

Green and roasted coffee extracts as antioxidants in β TC3 cells with induced oxidative stress and lipid accumulation inhibitors in 3T3L1 cells, and their bioactivity in rats fed high fat diet

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Abstract Chlorogenic acids from coffee show high antioxidant activity that contributes to inhibition of oxidative stress, which results in anticancer, antidiabetic and antiobesity coffee activities. Coffee roasting causes their partial decomposition and partial incorporation into Maillard reaction products also of high antioxidant activity, and it is still not clear whether more favorable impact on health is associated with the consumption of green or roasted coffee. The aim of the study was to evaluate the impact of coffee roasting process and also of the degree of chlorogenic acid esterification on bioactivity of coffee extracts. Extracts of green and roasted coffee and their fractions containing mono- and dichlorogenic acids (six extracts) were obtained by purification and optional fractionation of crude extracts using preparative centrifugal partition chromatography technique. The extracts were evaluated *in vitro* in terms of scavenging capacity, influence on lipids accumulation in 3T3L1 adipocytes, cytoprotective potential decreasing oxidative stress induced by cellular pro-oxidants in β TC3 cells, and *in vivo* by selected physiological indices in rats fed diets supplemented with the extracts. Roasted coffee extracts, in particular hydrophilic fraction containing monochlorogenic acids and Maillard reaction products, showed the highest scavenging capacity and contributed to the

highest level of antioxidants in the hydrophilic fraction of rats serum and may be potentially used as anticancer nutraceutical with oxidative stress inhibitor activity. Non-fractionated green coffee extract exhibited antidiabetic activities including inhibition of adipogenesis, while *in vivo* it significantly lowered the levels of triglycerides, glucose and oxidized glutathione.

Keywords Green and roasted coffee · Chlorogenic acids · Dichlorogenic acids · 3T3L1 · β TC3 · Rat model · Antidiabetic activity

Introduction

Green coffee contains hydroxycinnamic acids, such as caffeic and ferulic acid, mainly in a form of mono- and diesters with quinic acid, referred to as chlorogenic acids (CHAs) [1, 2]. CHAs exhibit a broad range of biological activities: antibacterial, antifungal, hepatoprotective, antithrombotic, antiinflammatory and antioxidant activities [3–8]. Antioxidant activity of CHAs decrease the risk of several oxidative stress-related diseases, including atherosclerosis, some kinds of cancer and Alzheimer's disease [9–11]. Consumption of coffee containing CHAs and other bioactive compounds also helps to fight against obesity and limits the effects of type 2 diabetes [12, 13]. The antioxidant activity of particular CHAs is similar, although diesters containing two caffeic acid molecules and hence four hydroxyl groups may have stronger antioxidant activity than monoesters. Roasting coffee causes significant losses of CHAs largely resulting from their incorporation into high molecular mass material, including melanoidins [14]. On the basis of available studies it is not clear, if green or roasted coffee is more beneficial for health, because both chlorogenic acids and

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products of their changes exhibit many bioactivities [15]. There is also no available research if the mixture of all coffee CHAs or fractions like more hydrophilic monoesters or more hydrophobic diesters are more bioactive. Dichlorogenic acids of green or roasted coffee were the subject of very few studies evaluating their in vitro and in vivo antioxidant and pro-healthy activities, although they might have remarkably high biological potential and could be used to obtain dietary supplements or food additives with strong health-promoting benefits.

The objective of the study was to evaluate the antioxidant activity (AA) of two fractions of coffee CHAs, i.e., monochlorogenic acids (MCHAs) and dichlorogenic acids (DiCHAs) from green and roasted coffee as radical scavengers, adipogenesis inhibitors and suppressors of oxidative stress in pancreas, and also in rats fed a high fat diet. Some amounts of soluble fiber and Maillard reaction products were left in fractions to include their bioactivities, but unlike most available studies the caffeine was removed from the extracts.

Materials and methods

Chemicals and raw materials

Analytical-grade ethanol, ethyl acetate and 30% hydrogen peroxide were purchased from Poch (Gliwice, Poland); HPLC-grade formic acid and 2,2-diphenyl-1-picrylhydrazyl (DPPH[•]) were purchased from Fluka (St. Louis, MO, USA); sodium salicylate (98%), 5-caffeoylquinic acid (99%), caffeic acid (99%), ferulic acid (99%), 3,4-dicaffeoylquinic acid (98%) and benzoic acid (99%) from Sigma Aldrich (St. Louis, MO, USA); 3-caffeoylquinic acid (99%), 4-caffeoylquinic acid (99%), 3,5-dicaffeoylquinic acid (99%) and 4,5-dicaffeoylquinic acid (99%) from PhytoLab (Vestenbergsgreuth, Germany); and nylon filters from Chromacol (Herts, UK). Ultrapure water (resistivity, 18.2 M Ω cm) was obtained from a Millipore Milli-Q Plus purification system (Bedford, MA, USA).

All cell culture reagents were obtained from the Life Technologies (Carlsbad, CA, USA), PrestoBlue cell viability reagent from Life Technologies (Van Allen Way, CA, USA) and tissue culture plastics from Greiner Bio-One GmbH (Frickenhausen, Austria). Mouse insulinoma β TC3 cells were purchased from Leibniz Institute DSMZ—German Collection of Microorganisms and Cell Cultures (Leibniz, Germany) and mouse 3T3L1 preadipocytes were purchased from ATCC—American Type Culture Collection (Manassas, VA, USA).

Green Robusta coffee beans (*Coffea canephora* L.) harvested in Brazil in 2014 and hulled by the dry method were purchased from Bero Polska (Gdynia, Poland).

Coffee beans roasting

Roasting was conducted in a convection automatic roaster CBR-101 with a load capacity of 200 g (Gene Café, Gyeonggi-Do, Korea). The temperature of roasting was 230 °C and the time was adjusted to achieve a weight loss of 17% and amounted to 12 min [16].

