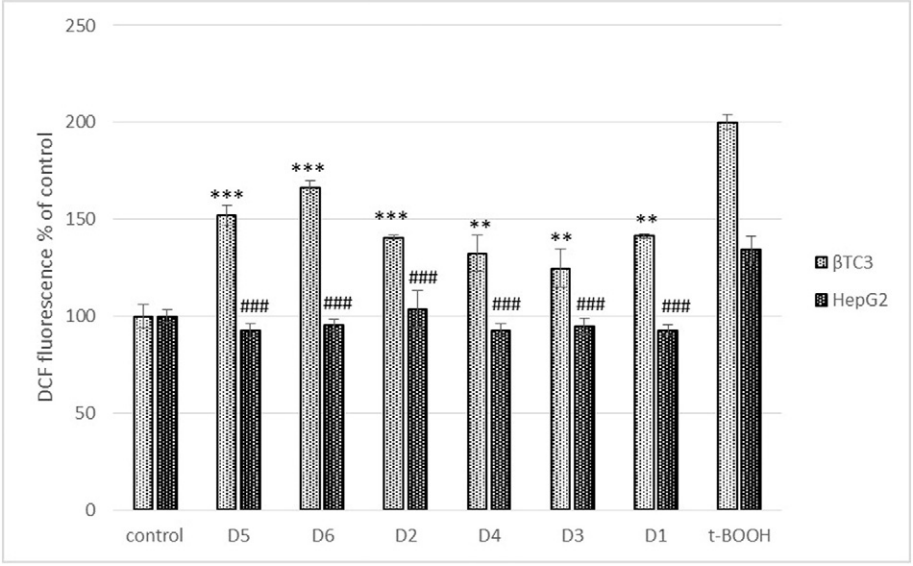

Fig. 3. Effect of *Forastero* preparations on intracellular ROS generation. β-TC3 and HepG2 cells were incubated with IC_0 of the preparations for 20 h and intracellular ROS production was evaluated after 40 min incubation with DCFH. Data represent the means ± SD from at least nine independent experiments vs untreated control cells (100%); *indicates $p < 0.05$ compared with the control cells; # indicates $p < 0.05$ compared with 500 μM t-BOOH treated cells.

cocoa bean (D1) > CBE from roasted cocoa bean (D6) > fraction of colored compounds from raw cocoa bean (D3) > procyanidins fraction (D4) > CBE from raw cocoa bean (D5) > monomeric flavan-3-ols fraction (D2). The observed biological effects of cocoa preparations depend on the structure and concentration of the compounds — in the fractions with the highest PTP1B inhibition activity there are MRPs present. There is also correlation between bioactivity and degree of polymerization: catechin monomers are less active then procyanidins. These observations are consistent with Dorenkott report (Dorenkott et al., 2014), which proved that among cocoa flavanol monomers, oligomers, and polymers the oligomer-rich fraction was the most effective in preventing weight gain, fat mass, impaired glucose tolerance, and insulin resistance in long-term high fat mice feeding study. However studies performed by Bitzer (Bitzer et al., 2015) on anti-inflammatory activities in models of colonic inflammation revealed that oligomeric procyanidins appeared to be the least effective. Recent data indicates that procyanidins exhibit selective effects between the different members of cellular protein phosphatases (Stadlbauer, Rios, Ohmori, Suzuki, & Köhn, 2015), thus relationship between the degree of flavanol polymerization and bioactivity appears to be system dependent.

Obesity is associated with increased flux of free fatty acids into the circulation, thereby leading to enhanced uptake of the fatty acids into multiple tissues, including the liver (Chen et al., 2015). More recent studies have revealed PTP1B as a new activator for hepatic lipogenesis essential for activation of SREBP1 transcription factor expression and fatty-acid and triglyceride synthesis. Elevated level of PTP1B is observed in mice fed a high-fat diet, but also in patients with nonalcoholic fatty liver disease (NAFLD), which is characterized by the accumulation of triglycerides in hepatocytes causing damage to the liver by forming fat droplets within the hepatic tissue (Cui et al., 2010). In addition, cytokines and reactive oxygen species create a pro-oxidant state that can activate stellate cells to produce fibrotic scar tissue. To evaluate whether cocoa preparations at IC_0 dosages prevent lipid accumulation HepG2 cells were coincubated for 24 h with 400 μM oleic acid. Intracellular lipid vacuole level was confirmed fluorimetrically with Nile red staining (Fig. 4). The increase in lipid droplets in HepG2 cells induced by oleic acid was reduced by 20% in the presence of fraction of colored compounds from roasted cocoa bean (D1), which is consistent with its PTP1B inhibition potential. According to the literature PTP1B inhibition diminishes hepatic expressions of genes involved in lipid and cholesterol synthesis, such as SREBPs, fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC) and 3-hydroxy-3-methylglutaryl-coenzyme A synthase 1 (HMGCS1) (Fabbrini, Sullivan, & Klein, 2010; Owen et al., 2013).

