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The protective effect of a mix of *Lactarius deterrimus* and *Castanea sativa* extracts on streptozotocin-induced oxidative stress and pancreatic β-cell death

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Abstract

Pancreatic β-cell death or dysfunction mediated by oxidative stress underlies the development and progression of diabetes mellitus. In the present study, we tested extracts from the edible mushroom *Lactarius deterrimus* and the chestnut *Castanea sativa*, as well as their mixture (MIX *Ld/Cs*), for potential beneficial effects on streptozotocin (STZ)-induced pancreatic β-cell death. Analysis of chelating effects, reducing power and radical-scavenging assays revealed strong antioxidant effects of the *C. sativa* extract and MIX *Ld/Cs*, while the *L. deterrimus* extract displayed a weak to moderate effect. The antioxidative effect of the chestnut extract corresponds with the high content of phenolics and flavonoids identified by HPLC analysis. In contrast, the mushroom extract contains relatively small amounts of phenols and flavonoids. However, both extracts, and especially their combination MIX *Ld/Cs*, increased cell viability after the STZ treatment as a result of a significant reduction of DNA damage and improved redox status. The chestnut extract and MIX *Ld/Cs* significantly lowered the STZ-induced increases in superoxide dismutase and catalase activities, while the mushroom extract had no impact on the activities of these antioxidant enzymes. However, the *L. deterrimus* extract exhibited good NO-scavenging activity. Different mechanisms that underlie antioxidant effects of the mushroom and chestnut extracts were discussed. When combined as in the MIX *Ld/Cs*, the extracts exhibited diverse but synergistic actions that ultimately exerted beneficial and protective effects against STZ-induced pancreatic β-cell death.

Key words: ***Lactarius deterrimus*: *Castanea sativa*: Rin-5F cells: Cytoprotection: Antioxidant activity**

Reactive oxygen (ROS) and nitrogen (RNS) species are products of normal aerobic metabolism and are continuously produced under physiological conditions. However, when they rise above their physiological concentrations, ROS and RNS are extremely toxic due to their ability to induce protein and DNA damage and lipid peroxidation. Consequently, organisms have developed antioxidant defence systems, i.e. antioxidative enzymes that work in synergy with non-enzymatic antioxidant systems that are produced in cells or ingested through the diet. In healthy individuals, there is a balance between ROS and RNS production and antioxidant defences. An imbalance provoked by either overproduction of reactive species or attenuation of the antioxidative system leads to a process called oxidative stress. Oxidative stress is implicated in the development of many diseases such as CVD, atherosclerosis, neurodegenerative diseases and diabetes mellitus[(1)].

Diabetes mellitus is a chronic metabolic disorder that continues to present a major health problem worldwide. It is characterised by hyperglycaemia resulting from deficiencies in insulin secretion, insulin action or both. Multiple biochemical pathways and mechanisms for glucose toxicity have been

Abbreviations: CAT, catalase; GSH, reduced glutathione; GSSG, glutathione disulfide; GSSP, glutathionylated proteins; MIX *Ld/Cs*, mixture of *Lactarius deterrimus* and *Castanea sativa*; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; RNS, reactive nitrogen species; ROS, reactive oxygen species; RPMI, Roswell Park Memorial Institute; SOD, superoxide dismutase; STZ, streptozotocin.

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suggested[2]. All of these pathways have in common the formation of ROS that when in excess cause chronic oxidative stress. It has been established that chronic hyperglycaemia and associated oxidative stress are linked to long-term damage, dysfunction and eventually the failure of organs, especially the eyes, kidneys, nerves, heart and vascular system that lead to diabetic complications[3]. As the final targets for clinical intervention, diabetic complications attract more attention than the insulin-secreting pancreatic β-cells that assume central place in the onset and development of diabetes mellitus. Though different aetiologies underlie type 1 and type 2 diabetes, β-cell death and/or their dysfunction are at the core of the pathophysiology of both types of disease[4]. There is convincing evidence that reactive species play an important role in the pathogenesis of pancreatic β-cell loss or dysfunction[5]. Namely, it has been documented that ROS and RNS are generated intracellularly when β-cells are under autoimmune attack mediated by pro-inflammatory cytokines in type 1 diabetes, or when exposed to a hyperglycaemic milieu which is β-cell toxic, as occurs in type 2 diabetes. Since β-cells are characterised by a constitutively low enzymatic antioxidative defence system, they are particularly susceptible to oxidative stress[6]. Therefore, antioxidants taken as a supplement or through the diet could be helpful in reducing oxidative stress, and thus in preventing or slowing down the process of β-cell loss. As recent studies have shown that certain synthetic antioxidants have toxic and carcinogenic effects, at present, there is considerable interest in finding natural antioxidants with less frequent side effects, to replace synthetic ones[7].