Obtaining, purification and fractionation of coffee extracts

Aqueous extracts from green and roasted coffee beans were obtained at 110 °C for 10 min in overpressure conditions as previously described [17]. The solutions were frozen at -80 °C, freeze-dried in a DELTA 1-24LSC Christ freeze drier (Osterode am Harz, Germany) and purified by centrifugal partition chromatography (CPC) with SPOT Prep II 50 chromatograph (Armen Instrument, Saint-Avé, France) integrated with UV/VIS detector and a fraction collector. Briefly, the two-phase system of solvents was prepared from water, ethanol and ethyl acetate (5:1:4, v/v/v). Elution of CHAs for both green and roasted coffee extracts occurred from 19 to 24 min (hydrophobic fraction) and from 27 to 31 min (hydrophilic fraction) of the analysis, detected by high UV absorption at 320 nm (Fig. 1). The fractions were collected and analyzed separately or combined after another run.

The fractions and the whole purified extracts were concentrated in a ScanMaxiVac concentrator Labogene

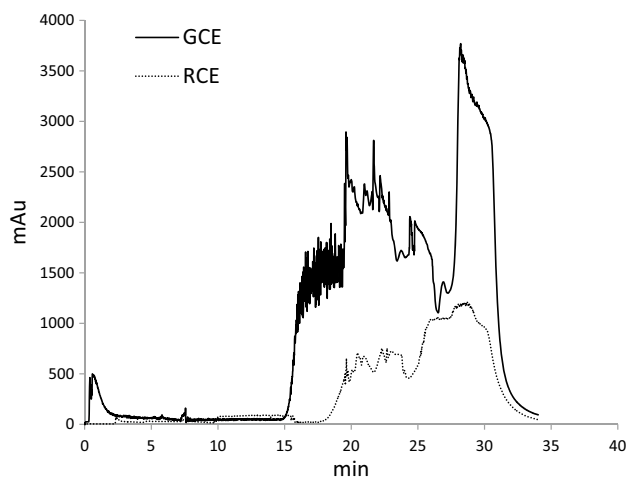
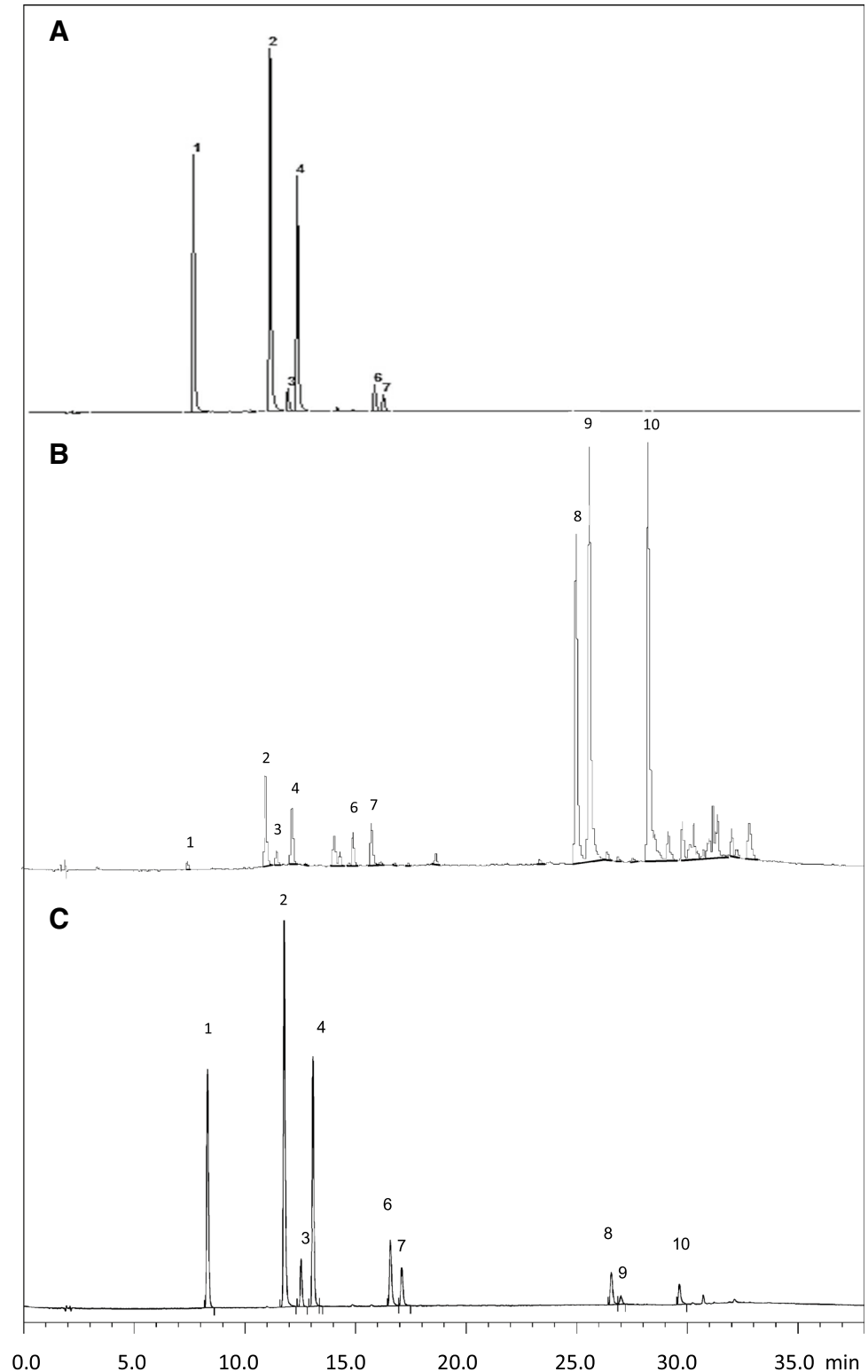


Fig. 1 Centrifugal partition chromatogram (CPC) of crude aqueous green (GCE) and roasted (RCE) coffee extracts (Budryn et al. [2]). CHAs detected by high UV absorption at 320 nm eluted from 19 to 24 min (less hydrophilic) are denoted as dichlorogenic acids (DiCHAs) and from 27 to 31 min (more hydrophilic) as monochlorogenic acids

(Lyngø, Denmark) and again freeze-dried. The profile of CHAs in fractions of both green and roasted coffee extracts was analyzed by HPLC–UV/MS method with details in accordance with the previous study [2]. Briefly, solutions of extracts in ultrapure water (20 mg/mL) with

an added internal standard (benzoic acid, 1 mg/mL) were filtered through a 0.20 µm nylon syringe filter. Chromatographic analysis was carried out using UHPLC⁺ Ultimate 3000 system with a UV/DAD detector from Dionex (Sunnyvale CA, USA) (Fig. 2) and with an ultrahigh