3.4. Animal study

The daily dietary intake was the highest in the control group ($p \le 0.05$ vs. other groups except HF_{D2}), while the lowest in the HF_{D6} treatment ($p \le 0.05$ vs. C and HF; Table 5). The body weight gain (BWG) of rats from HF dietary treatment was the highest among treatments ($p \le 0.05$ vs all other groups). Additionally, the BWG of HF_{D2} rats was significantly higher than that of rats from groups C, HF_{D5} and HF_{D6}. The final BW of rats from HF and HF_{D2} dietary treatments was significantly higher than that of rats from groups C, HF_{D5} and HF_{D6}. The rats fed high-fat diet without cocoa extract supplementation were characterized by the highest relative content of fat tissue ($p \le 0.05$ vs C and HF_{D6}) and the lowest relative content of lean tissue ($p \le 0.05$ vs C, HF_{D5} and HF_{D6}) in the body.

The HF treatment was characterized by the lowest HDL-C serum concentration ($p \le 0.05$ vs HF_{D5}) and HDL-C profile value as well as the highest value for atherogenic index [(TC-HDL)/HDL] (in both latter parameters $p \le 0.05$ vs C and HF_2). The lowest serum concentrations of TC and non-HDL fraction were noted in the HF_{D2} group. The highest values for serum ACW and ACL followed the dietary treatments with extract D5 and D2, respectively. The statistical tendency towards higher serum concentration of uric acid was observed in group HF_{D5} ($p = 0.091$ vs HF). The TBARS concentration in the kidney tissue was significantly reduced by consumption the diet with D6 cocoa extract in comparison to the rats from HF group. The highest value for liver TBARS concentration was found in the HF_{D6} group, but it differed statistically at $p \le 0.05$ only from the control C rats. The GSH:GSSG ratio in the liver tissue was found to be highest in the HF_{D2} group ($p \le 0.05$ vs all other treatments).

According to the accepted hypotheses, the results obtained in *in vivo* experiment clearly showed that all the cocoa extracts D2, D5, and D6 positively modulated physiological rat response but they acted through different metabolic ways. The dosage of polyphenols used in the present study, ca 15 mg/kg of BW, may reflect a consumption of 0.6 g cocoa polyphenols by an adult weighing 65 kg (calculated using the Body Surface Area method). The health promoting properties of ingested cocoa is well known (Corti, Flammer, Hollenberg, & Luscher, 2009; Mathur, Devaraj, Grundy, & Jialal, 2002; Vinson et al., 2006), but it seems to be of paramount importance to get the knowledge what biologically active