The antioxidative properties for a large number of natural compounds that are synthesised by higher plants and fungi as secondary metabolites have been demonstrated. Phytochemicals with antioxidative effects include a variety of phytosterols, terpenes and especially polyphenols, such as flavonoids, tannins and phenylpropanoids. A direct correlation between the total phenolic content and antioxidant capacity has been established and explained through a number of different mechanisms, such as free radical scavenging, metal ion chelation and hydrogen donation[8,9]. Therefore, numerous scientific efforts are directed at discovering plants rich in antioxidant compounds. As it has been suggested that plant extracts possess higher antioxidant activities than pure molecules, there is a growing interest for the use of plant extracts as an adjunct in the therapy of oxidative stress-related diseases[10].

In the present study, two extracts and their combination were tested for antioxidant properties and potential protective effects against rat pancreatic β-cell (Rin-5F cells) death induced by streptozotocin (STZ). One of the examined extracts was obtained from spiny burrs, which are the less studied part of the sweet chestnut (*Castanea sativa*), known for its antioxidant properties. Considering the growing interest for mushrooms, as a dietary source for human consumption and as a source of physiologically beneficial components, we studied the extract from the edible mushroom *Lactarius deterrimus*. Although we examined protective effects of both chestnut and mushroom extracts on β-cell death induced by oxidative stress, our main goal was to establish potential beneficial effects of *L. deterrimus* and *C. sativa* extracts on β-cell survival when used in combination (MIX *Ld*/*Cs*).

Experimental methods

Plant material and extraction procedure

The mushroom *L. deterrimus* was collected near the village Mune, the Istra region in Croatia, in the summer of 2008. Fruiting bodies were gently cleansed of any residual compost. Fresh mushrooms were air-dried and stored in airtight plastic bags at room temperature. Samples of spiny burrs of the sweet chestnut (*C. sativa* Mill.) were collected in the Bihać region in western Bosnia and Herzegovina. The chestnut samples were harvested during the chestnut-ripening season, from the middle of September to the end of October 2006. The collected samples were kept at −20 °C and protected from light before further use.

The dried mushroom samples and spiny-burrs chestnut samples were milled in a blender before extraction with 50 % ethanol (the sample:solvent ratio was 1:5 for the chestnut extract and 1:10 (w/v) for the mushroom extract). The extraction process was carried out using an ultrasonic bath (B-220; Branson and SmithKline Company) at 45 °C for 40 min for the mushroom extract, and at room temperature for 30 min for the chestnut extract. After filtration, the extraction solvent was removed by a rotary evaporator (Devarot; Elektromedicina) under vacuum. The obtained extracts were then dried at 60 °C to a constant mass and stored in glass bottles at −80 °C to prevent oxidative damage.

HPLC analysis

In brief, 50 mg of dry extracts were dissolved in 1·0 ml of methanol using an ultrasonic bath. The samples were filtered with a 0·45 μm filter and injected in LC/MS or HPLC/diode array detection (DAD) systems.

HPLC analysis of the extracts was performed using an Agilent 1200 series HPLC with a RP Zorbax Eclipse Plus C18 column (1·8 μm, 150 × 4·6 mm). The mobile phase A was 0·2 % formic acid in water, and the mobile phase B was acetonitrile. The injection volume was 3 μl for the mushroom sample and 1 μl for the chestnut sample. The samples were eluted at a rate of 0·95 ml/min, with the following gradient programme: 0–20 min 5–16 % B; 20–28 min 16–40 % B; 28–32 min 40–70 % B; 32–36 min 70–99 % B; 36–45 min 99 % B; 45–46 min 99–5 % B. Quantification was based on the measured integration area of the peaks of interest compared with the calibration value of the corresponding standards.