Fig. 2 HPLC chromatogram detected by UV absorption at 320 nm of two fractions and whole green coffee extract containing chlorogenic acids designated as **a** monoesters (MGCE); **b** diesters (DiGCE); **c** whole extract (GCE); **1** 3-caffeoylquinic acid (3-CQA); **2** 5-CQA; **3** 3-feruloylquinic acid (FQA); **4** 4-CQA; **6** 5-FQA; **7** 4-FQA; **8** 3,4-diCQA; **9** 3,5-diCQA; **10** 4,5-diCQA. MSⁿ spectra were analyzed to identify the chlorogenic acids [2]



resolution hybrid quadrupole/time-of-flight mass spectrometer (UHR-Q-TOF-MS/MS, Bruker Daltonics GmbH, Bremen, Germany) using an ESI source [2, 18]. As a result of purification and fractionation, six different extracts were obtained. They included: green coffee extract (GCE), hydrophilic fraction of monochlorogenic acids from green coffee extract (MGCE), hydrophobic fraction of dichlorogenic acids from green coffee extract (DiGCE), roasted coffee extract (RCE), hydrophilic fraction of monochlorogenic acids from roasted coffee extract (MRCE) and hydrophobic fraction of dichlorogenic acids from roasted coffee extract (DiRCE). Additionally in extracts, the concentration of soluble dietary fiber was determined according to the AOAC 991.19 official method. Coffee dietary fiber included mostly hemicelluloses or Maillard reaction products in case of green and roasted coffee, respectively.

Radical-scavenging capacity

Scavenging capacity of DPPH radicals was determined as described by Scherer et al. [19]. The test was carried out using aqueous solutions of extracts with concentrations in the range of 0.5–2.0 mg/mL. 0.1 mL of each solution was reacted with 3.9 mL of methanolic radical solution (DPPH':methanol, 5 mg/100 mL). 0.1 mL of water with 3.9 mL of methanol was used as a blank sample or with 3.9 mL of methanolic DPPH' solution as a control. The absorbance at 517 nm was measured (UV/VIS spectrophotometer U-2800 A Hitachi, Tokyo, Japan) after 30 min. Subsequently, a calibration curve of sample concentration versus scavenging capacity (AA%) was obtained, where $AA\% = [(A_{\text{control}} - A_{\text{test}}) / A_{\text{control}}] \times 100\%$. Then the concentrations of extracts, at which the initial radical form was scavenged by 50% ($IC_{50} = AA_{50\%}$), were calculated.

The scavenging capacity of hydroxyl radicals (OH^{\cdot}) by extracts was performed according to Sudha et al. [20]. Briefly, 1 mL of 1.5 mM iron (II) sulfate was combined with 0.7 mL of 6 mM hydrogen peroxide, 0.3 mL of 20 mM sodium salicylate and 1 mL of extracts with concentrations from 0.5 to 2.0 mg/mL. Samples were incubated for 1 h at 37 °C and the absorbance was measured at 562 nm. Scavenging capacity was calculated as $AA\% = [1 - (A_1 - A_2) / A_0] \times 100$, where A_0 is the absorbance of the control (with water instead of extracts), A_1 —in the presence of extracts, and A_2 —with water instead of sodium salicylate.

Viability assay of β TC3 cell culture oxidized with *t*-BOOH

Mouse insulinoma β TC3 cells were maintained at 37 °C in a humidified incubator containing 5% CO_2 and 95% air.

They were grown in Dulbecco's modified Eagle's medium (DMEM; D5030; Sigma Aldrich; St. Louis, MO, USA) with 25 mM glucose, supplemented with 15% horse serum and 3% fetal bovine serum (FBS), 1% Hepes, 1 mM sodium pyruvate, 100 μ g/mL ampicillin and 100 μ g/mL streptomycin. Cells were seeded into 96-well plates with a number of 10^4 per well in complete medium and grown for 20 h and then incubated in the presence of coffee extracts differentially diluted in culture medium for a further 24 h. In the preliminary step of extract toxicity evaluation, following 10 min incubation with 10 μ L of PrestoBlue, 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 the viability of cells treated with coffee extracts as a percent of the viable control cells and the maximum nontoxic concentrations (IC_0) that was the highest used while not decreasing the viability of the cells ($p \leq 0.05$). IC_{50} value was also calculated. To evaluate the protective effect of coffee extracts against oxidative stress with *tert*-butylhydroperoxide (*t*-BOOH), cells were preincubated with nontoxic concentration of extracts. To induce the oxidative stress condition after 24 h pre-incubation with coffee extracts, 500 μ M *t*-BOOH was added for 2 h and then the cell viability was measured [21].

Detection of intracellular reactive oxygen species (ROS)

To determine the effect of extracts on the intracellular ROS generation, the dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay was performed. After treatment with extracts for 20 h, β TC3 cells were washed with phosphate-buffered saline (PBS) and loaded with the DCFH-DA dye at a final concentration of 1 μ M 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 generation was evaluated and expressed as fluorescence % of control (cells without coffee extracts). As a positive oxidative stress inducer, 500 μ M *t*-BOOH was used [22]. The assay was performed in the presence of serum-free medium with phenol red. However in the described experiment, beyond wells with control cells (F_{contr}) and wells with cells treated with compounds (F_{treated}), there were also present blank wells (without DCFH-DA) with media (F_{blank}) and blank wells with studied compounds only ($F_{\text{blankcomp}}$). Thus the final results were calculated as:

$$100\% \times ((F_{\text{treated}}) - (F_{\text{blankcomp}})) / (F_{\text{contr}}) - (F_{\text{blank}}) = \text{ROS generation}$$

Lipid accumulation assay

For adipocyte differentiation, confluent culture of 3T3L1 cells were grown for 2 days in preadipocyte medium

(DMEM with 10% calf serum) than cells stimulated by differentiation mixture containing 10% FBS, 1.0 μM dexamethasone, 0.5 mM methylisobutylxanthine and 1.0 $\mu\text{g}/\text{mL}$ insulin for 2 days. After 48 h of incubation, the differentiation medium was replaced with DMEM containing 10% FBS and 1.0 $\mu\text{g}/\text{mL}$ insulin. Analysis was performed on the 8th day after differentiation. As a positive lipid drops formation inducer, 400 μM oleic acid was added. To observe the influence of extracts on lipid drops formation in 3T3L1 cell culture, the cells were treated with extracts for 48 h at the early stage of the differentiation process. After treatment, the cells were washed with cold PBS, fixed in 5% paraformaldehyde for 30 min and stained with Nile red solution (1 $\mu\text{g}/\text{mL}$) for 40 min at 37 °C and 5% CO_2 ; the lipid-bound Nile red fluorescent signal F485/530 was measured. Lipid accumulation was evaluated and expressed as fluorescence % of control (cells without coffee extract treatment) [22].