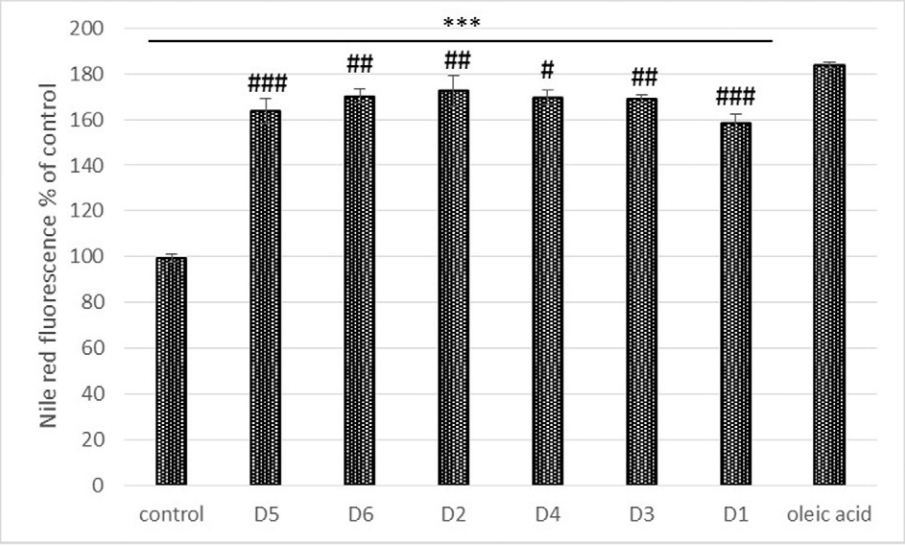

Fig. 4. Effect of *Forastero* preparations on the lipid accumulation in HepG2 cells. Cells were incubated with IC_0 of the preparations for 24 h with intracellular lipid vacuole inducer — 400 μM oleic acid. Lipid accumulation was determined by Nile red fluorimetric assay. Data represent the means ± SD from at least nine independent experiments vs untreated control cells (100%); *indicates $p < 0.05$ compared with the control cells; # indicates $p < 0.05$ compared with the oleic acid treated cells.

Table 5
Diet intake, body weight (BWG), body fat/lean mass and indices of serum lipid profile and antioxidant status in rats (for all parameters n = 8).

	Group[1]					Pooled SEM	p value
	C	HF	HF_{D5}	HF_{D6}	HF_{D2}		
Diet intake (g/day)	20.0^{a}	19.8^{b}	18.9^{bc}	18.5^{c}	19.5^{abc}	0.167	0.006
BWG (g)	42.2^{c}	66.1^{a}	46.0^{c}	40.0^{c}	56.4^{b}	2.111	<0.001
Relative fat tissue (% of BW)	31.0^{b}	34.2^{a}	32.5^{ab}	31.9^{b}	32.9^{ab}	0.349	0.007
Relative lean tissue (% of BW)	40.0^{a}	34.8^{c}	39.0^{ab}	40.0^{a}	36.5^{bc}	0.523	0.003
Serum parameters							
GL (mmol/L)	9.57	10.1	10.6	9.09	9.71	0.285	0.156
TC (mmol/L)	1.93^{ab}	1.93^{ab}	2.09^{a}	2.11^{a}	1.81^{b}	0.037	0.015
HDL-C (mmol/L)	0.95^{ab}	0.81^{b}	0.98^{a}	0.95^{ab}	0.86^{ab}	0.025	0.044
HDL-C profile (% of TC)	49.1^{a}	41.4^{b}	46.8^{ab}	45.2^{ab}	47.7^{a}	0.925	0.015
Non-HDL-C (mmol/L)	0.98^{bc}	1.12^{ab}	1.11^{abc}	1.16^{a}	0.95^{c}	0.027	0.016
(TC-HDL)/HDL index	1.05^{b}	1.45^{a}	1.18^{ab}	1.24^{ab}	1.11^{b}	0.050	0.040
ACW (μg/mL)	4.01^{b}	3.69^{b}	6.01^{a}	4.51^{ab}	5.45^{ab}	0.285	0.015
ACL (μg/mL)	18.1^{b}	18.1^{b}	18.6^{b}	18.6^{b}	21.7^{a}	0.465	0.018
Uric acid (μmol/L)	29.7	23.4	36.9	24.6	26.0	2.215	0.091
Kidney parameter							
TBARS[3] (ng/g)	846^{ab}	896^{a}	817^{ab}	690^{b}	861^{ab}	29.64	0.050
Liver parameters							
Mass (g/100 g BW)	3.52	3.68	3.43	3.35	3.61	0.049	0.065
TBARS[3] (ng/g)	408^{b}	462^{ab}	432^{ab}	516^{a}	412^{b}	13.51	0.018
GSH (μmol/g)	44.4	49.9	46.9	46.2	51.9	1.207	0.084
GSSG (μmol/g)	5.24^{b}	6.37^{ab}	8.37^{a}	5.41^{b}	3.12^{c}	0.421	<0.001
GSH:GSSG ratio	8.55^{bc}	9.84^{b}	5.79^{c}	8.64^{bc}	17.1^{a}	0.789	<0.001

^{abc} Mean values not sharing the same superscript letters within a row are significantly different at $p \leq 0.05$. ACW, antioxidant capacity of water-soluble substances; ACL, antioxidant capacity of lipid-soluble substances; BWG, body weight gain; GL, glucose; GSH, reduced glutathione; GSSG, oxidized glutathione; HDL, HDL-cholesterol; non-HDL, non-HDL-cholesterol; SEM, standard error of the mean; TBARS, thiobarbituric acid-reactive substances; TC, total cholesterol.