LC/MS analysis was performed on an Agilent MSD TOF coupled to an Agilent 1200 series HPLC, using the same column and gradient programme as those for HPLC/DAD analysis. Mass spectra were acquired using an Agilent electrospray ionisation (ESI)/MSD TOF. Drying gas (N_2) flow was 12 litres/min; nebuliser pressure was 45 psig; drying gas temperature was 350°C. For ESI analysis, the parameters were as follows: capillary voltage – 4000 V; fragmentor – 140 V;

skimmer – 60 V; Oct RF V – 250 V, for positive and negative modes. The mass range was from 100 to 2000 *m/z*.

Biochemical assays

All biochemical assays were performed with *L. deterrimus* and *C. sativa* extracts as well as with their combination MIX *Ld/Cs*. The concentrations of individual extracts used to define MIX *Ld/Cs* are given in detail in the Cell culture and treatment section.

2,2-Diphenyl-1-picrylhydrazyl free radical-scavenging assay

The free radical-scavenging activity of the *L. deterrimus* and *C. sativa* extracts were measured by the decrease in the absorbance of ethanolic 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution at 517 nm in the presence of the extract, following the standard method[11]. The inhibitory percentage of DPPH was calculated according to the formula: percentage of inhibition $= ((A_{blanc} - A_{test})/A_{blanc}) \times 100$, where A_{blanc} is the absorbance of the ethanolic DPPH solution and A_{test} is the absorbance of DPPH in the solution with the extract or a standard (ascorbic acid).

Total reducing power

The reducing power of the *L. deterrimus* and *C. sativa* extracts was determined by using the potassium ferricyanide–ferric chloride method according to Oyaizu[12]. The absorbance was measured at 700 nm. Ascorbic acid was used as a positive control.

Hydrogen peroxide-scavenging activity

The H_2O_2-scavenging ability of the *L. deterrimus* and *C. sativa* extracts was determined by the decrease in the absorbance (A) of H_2O_2 solution at 230 nm in the presence of the extract, according to the method of Ruch *et al.*[13]. The percentage of H_2O_2 scavenging of the extracts and standard compound (ascorbic acid) was calculated as: H_2O_2-scavenging effect $(\%) = ((A_{control} - A_{sample})/A_{control}) \times 100$.

Ferrous ion chelation

The ferrous ion-chelating activity of the *L. deterrimus* and *C. sativa* extracts was measured by the decrease in absorbance (A) at 562 nm of the Fe(II)-ferrozine complex in the presence of different concentrations of the extracts, according to Dinis *et al.*[14]. The percentage of inhibition of the ferrozine–Fe^{2+} complex formation was calculated using the following formula: ferrous ion-chelating effect $(\%) = ((A_{control} - A_{sample})/A_{control}) \times 100$. EDTA was used as a positive control.

Nitric oxide radical scavenging

NO-scavenging potential of the examined extracts was estimated by the Griess–Ilosvay reaction[15]. At physiological pH, NO generated from aqueous sodium nitroprusside solution interacts with oxygen to produce nitrite ions, which may be quantified by the Griess–Ilosvay reaction. The percentage of NO scavenging of the extracts and standard compound (curcumin) was calculated from the following formula: NO-scavenging effect $(\%) = ((A_{control} - A_{sample})/A_{control}) \times 100$, where A is the absorbance at 540 nm.

Cell culture and treatment

Rin-5F cells (ATCC 11 605) were cultivated at 37°C under 5 % CO_2 in a humidified atmosphere in Roswell Park Memorial Institute (RPMI) medium, supplemented with 10 % fetal calf serum, 100 U penicillin/ml and 100 μg streptomycin/ml. The medium was exchanged every 72 h. For the treatment, cells were incubated for 6 h with 7·5 mM-STZ (MP Biomedicals, 100557), unless otherwise indicated, dissolved in citrate buffer (pH 4·5). After the incubation with STZ, cells were processed immediately. The mushroom and chestnut extracts were dissolved in the RPMI medium for the Rin-5F cell treatment, at concentrations corresponding to the calculated IC_{50} values obtained from the DPPH assay, but adjusted with respect to the actual concentrations in the cuvette and taking into consideration the factor of dilution[16]. Therefore, the *C. sativa* extract was used at a concentration of 0·02 mg/ml, whereas the concentration of the extract from *L. deterrimus* was 0·4 mg/ml. The same concentrations of individual extracts were mixed and used in experiments as a combination, abbreviated as MIX *Ld/Cs*. Depending on the experimental design, the extracts were combined with 7·5 mM-STZ before applying to the Rin-5F cells.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide viability test

Rin-5F cell viability was estimated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) viability assay. Rin-5F cells were grown in ninety-six-well plates, treated with 7·5 mM-STZ in combination with different extracts, and after 6 h, 200 μl MTT (Sigma, M5655) at a concentration of 0·5 mg/ml in the RPMI medium was added to each well. After incubation for 2 h in the dark, the insoluble purple formazan products formed in living cells were dissolved in dimethyl sulfoxide. Formazan product formation was quantified by measuring the absorbance at 570 nm. Cell viability was expressed in percentages after comparison with control cells that were assumed to be 100 % viable.