Animal study

Studies were conducted according to the proposal approved by the Local Institutional Animal Care and Use Committee (permission no. 71/2014; Olsztyn, Poland) on selected Wistar rats of similar age of 8 weeks and body weight about 165 g. The experiment lasted 4 weeks. Experimental groups were composed of eight male rats. One group was fed one experimental diet. The basic diet was semi-synthetic modification proposed by Reeves [23] of AIN-G93G diet developed by the American Institute of Nutrition. Diets CS (standard) and CHF (oxidative—high fat) were treated as controls, where CS provided an adequate level of dietary fiber (5% cellulose), right share of energy from

fat (7% rapeseed oil) and highly digestible carbohydrates (10% sucrose and 53% cornstarch). CHF diet was adapted to the needs of the ongoing research, in which the important aspect was to verify the impact of coffee extracts on indices of oxidative stress. As a prooxidant factor, palm oil with a high ratio of n-6/n-3 acids (>122:1) was used. CHF diet contained 14% palm oil at the expense of cornstarch (11%) and cellulose (3%). Modified HF diets contained 0.5% of coffee extracts at the expense of cornstarch. Energetic values for experimental CS and CHF diets were estimated according to Research Diets, Inc. (New Brunswick, NJ, USA). The detailed composition of each diet is given in Table 1. The compared diets were administered for 4 weeks with everyday control of feed intake. The rats were used in compliance with the European guidelines for the care and use of laboratory animals. The animals were maintained individually in metal cages under a stable temperature (21–22 °C), a 12-h light/12-h dark cycle and a ventilation rate of 15 air changes per hour.

After experimental feeding, the rats were weighed and anesthetized with sodium pentobarbital (50 mg/kg body weight) [24]. The body fat and lean mass was determined by time-domain nuclear magnetic resonance using a minispec LF 90II analyzer (Bruker, Karlsruhe, Germany). The blood was collected from the caudal vein, whereas the liver, heart and kidney were removed, immersed in liquid nitrogen and stored at $-20\text{ }^\circ\text{C}$ until analysis. The blood was centrifuged for 15 min at $380\times g$ and the obtained serum was stored at $-70\text{ }^\circ\text{C}$ until analysis. In the blood serum, the antioxidant capacities of water-soluble and lipid-soluble substances (ACW and ACL, respectively) were determined by a photochemiluminescence detection method using a Photochem and respective kits (ACW-Kit and ACL-Kit,

Table 1 Comparison of the diets fed to rats (%)

	Group							
	CS	CHF	GCE	MGCE	DiGCE	RCE	MRCE	DiRCE
Caseine	20	20	20	20	20	20	20	20
DL-Methionine	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Rapeseed oil	7	7	7	7	7	7	7	7
Palm oil	–	14	14	14	14	14	14	14
Cellulose	5	2	2	2	2	2	2	2
Sucrose	10	10	10	10	10	10	10	10
Mineral mix	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5
Vitamin mix	1	1	1	1	1	1	1	1
Choline chloride	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Coffee extract	–	–	0.5	0.5	0.5	0.5	0.5	0.5
Cornstarch	53.0	42.0	41.5	41.5	41.5	41.5	41.5	41.5
Gross energy MJ/kg	16.7	19.6	19.6	19.6	19.6	19.6	19.6	19.6

CS control, CHF control high fat; diets supplemented with coffee extracts: GCE green coffee extract, RCE roasted coffee extract, M monoesters of caffeic and ferulic acid with quinic acid, Di diesters of caffeic acid with quinic acid

Analytik Jena AG, Germany). In the photochemiluminescence assay, the generation of free radicals was partially eliminated through reactions with antioxidants present in the serum samples, and the remaining radicals were quantified by luminescence generation. Ascorbate and tocopherol calibration curves were used to evaluate ACW and ACL, respectively. The serum concentration of glucose (GL), triglycerides (TG), total cholesterol (TC) and high-density lipoprotein cholesterol fraction (HDL) were estimated using a biochemical analyzer (Horiba, Pentra C200, Kyoto, Japan). The non-HDL fraction was calculated by subtracting HDL from TC. In the liver, heart and kidney, thiobarbituric acid-reactive substances (TBARS) were determined as an indicator of lipid peroxidation in these organs. A procedure developed by Botsoglou et al. [25] was used in this assay, and the TBARS concentrations were determined spectrophotometrically at 532 nm and expressed in nanogram of malondialdehyde per gram of a tissue. The hepatic concentration of reduced and oxidized glutathione (GSH and GSSG, respectively) was determined by using an enzymatic recycling method described by Rahman et al. [26].

Statistical analysis

Data (excluding animal assay) were expressed as mean \pm standard deviation (SD) of three independent experiments. Each experiment was performed at least in triplicate. Statistical values comparing sample groups were determined using the Student's *t* test and one-way analysis of variance ANNOVA using Statistica 10.0 software at the significance level of $p \leq 0.05$ [22].