[1] C, control; HF, high-fat; HF_{D5}, high-fat supplemented with CBE from raw cocoa bean; HF_{D6}, high-fat supplemented with CBE from roasted cocoa bean; HF_{D2}, high-fat supplemented with monomeric flavan-3-ols fraction of CBE.

compounds from cocoa bean are responsible for those beneficial effects. Some of the authors point at proanthocyanidins (Tomaru et al., 2007) but recently more and more attention has been paid to Maillard reaction products (MRP) and their antioxidant activity (Amarowicz, 2009). As expected even though HF rats ate less food (vs control), the rats on HF diet gained significantly more, and it was the fat tissue at the expense of lean one. The reason was that one gram of the HF diet provided far more energy that the control one. Among the experimental cocoa groups, the final BWG of rats from HF_{D5} and HF_{D6} but not those from HF_{D2} was comparable to the control. These findings were also reflected in the body composition, since the percentages of fat and lean tissue in HF_{D2} rats did not differ statistically in comparison to the HF group. This points that cocoa MRP acts better as preventing agents against high-fat diet-induced obesity than polyphenolic compounds. Matsui et al. (2005) proposed that ingested cocoa can prevent obesity by modulating lipid metabolism, especially by decreasing fatty acid synthesis and transport systems and enhancement of part of the thermogenesis mechanism in liver and white adipose tissue. In fact in our experiment the consumption of cocoa extracts positively modulated blood serum HDL-cholesterol concentration as well as dietary atherogenic index (TC-HDL)/HDL. Some other authors reported beneficial effects of cocoa extracts on lipid profile in hypercholesterolemic (Lecumberri et al., 2007) and diabetic (Ruzaidi, Amin, Nawalyah, Hamid, & Faizul, 2005) rats, but without pointing at most active cocoa compounds. By comparing the experimental treatments it could be also asserted that cocoa polyphenols, monomeric flavan-3-ols, predominantly as compared to proanthocyanidins and MRP are responsible for beneficial changes in the lipid profile.

It has been reported that oxidative stress and reduced antioxidant defense play a crucial role in pathogenesis of several diseases (Corti et al., 2009). In the case of cocoa properties, not only polyphenols but also other macronutrients and micronutrients in addition to flavanols may affect the total antioxidative capacity of body tissues (Corti et al., 2009; Sies, 2007). Although the results of a present study showed that the high-fat dietary treatment did not strongly disturbed the antioxidant defense of the body, the treatments with cocoa extracts beneficially improved several parameters describing multidirectional mechanisms associated with final antioxidative properties of the product. Among cocoa groups, the extract obtained from raw cocoa beans (D5) enhanced serum ACW and uric acid concentration while the D6 extract from roasted beans beneficially lowered TBARS values in kidney tissue (please note but not in the liver). But the most evident antioxidant action followed the consumption of the D2 extract containing the monomers of flavan-3-ols including epicatechin and catechin, and no MRP. The HF_{D2} treatment was accompanied by most desirable values for serum ACL, liver TBARS, and liver GSH:GSSG ratio, as compared to other experimental treatments.

4. Conclusions

In the present study extracts obtained from raw and roasted cocoa bean of *Forastero* variety (full, fractionated and purified) were screened as potential protein phosphatase PTP1B inhibitors and protective agents against oxidative stress in human hepatoma HepG2 and mouse insulinoma β-TC3 cells. Additionally their influence on oxidative stress and lipid metabolism in rats fed high-fat diet was studied. Cocoa bean polyphenolic content is already known but for the first time we have characterized full and fractioned cocoa preparations via CPC method. It is known that thermal treatment during cocoa bean processing causes the formation of products of the Maillard reactions — melanoidins and despite the documented antidiabetic and antihypertensive potential of *T. cacao* no previous report has been given on the anti-obesity properties of pigment's fraction of roasted beans (with melanoidins). The results clearly showed that the separate mechanisms leading to beneficial effects upon dietary administration of cocoa bean extracts could be ascribed to different biologically active compounds of the extract, e.g. MRP (decreased fat tissue, PTP1B inhibition, hepatic steatosis attenuation, protection against oxidative stress) or flavan-3-ol monomers (improved serum lipid profile, increased serum ACL and liver GSH:GSSG ratio). It is worth mentioning that roasting processing of cocoa beans did not diminished the health promoting properties of the cocoa extracts. Quite the opposite, this study for the first time reports potential anti-obesity properties of roasted cocoa bean extract rich in MRP, which makes this extract as promising candidate for obesity prevention and associated metabolic disorder. The results presented

in this article are being the base for our further studies to estimate an influence of cocoa preparations, especially MRP, on the expression of genes involved in the metabolism of carbohydrates and lipids in human cell cultures.

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