Comet assay

The level of DNA damage produced by STZ was examined using the alkaline Comet assay according to Singh *et al.*[17]. Control and Rin-5F cells incubated with 7·5 mM-STZ and STZ/extracts were mixed with low-melting agarose and

applied to a microscope slide. After lysis and electrophoresis, the slides were stained with Sybr Green I (Sigma-Aldrich, S9430). DNA damage was quantified by measuring the displacement between the genetic material of the nucleus (comet 'head') and the resulting comet 'tail'. The tail moment has been suggested to be an appropriate index of induced DNA damage, considering both the migration of the genetic material and the relative amount of DNA in the tail. Images were analysed with TriTekCometScore™ Freeware version 1.5 (available at http://www.AutoComet.com).

Thiobarbituric acid-reactive substance assay

Lipid peroxidation was assessed using the thiobarbituric acid-reactive substance assay. The following chemicals were used: thiobarbituric acid (Sigma, T5500); tetramethoxypropane (Sigma, 108383); pyridine (Sigma, 320498). Untreated, STZ- and STZ/extract-treated Rin-5F cells (5×10^6 cells) were homogenised using a Potter–Elvehjem Teflon-glass homogeniser in ice-cold KCl buffer (1·15 M-KCl, pH 7·4) to produce a 1:2 (w/v) cell homogenate. An aliquot (0·1 ml) of the homogenate was mixed with 0·2 ml of 8·1 % SDS, 1·5 ml of 20 % acetic acid (pH 3·5), 1·5 ml of 0·8 % thiobarbituric acid and 0·7 ml of water. The mixture was heated at 95 °C for 60 min and then supplemented with 1 ml of water and 5 ml of *n*-butanol–pyridine (15:1, v/v), mixed and centrifuged at 3000 ***g*** for 10 min. The absorbance of the supernatants was measured at 532 nm and the total amount of Malondialdehyde (MDA) was calculated from the MDA standard curve (the concentrations of MDA were from 12·5 nM to 1 μM).

Measurement of reduced glutathione, glutathione disulfide and glutathionylated proteins

Untreated, STZ- and STZ/extract-treated Rin-5F cells (1×10^6 cells) were resuspended in 2·5 % sulfosalicylic acid and homogenised using a Potter–Elvehjem Teflon-glass homogeniser. After incubation for 5 min at room temperature, the homogenate was centrifuged at 5000 ***g*** for 5 min at 4°C. An aliquot of the supernatant was analysed for the content of glutathione, while acid-precipitated proteins were used for glutathionylated proteins (GSSP) measurement. Before the estimation of glutathione, triethanolamine was added in deproteinated samples (0·2 M) to increase the pH of the samples. Thereafter, 50 μl of the sample and 150 μl of the reaction mixture (0·1 M-sodium phosphate buffer (pH 7·5) containing 1 mM-EDTA, 0·3 mM-5,5′-dithiobis-(2-nitrobenzoic acid), 0·4 mM-NADPH and 1 U/ml glutathione reductase I) were incubated for 30 min at room temperature. The reaction rate was monitored by measuring the absorption at 412 nm. Reduced glutathione (GSH) contents were evaluated using a calibration curve. Glutathione disulfide (GSSG) was quantified after derivatisation of GSH with 10 mM-2-vinylpyridine. After a 1 h incubation at room temperature, 2-vinylpyridine-treated samples and standards were assayed as described above using a calibration curve.

To measure GSSP, acid-precipitated proteins were resuspended and washed twice with 1·5 % TCA. After washing, the proteins were resuspended in 0·5 ml of basic solution (9:1, v/v, 0·1 mM-phosphate buffer (pH 7·4):0·25 mM-NaOH), and stirred for 30 min at room temperature. Then, 40 μl of 60 % TCA were added to precipitate the proteins; GSH was determined in the supernatant, as described above.