Results and discussion

CHAs profile of green and roasted coffee extracts and their fractions

Crude extracts of green and roasted coffee were purified by preparative CPC chromatograph connected with a fraction collector. Fractions rich in CHAs selected based on high UV absorbance at 320 nm were collected to give purified extracts of green and roasted coffee (GCE and RCE, respectively) [27]. In another run, fractions were separated due to the content of monoesters (MCHAs) and diesters of chlorogenic acid (DiCHAs) (Fig. 1) and denoted, respectively, as MGCE, DiGCE, MRCE and DiRCE. The profiles of CHAs in the six studied extracts and their fractions, generally referred to as extracts, are shown in Table 2. MCHAs contained 3-, 4- and 5-caffeoylquinic acids, as well as 3-, 4- and 5-feruloylquinic acids (Fig. 2a). Figure 2b shows the content of slight amounts of the mentioned MCHAs besides 3,4-, 3,5- and 4,5-dicaffeoylquinic acids in the fraction characterized as DiCHAs of green coffee extract. The extracts of green coffee were characterized by higher contents of CHAs compared to the roasting material. Within extracts of green and roasted coffee, higher contents of CHAs were observed for fractions containing monochlorogenic acids compared to fractions of dichlorogenic acids and also compared to the unfractionated extracts. Due to the purification, the concentration of CHAs increased and caffeine was removed from the crude extracts as proved in a previous study [2]; hence, concentrated formulations can be commercially used without the risk of excessive caffeine supply.

Table 2 Concentration of chlorogenic acids in extracts from green and roasted coffee and their fractions (%)

Chlorogenic acid	GCE	MGCE	DiGCE	RCE	MRCE	DiRCE
3- <i>O</i> -Caffeoylquinic acid	11.02 \pm 0.87**	19.22 \pm 1.13**	0.08 \pm 0.01**	2.84 \pm 0.14**	4.94 \pm 0.31**	0.02 \pm 0.00**
5- <i>O</i> -Caffeoylquinic acid	16.82 \pm 1.12**	23.86 \pm 1.70**	0.91 \pm 0.06**	3.86 \pm 0.26**	9.81 \pm 0.76**	0.09 \pm 0.01**
4- <i>O</i> -Caffeoylquinic acid	13.28 \pm 0.93**	18.56 \pm 1.05**	0.75 \pm 0.04**	2.56 \pm 0.18**	5.69 \pm 0.40**	0.03 \pm 0.00**
3- <i>O</i> -Feruloylquinic acid	2.38 \pm 0.15*	3.09 \pm 0.21*	0.22 \pm 0.03 ^a	0.24 \pm 0.02 ^a	0.47 \pm 0.09**	0.07 \pm 0.01**
5- <i>O</i> -Feruloylquinic acid	1.98 \pm 0.12*	4.01 \pm 0.29**	0.43 \pm 0.05 ^a	0.41 \pm 0.05 ^a	1.50 \pm 0.11*	0.03 \pm 0.00**
4- <i>O</i> -Feruloylquinic acid	2.51 \pm 0.19*	3.40 \pm 0.33*	0.50 \pm 0.07 [#]	0.60 \pm 0.08 [#]	1.42 \pm 0.07**	0.04 \pm 0.00**
3,4-di- <i>O</i> -Caffeoylquinic acid	3.79 \pm 0.24**	nd	5.68 \pm 0.44*	0.12 \pm 0.01*	nd	2.01 \pm 0.17**
3,5-di- <i>O</i> -Caffeoylquinic acid	0.83 \pm 0.07**	nd	7.82 \pm 0.61**	nd	nd	2.76 \pm 0.14**
4,5-di- <i>O</i> -Caffeoylquinic acid	1.73 \pm 0.10**	nd	7.97 \pm 0.55**	0.06 \pm 0.01*	nd	0.02 \pm 0.00*
Total chlorogenic acids	54.35 \pm 3.12**	72.14 \pm 4.22**	24.36 \pm 1.63 ^a	13.06 \pm 0.74**	23.85 \pm 1.65 ^a	7.42 \pm 0.48**
Soluble dietary fiber	18.03 \pm 0.84**	15.46 \pm 0.38**	27.22 \pm 1.03	48.92 \pm 3.23**	31.71 \pm 2.85**	54.76 \pm 2.19**

Values expressed as mean \pm standard deviation; different letters in one row or lack of them correspond to significant differences (* or [#] $p < 0.05$ and ** $p < 0.01$ for the values within groups with the same superscript); $n = 9$

nd not detected, GCE green coffee extract, RCE roasted coffee extract, M monoesters of caffeic and ferulic acid with quinic acid, Di diesters of caffeic acid with quinic acid

Antioxidant properties of green and roasted coffee extracts and their fractions

Extracts were tested for DPPH and OH radicals scavenging capacity. The last one was carried out at 37 °C, which simulates the conditions in the human body. The results obtained in DPPH[•] scavenging capacity test for both green and roasted coffee extracts showed higher antioxidant activity (AA) of the fractions of mono- above dichlorogenic acids ($p < 0.01$) (Table 3). MRCE exhibited the highest AA against DPPH radicals ($p < 0.01$). A similar trend was obtained also in the test with OH[•], where the most active was also MRCE.

This trend might result from the fact that the fractions containing monoesters were characterized by higher concentrations of CHAs. However, the highest AA showed MRCE, although it contained nearly three times less amounts of CHAs than MGCE, which was the second most active radicals scavenger. Therefore it could be concluded that in case of MRCE, high AA was caused not only by CHAs, but also by other compounds with antioxidant properties, i.e., hydrophilic Maillard reaction products (MRP), which accompanied the elution of MCHAs. It is however known that MRP of roasted coffee also contains incorporated CHAs [28]. The studies by Sulaiman et al., Liu and Kitts, as well as Del Castillo with DPPH[•] and whole roasted coffee extracts exhibited higher AA than those obtained from green coffee [29–31], which confirms the trend obtained in our study. Chu et al. [28] showed also that decaffeinated coffee has a higher AA than regular coffee suggesting that caffeine is not an effective radical scavenger and removing it from coffee extract does not decrease its antioxidant activity.