Measurement of nitrite by the Griess reaction method

From the control and treated Rin-5F cells, 200 μl of supernatant medium were deproteinised in 5 % sulfosalicylic acid. The deproteinised samples were neutralised with 17·4 mM-NaOH and 100 μl were transferred to ninety-six-well plates. Then 100 μl of Griess reagent were added to each well stepwise: first 50 μl of 1 % sulphanilamide (Griess A) and after 5 min of incubation, 50 μl of 0·1 % naphthylethylenediamine dihydrochloride, prepared in 5 % H_3PO_4 (Griess B), was added to each well. The reagents were mixed for 1 min at 185 rpm on a shaker and the plates were incubated for additional 5 min at room temperature in the dark. The absorbance was measured at 540 nm.

Superoxide dismutase and catalase activities

Rin-5F cells were rinsed with the RPMI medium and scraped with a sterile rubber scraper. The cells were centrifuged for 10 min at 200 ***g***, resuspended in 50 mM-potassium phosphate buffer and centrifuged for 10 min at 200 ***g***. After sonication on ice by a 30 s burst, the homogenates were centrifuged for 1 min at 14 000 ***g*** at 4°C, and the supernatant was collected for the determination of catalase (CAT) and superoxide dismutase (SOD) activities. Protein concentrations were determined according to the method of Lowry *et al.*[18]. Total SOD, based on its capacity to inhibit the auto-oxidation of epinephrine to adrenochrome, was measured by the epinephrine method[19]. SOD activity was expressed as U/mg protein. MnSOD activity was assessed after preincubation with 8 mM-KCN, while CuZn-SOD activity represented the calculated difference between total SOD and MnSOD activities. CAT activity was measured by the rate of H_2O_2 decomposition and expressed as μmol H_2O_2/min per mg protein[20].

Immunoblot analysis

In brief, 20 μg of the cell lysate isolated from the Rin-5F cells or proteins precipitated from the cell-culture medium after the cell treatment were separated by 12 % SDS-PAGE and electroblotted onto a polyvinylidene difluoride membrane. Immunoblot analysis was performed using rabbit polyclonal anti-NF-κB p65 antibody (1:1000, C-20; Santa Cruz), rabbit polyclonal anti-pNF-κB p65 antibody (1:1000, Ser-311; Santa Cruz), anti-MnSOD antibody (1:1000, FL-222; Santa Cruz), goat polyclonal anti-Cu/ZnSOD antibody (1:750, C-17; Santa Cruz) and mouse polyclonal anti-tubulin-α antibody (P-16; Santa Cruz). The blots were then probed with appropriate horseradish peroxidase-conjugated IgG (all from Santa Cruz). Staining was performed by the chemiluminescent technique according to the manufacturer's instructions (Santa Cruz Biotechnology).

Statistical analysis

In every experiment, three different samples were used and all the assays were carried out in triplicate. All results are expressed as mean values, with their standard errors. Differences between the corresponding means were verified by the Mann–Whitney *U* test. A *P* value of less than 0·05 was considered to be statistically significant.

Results

Qualitative and quantitative determinations of the extracts' contents

It has been reported that the antioxidant activity of plant material correlates with the content of their phenolic compounds[21]. The presence of aromatic ring structures and the number and relative positions of hydroxyl groups provide antioxidant potential to many phenolic compounds[22]. The qualitative and quantitative analysis of the compounds present in the *C. sativa* and *L. deterrimus* extracts was carried out using a HPLC/DAD method (Table 1). For the *C. sativa* extract, UV/vis maximums, which are characteristic for various classes of polyphenolic compound, were compared with the literature data[23,24]. The identity of tryptophan and *p*-hydroxybenzoic acid from the *L. deterrimus* extract was confirmed by comparison of retention times with those of standard compounds. The identified compounds from the *C. sativa* extract could be categorised as ellagic acid/ellagic acid derivatives, gallic acid/gallic acid derivatives and flavonoid structures, with the highest content of ellagic acid structures (100·4 mg/g), followed by gallic acid derivatives (59·6 mg/g) and flavonoid structures (24·1 mg/g). In the *L. deterrimus* extract, the only detected compounds with a phenolic structure were tryptophan and *p*-hydroxybenzoic acid, present in quantities of 0·07 and 0·034 mg/g, respectively, while other detected compounds were unsaturated and oxy(hydroxy- or epoxy-) fatty acids.