Effect of coffee extracts on viability and level of intracellular oxidative stress in β TC3 cells and lipid accumulation in 3T3L1 adipocytes

Insulin resistance, obesity and type 2 diabetes symptoms are closely linked to the metabolic activity of pancreatic β cells and adipose tissue [32]. Decrease in insulin secretion, which causes hyperglycemia, is one of the main effects arising from the excessive oxidative stress in β cells. Typical low level of antioxidant defense in β

cells prompts searching for cytoprotective drugs of high radical-scavenging capacity. Plant polyphenols as chlorogenic acids exhibit activity as radical scavengers. To evaluate the cytoprotective/cytotoxic activity of coffee extracts in cell cultures, the influence of their different concentrations (0.05–7.50 mg/mL) on β TC3 cells metabolic activity was studied with Presto Blue reagent and the highest nontoxic concentrations (IC_{0}) were calculated from the viability curves. The obtained IC_{0} values ranged from 0.05 to 0.85 mg/mL and IC_{50} from 2.74 to 7.63 mg/mL (Table 4). The comparison of IC_{50} parameters revealed that DiGCE was the most toxic against β cells, and the least toxic was MGCE. This suggest that generally much lower concentrations of DiGCE compared to other analyzed extracts exhibited effective bioactivity and that diesters of chlorogenic acids have a high impact on cell activity. IC_{50} values were taken to prepare a solution for further antioxidative stress assays. To compare the protective effects of coffee extracts against strong pro-oxidant *t*-BOOH, β TC3 cells were preincubated with IC_{0} doses of the six extracts. The untreated cells were the control samples. *t*-BOOH at 500 μ M concentration decreased control β TC3 cell viability almost by 30%. Preincubation of the cells with coffee extracts prior to

Table 4 Biological activity of coffee extracts and their fractions against β -TC3 cells

Coffee extract	IC_{0} (mg/mL)	IC_{50} (mg/mL)	% of cell viability ¹
<i>t</i> -BOOH	–	–	70.96 ± 1.97**
GCE	0.60 ± 0.04**	4.25 ± 0.26 ^a	105.70 ± 1.80 ^a
MGCE	0.85 ± 0.07**	7.63 ± 0.52*	104.47 ± 2.22 ^a
DiGCE	0.05 ± 0.01 ^{##}	2.74 ± 0.34**	84.45 ± 3.48 ^b
RCE	0.30 ± 0.02*	4.15 ± 0.61 ^a	101.21 ± 2.18**
MRCE	0.10 ± 0.02*	3.25 ± 0.62*	85.41 ± 3.73 ^b
DiRCE	0.15 ± 0.01 ^{##}	3.75 ± 0.41**	93.70 ± 2.17**

Values were determined by regression analyses and expressed as mean ± SD; different letters in one column or lack of them correspond to significant differences (* $p < 0.05$ and ** or ^{##} $p < 0.01$ for the values within groups with the same superscript); $n = 9$; for coffee extracts abbreviations see legend below Table 1

¹The influence of cells preincubation with nontoxic concentrations of extract (IC_{0}) for 2 h against oxidative stress induced by 500 μ M *t*-BOOH on metabolic activity measured with PrestoBlue assay

Table 3 Antioxidant activity of coffee extracts and their fractions expressed as radical-scavenging capacity (%)

Radical	GCE	MGCE	DiGCE	RCE	MRCE	DiRCE
OH [•]	45.49 ± 2.48 ^a	52.26 ± 3.55**	37.05 ± 2.38*	49.12 ± 1.99 ^a	72.15 ± 5.26**	30.09 ± 2.18*
DPPH [•]	57.33 ± 3.17 ^{a,b}	59.67 ± 4.09 ^b	53.35 ± 4.01 ^a	41.60 ± 2.73**	70.46 ± 3.92**	15.66 ± 1.33**

Values expressed as mean ± standard deviation; different letters in one row or lack of them correspond to significant differences (* $p < 0.05$ and ** $p < 0.01$ for the values within groups with the same superscript); $n = 9$; for coffee extracts abbreviations see legend below Table 1

t-BOOH injection allowed to maintain their metabolic activity on a higher level that proves the cytoprotective effects of coffee extract components including CHAs. The activity of DiGCE and MRCE in reducing the oxidative stress in β TC3 cells incubated with the pro-oxidant was rather low, and cell viability increased to 84.5 and 85.4%, respectively. The activity of DiRCE, GCE, RCE and MGCE was higher ($p < 0.01$) and enabled maintaining the cells viability in the range of 93.7–105.7%.

Further studies evaluated coffee extracts as protective agents against oxidative stress in β TC3 cells induced by intracellular ROS production. ROS have been implicated in a number of processes including cell proliferation, DNA damage and apoptosis [33]. At low levels, ROS modulate gene expression by acting as second messengers, but at high levels they cause oxidative injury leading to cell death. To evaluate whether the coffee extracts prevent the generation of oxidative stress, the intracellular ROS production was quantified by measurement of DCFH-DA fluorescence. β TC3 cells with induced oxidative stress by *t*-BOOH addition were characterized by 176% higher ROS production than the control (Fig. 3a). Studies revealed that all coffee extracts decreased ROS generation by 28.6–61.0% compared to the control and by 59.1–77.8% compared to cells with induced oxidative stress. The results suggest that coffee extracts decreased the level of ROS even in cells representing physiological amounts of ROS due to their high scavenging and oxidative stress inhibition activities [34, 35]. The higher activity to decrease ROS generation showed DiRCE. The results confirmed the observations made by Liang et al., who suggested on the basis of PCA analysis that dichlorogenic acids exhibit high potential to scavenge intracellular ROS [36].

It is known that adipose tissue secretes adipocytokines, growth factors and hormones involved in energy homeostasis and insulin sensitivity maintenance, and increased adiposity is regarded as one of the most important risk factors of insulin resistance—type 2 diabetes and obesity [37]. To evaluate the influence of CHAs on adipogenesis, 3T3L1 cells were incubated with coffee extracts during the whole process of preadipocytes differentiation. The level of lipid accumulation was evaluated with Nile red staining and oleic acid was used as a positive control, which caused increase of lipid accumulation by 47.2% compared to the control. It was found that all the studied coffee extracts decreased lipid accumulation compared to the untreated mature cells by 20.3–45.4% and by 38.5–61.5% compared to the positive control. Similar observation were previously made for green coffee by Kim et al. [38]. In our study additional fractionation of extracts proved that DiRCE was the most active (Fig. 3b).