Analysis of the antioxidative properties of the examined extracts

In evaluating the possible antioxidant action of a compound or extract on a biological system, it is a standard practice to start with biochemical assays since a compound that exhibits poor antioxidant activity in assays *in vitro* is unlikely to be better *in vivo*. The antioxidant activities of the *C. sativa* and *L. deterrimus* extracts were examined as a function of their concentrations in several biochemical assays (Fig. 1). Each assay was performed for two separate extracts and their combination (MIX *Ld*/*Cs*), and the obtained results were compared with standard substances at the same concentration. The concentration ranges differed for the two extracts and were

Table 1. Quantitative HPLC/diode array detection and LC/MS data of *Lactarius deterrimus* and *Castanea sativa* extracts

Extract	RT (min)	UV λ_{max} (nm)	Molecular mass (Da)	Molecular formula	Compound	Content (mg/g)
C. sativa	8·61	254, 374	596·0414	$C_{27}H_{16}O_{16}$	Ellagitannin	8·9
	9·53	218, 276	498·1002	$C_{21}H_{22}O_{14}$	Gallic acid derivative	11·9
	10·00	218, 272	184·0367	$C_8H_8O_5$	Methyl gallate	30·8
	11·39	216, 276	498·1008	$C_{21}H_{22}O_{14}$	Gallic acid derivative	8·8
	12·00	224, 268sh	1130·1081	$C_{50}H_{34}O_{31}$	Ellagitannin	2·9
	13·02	224, 268sh	1116·0927	$C_{49}H_{32}O_{31}$	Ellagitannin	4·5
	15·16	218, 256, 298, 366	470·0127	$C_{21}H_{10}O_{13}$	Flavogallonic acid	9·3
	17·92	216, 276	498·1006	$C_{21}H_{22}O_{14}$	Gallic acid derivative	1·8
	18·21	218, 256, 298, 366	484·0275	$C_{22}H_{12}O_{13}$	Flavogallonic acid methyl ester	3·5
	18·53	278, 344	306·0376	$C_{14}H_{10}O_8$	Protocatechuic acid derivative	2·7
	21·16	224, 272	366·0585	$C_{16}H_{14}O_{10}$	Dehydrodigallic acid, dimethyl ester	6·3
	22·34	254, 368	302·0060	$C_{14}H_6O_8$	Ellagic acid	84·1
	24·91	220, 254, 302, 364	484·0271	$C_{22}H_{12}O_{13}$	Valoneic acid dilactone, methyl ester	73·7
	27·84	202, 256, 372	302·0423	$C_{15}H_{10}O_7$	Quercetin	12·7
	28·41	228, 298, 310	178·0626	$C_{10}H_{10}O_3$	*p*-Methoxycinnamic acid	20·5
	29·72	196, 222sh, 264, 368	286·0478	$C_{15}H_{10}O_6$	Kaempferol	7·4
	30·2	254, 266sh, 306sh, 326sh, 370	316·0575	$C_{16}H_{12}O_7$	Isorhamnetin	4·0
L. deterrimus	7·6	220, 268sh, 278, 288sh	204·0889	$C_{11}H_{12}N_2O_2$	Tryptophan	0·0695
	8·9	256	138·0336	$C_7H_6O_3$	*p*-Hydroxybenzoic acid	0·0338
	30·7	–	330·2575	$C_{22}H_{34}O_2$	22 : 5 fatty acid	–
	30·8	–	330·2567	$C_{22}H_{34}O_2$	22 : 5 fatty acid	–
	32·4	–	330·2571	$C_{22}H_{34}O_2$	22 : 5 fatty acid	–
	32·6	–	330·2567	$C_{22}H_{34}O_2$	22 : 5 fatty acid	–
	32·7	–	332·2719	$C_{22}H_{36}O_2$	22 : 4 fatty acid	–
	33·3	–	332·2723	$C_{22}H_{36}O_2$	22 : 4 fatty acid	–
	34·9	–	172·1458	$C_{10}H_{20}O_2$	Decanoic acid	–
	35·3	–	296·2323	$C_{18}H_{32}O_3$	18 : 2 oxy-fatty acid	–
	35·5	–	296·2331	$C_{18}H_{32}O_3$	18 : 2 oxy-fatty acid	–
	36·2	–	294·2202	$C_{18}H_{30}O_3$	18 : 3 oxy-fatty acid	–
	37·3	–	294·2214	$C_{18}H_{30}O_3$	18 : 3 oxy-fatty acid	–
	37·7	–	298·2519	$C_{18}H_{34}O_3$	18 : 1 oxy-fatty acid	–

RT, retention time, sh, wavelength shift.

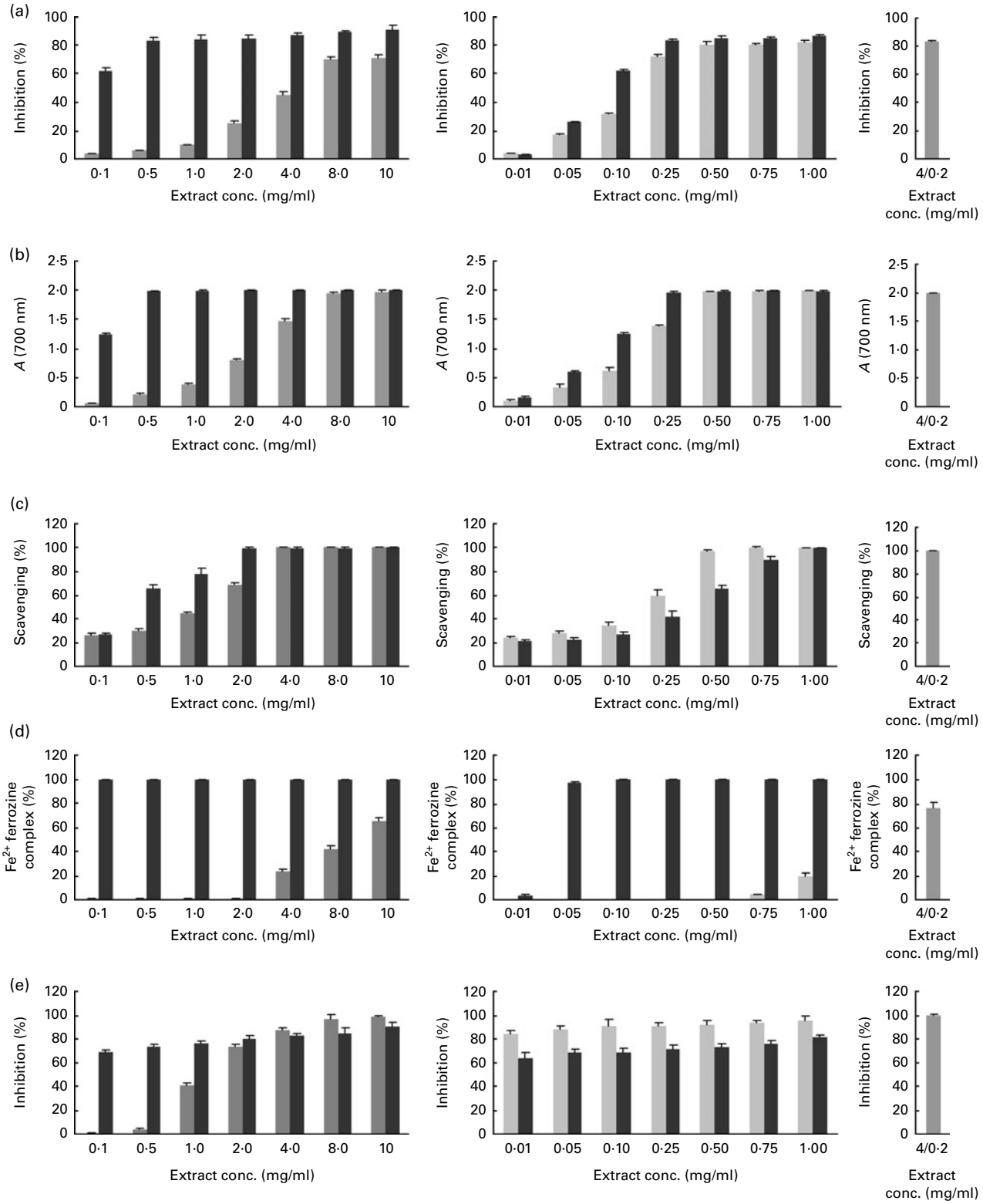

Fig. 1. Antioxidant and scavenging properties of *Lactarius deterrimus* and *Castanea sativa* extracts and their combination (MIX *Ld*/*Cs*). (a) Radical-scavenging activity on the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical as a function of extract concentration (conc.). (b) Reducing power as a function of extract concentration. (c) H_2O_2-scavenging activity as a function of extract concentration. (d) Chelating effect of the extracts as a function of their concentration. (e) NO-scavenging activity as a function of extract concentration. (a–c) ■, *L. deterrimus*; ■, ascorbic acid; ■, *C. sativa*; ■, MIX *Ld*/*Cs*. (d) ■, *L. deterrimus*; ■, EDTA; ■, *C. sativa*; ■, MIX *Ld*/*Cs*. (e) ■, *L. deterrimus*; ■, curcumin; ■, *C. sativa*; ■, MIX *Ld*/*Cs*. *A*, absorption.