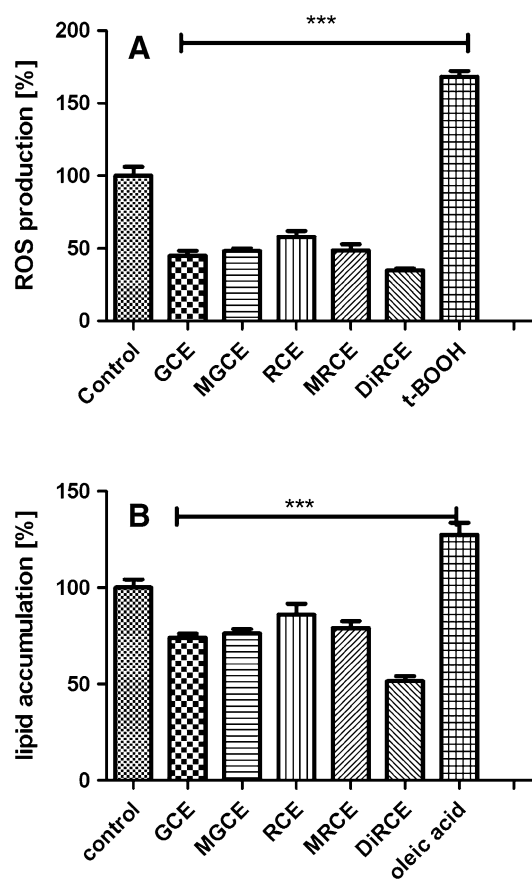


Fig. 3 Biological activity of coffee extracts and their fractions: **a** the influence of β TC3 cells preincubation with nontoxic extract concentrations (IC_0) for 24 h on intracellular ROS production measured with DCFH-DA; **b** the influence of 3T3L1 adipocytes preincubation with nontoxic extract concentrations (IC_0) for 24 h on lipid accumulation measured with Nile red staining; data represent the means \pm SD from nine independent experiments vs untreated control cells (100%); *** $p < 0.001$ compared with the control cells

Animal study

Studies in rats were focused on the evaluation of the antioxidant, antiobesity and antidiabetic properties of coffee extracts, which were administrated with a diet. The effects of feeding rats diets supplemented with coffee extracts were evaluated by indices summarized in Table 5. It was found that all the studied diets supplemented with coffee extracts decreased rats' BWG compared to CHF by 2.3–20.5%, which is in accordance with the study by Kim et al. on green coffee extract [38], proving that CHAs and not caffeine may be responsible for the antiobesity activity of coffee. The diets modified by adding MRCE as well as GCE and DiGCE caused the highest statistically significant decrease of this index ($p < 0.05$). RTF was also lowered by coffee extracts in the range from 1.2 to 5.8%. MRCE contributed to the smallest share of fat in the total mass of the animals.

Table 5 Body weight gain, relative fat tissue, indices of serum lipid and glucose profile and antioxidant status in rats

	CS	CHF	GCE	MGCE	DiGCE	RCE	MRCE	DiRCE	SEM
BWG (g)	41.9*	65.3 ^b	54.4 ^a	63.4 ^b	54.8 ^a	60.2*	51.9*	63.8 ^b	1.5
RFT (% of BW)	31.2 ^a	34.4 ^e	32.4 ^b	32.4 ^b	33.5 ^{c,d}	32.9 ^{b,c}	31.9 ^{a,b}	34.0 ^{d,e}	0.3
Serum indices									
GL (mmol/L)	9.59 ^b	10.40 ^{d,e}	8.88 ^a	9.06 ^{a,b}	10.53 ^c	9.73 ^{c,d}	10.70 ^e	9.69 ^{b,c}	0.22
TC (mmol/L)	1.93 ^b	1.96 ^{b,c}	1.94 ^b	1.90 ^{a,b}	1.86 ^a	1.95 ^b	2.00 ^c	1.71*	0.03
HDL (mmol/L)	0.933 ^d	0.861 ^a	0.896 ^b	0.899 ^{b,c}	0.899 ^{b,c}	0.903 ^{c,d}	0.930 ^d	0.878 ^{a,b}	0.020
HDL profile (% of TC)	48.4 ^{b,c}	43.4*	46.3 ^a	47.2 ^b	47.8 ^b	46.3 ^a	46.7 ^b	50.2*	0.8
Non-HDL (mmol/L)	0.99 ^a	1.10 ^c	1.04 ^b	1.00 ^{a,b}	0.98 ^a	1.05 ^b	1.07 ^{b,c}	0.86*	0.02
TG (mmol/L)	1.32 ^{a,b}	1.63 ^d	1.29 ^a	1.44 ^b	1.68 ^{d,e}	1.75 ^{e,f}	1.88 ^f	1.40 ^b	0.08
ACW (µg/mL)	4.08 ^a	4.19 ^{a,b}	3.75*	4.19 ^{a,b}	4.79 ^c	4.21 ^b	5.37 ^d	5.05 ^{c,d}	0.21
ACL (µg/mL)	18.0 ^a	18.1 ^a	21.9*	18.6 ^{a,b}	34.9	19.0 ^{b,c}	19.6 ^c	20.1*	0.4
Liver indices									
GSH (µmol/g)	43.6**	48.4 ^a	48.5 ^a	54.2 ^b	72.9**	54.4 ^b	64.6**	57.8**	1.5
GSSG (µmol/g)	5.00 ^{a,b}	6.41 ^{e,f}	1.91	5.33 ^{b,c}	6.84 ^f	4.71 ^a	5.47 ^{c,d}	5.79 ^{d,e}	0.29
GSH/GSSG	8.92 ^a	9.30 ^{a,b}	28.00	10.30 ^{b,c}	12.82 ^e	11.70 ^c	11.90 ^{c,d}	12.42 ^{d,e}	1.18
TBARS (ng/g)	409 ^a	462 ^{d,e}	422 ^{a,b}	438 ^{b,c}	489 ^e	343*	307*	449 ^{c,d}	12
Other indices									
TBARS heart (ng/g)	315 ^{a,b}	348**	312 ^a	311 ^a	332*	305*	287**	323 ^b	3
TBARS kidney (ng/g)	847*	896 ^a	814*	839*	918*	859*	826*	890 ^a	16

Different letters in one row or lack of them correspond to significant differences (* $p < 0.05$ and ** $p < 0.01$ for the values within groups with the same superscript)

BWG body weight gain, *RFT* relative fat tissue, *GL* glucose, *TC* total cholesterol, *TG* triglycerides, *ACW* antioxidant capacity of water-soluble substances, *ACL* antioxidant capacity of lipid-soluble substances, *GSH* reduced glutathione, *GSSG* oxidized glutathione, *HDL* HDL-cholesterol, *non-HDL* non-HDL-cholesterol, *TBARS* thiobarbituric acid-reactive substances, *SEM* summed standard error for all rats divided by the square root of the total number of rats, $n = 64$; Diets: *CS* control standard, *CHF* control high fat; for diets with 0.5% coffee extracts abbreviations see legend below Table 1; $n = 8$

GL level was decreased after feeding animals a diet supplemented with GCE and RCE and also some of their fractions—MGCE and DiRCE by 6.4 and 14.6%, respectively, but supplementing the diet with DiGCE and MRCE did not influenced the GL level compared to CHF ($p < 0.05$). The TC level did not change after adding GCE, MGCE, RCE and MRCE to the CHF diet, and supplements containing high concentrations of diesters caused its decrease from 4.1% (DiGCE) to 12.8% (DiRCE). A positive trend was observed in the concentration of beneficial HDL after feeding rats supplemented diets, where DiRCE caused its increase by 2.0%, while the other extracts by 4.1–4.9% and MRCE up to 8.0%. The supplements influenced lowering non-HDL levels from 2.7 to 21.8% compared to CHF, and the most active was DiRCE. TG was beneficially lowered up to 20.8% by a diet containing GCE compared to CHF; for most other extracts the decrease of TG level amounted to 11.7–14.1%, but it was increased by the diets with RCE and MRCE by 7.4 and 15.3%, respectively.

The evaluation of serum antioxidants revealed that ACW was beneficially increased by MGCE, MRCE and DiRCE by 14.3, 28.2 and 20.5%, respectively. ACL changes were characterized by much higher increase for two extracts,

i.e., from 21.0% for GCE to 92.8% for DiGCE. The other extracts caused its increase by 2.8–11.1%. Promising effects were observed after feeding animals coffee extracts in terms of glutathione profile. GSH increased after feeding all modified diets compared to CHF, from 12.0 for MGCE to 50.6% for DiGCE, respectively. For GCE, the effect was statistically insignificant ($p \geq 0.05$), but this extract caused the highest decrease of GSSG hepatic level (by 70.2%). For other extracts, GSSG decreased from 9.7 to 26.5%, but increased by 6.7% for DiGCE. GSH/GSSG profile was the most beneficial for the diet with GCE and increased by 201% compared to CHF, and for the other extracts it increased by 10.8–37.9%. TBARS was the highest in animals fed a diet supplemented with DiGCE and the beneficial low levels of TBARS in liver and heart were determined after feeding rats a diet with MRCE. The decrease amounted to 33.6 and 17.6%, respectively. GCE and MRCE caused the highest decrease of TBARS in kidney by 9.2 and 7.8%, respectively.

The results of in vivo tests indicated that MRCE contributed to the smallest share of fat in the total mass of the animals. Feeding rats a diet with this extract also caused the lowest level of TBARS in the liver and heart,

the highest ACW and the highest level of HDL ($p < 0.01$). All of these four indices were more beneficial compared to CHF and even to CS and diet. MRCE also contributed to the highest AA evaluated as radical-scavenging capacity in vitro in aqueous solutions (in tests with DPPH and OH radicals). Therefore, research on rats confirmed the highest antioxidant efficacy of MRCE. This hydrophilic extract contained MCHAs and hydrophilic MRP and this explains its high activity in the aqueous fraction of serum. High AA of roasted coffee was previously postulated by Daglia et al. [39]. Vicente et al. [40] demonstrated the increase of antioxidant enzyme activity in rats fed roasted coffee and postulated that coffee may reduce the overall oxidative stress and the risk of associated degenerative disorders. In another study, hydrophobic MRP was proved to inhibit in vitro and in vivo lipid peroxidation and formation of lipoxidation products [41]. In our study, hydrophobic MRP was contained in DiRCE, and the rats fed a diet supplemented with this extract had the lowest level of non-HDL cholesterol. The above conclusions suggest that RCE and its fractions could be used as food supplements of antioxidant and antiobesity bioactivity.

Several other indices exhibited favorable levels among the animals fed GCE-supplemented diet. GCE added to CHF decreased the level of TBARS in kidney and resulted also in the lowest level of TG, GSSG and the highest GSH/GSSG ratio ($p < 0.01$). The diet modified with GCE caused also the highest decrease of GL level in the rat blood serum. The highest concentration of GSH and the highest level of ACL were proved for animals fed the diet with addition of DiGCE. Therefore, green coffee supplements may be useful in diets preventing the symptoms of type 2 diabetes which was previously postulated by Ahmed et al. [42]. Iwai et al. [43] suggested that green coffee extracts inactivate enzymes hydrolyzing complex carbohydrates as a possible reason for reducing postprandial hyperglycemia. Nam et al. [44] explained instead the antidiabetic activity of green coffee with the effect of β cells regeneration resulting from blocking ATP-sensitive K^+ channels in vascular cells.

In conclusion, the results of the study showed a high and diverse biological activity of green and roasted coffee. The extract of roasted coffee, particularly the fraction containing monochlorogenic acids and more polar MRP, caused an increase of antioxidant potential evaluated as high radical-scavenging capacity and a high level of hydrophilic antioxidants in rat serum and may be potentially used as a nutraceutical of anticarcinogenic properties due to inhibition of oxidative stress resulting from the antioxidant capacity [34]. The results related to the antidiabetic activity of coffee, including protection against oxidative stress of β cells and the low levels of triglycerides, glucose and oxidized glutathione levels in rats, showed a beneficial

effect of non-fractionated GCE. This may indicate a synergistic effect of MCHAs, DiCHAs and perhaps soluble dietary fiber, and support the use of green coffee extract in functional food and food supplement developed to decrease metabolic syndrome including type 2 diabetes. Further research should include the analysis of non-phenolic antioxidants and other biologically active compounds of coffee extracts and their fractions.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Compliance with ethics requirements All institutional and national guidelines for the care and use of laboratory animals were followed.

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