defined in preliminary assays. A strong correlation with the phenolic/flavonoid content was observed and accordingly, the *L. deterrimus* extract was used at a concentration range that was 10-fold higher than that of the *C. sativa* extract.

The radical-scavenging activity of the extracts was assessed by the DPPH assay (Fig. 1(a)). Although the scavenging activity of both extracts increased with concentration, the DPPH assay revealed that the *L. deterrimus* extract possessed a relatively low scavenging activity. The *C. sativa* extract was much more effective, and it almost reached the value of ascorbic acid that was used as a reference compound. The combination of the extracts, MIX *Ld/Cs*, did not exhibit a stronger scavenging activity than the individual extracts, since its scavenging potential was at the level of the chestnut extract which was already at the level of the reference compound.

The reducing power of the extracts increased in a concentration-dependent manner (Fig. 1(b)). Both extracts were capable of reducing Fe(III), suggesting the potential to block free radical chain reactions. The mushroom extract reached the ascorbic acid value (the reference substance) at a concentration of 8 mg/ml, while the chestnut extract accomplished the same effect at 0·5 mg/ml. MIX *Ld/Cs* was effective as ascorbic acid.

Since H_2O_2 can generate highly reactive hydroxyl radicals through an interaction with transition metal ions, the generation of hydroxyl radicals can be prevented by scavenging of H_2O_2 or by chelation of metal ions. The chestnut and mushroom extracts were further tested for ability to block the Fenton reaction. Both extracts exhibited a H_2O_2-scavenging activity that depended on their concentration (Fig. 1(c)). The mushroom extract reached 100 % of scavenging at a concentration of 4 mg/ml, whereas the chestnut extract scavenged H_2O_2 even more effectively than ascorbic acid, reaching complete scavenging at 0·75 mg/ml. Conversely, the two extracts exhibited very poor metal-chelating capability (Fig. 1(d)). Detectable chelation was observed only above 4 mg/ml for the mushroom extract, and at 0·75 mg/ml for the chestnut extract. Surprisingly, the combination of the extracts MIX *Ld/Cs* possessed a strong chelating activity, providing nearly 80 % chelation of the ferrous ion.

Besides ROS, RNS are also responsible for altering the structural and functional behaviour of many cellular components. The two extracts and MIX *Ld/Cs* were therefore tested for NO-scavenging activity (Fig. 1(e)). The scavenging of NO by extracts increased in a concentration-dependent manner. Complete scavenging was achieved at 8 mg/ml for the mushroom extract and 1 mg/ml for the chestnut extract, which exhibited a better scavenging activity than the referent compound curcumin. MIX *Ld/Cs* scavenged 100 % of NO, as expected.

In conclusion, the biochemical assays revealed that both *C. sativa* and *L. deterrimus* extracts possess antioxidant activities, albeit to different extents. While the extract obtained from the mushroom had moderate antioxidant activity, the chestnut extract showed excellent antioxidant properties. The best antioxidative activity was obtained when the extracts were combined as in the MIX *Ld/Cs*.

The protective effects of the extracts on pancreatic β-cell survival

Our next aim was to examine the ability of the extracts to increase the survival of pancreatic β-cells after the STZ treatment. Pancreatic Rin-5F cells were treated with STZ that is taken up preferentially by pancreatic β-cells through the GLUT2 transporter. The mechanism of STZ-induced β-cell toxicity relies on its alkylating properties and the synergistic actions of both ROS and RNS, which are generated as a result of the STZ action on mitochondria and the increased activity of xanthine oxidase[25]. To estimate STZ toxicity, Rin-5F cells were treated with increasing concentrations of STZ for 6 h. Cell viability was assessed by the MTT viability assay (Fig. 2(a)). The number of viable cells gradually decreased with increasing STZ concentration. Treatment with

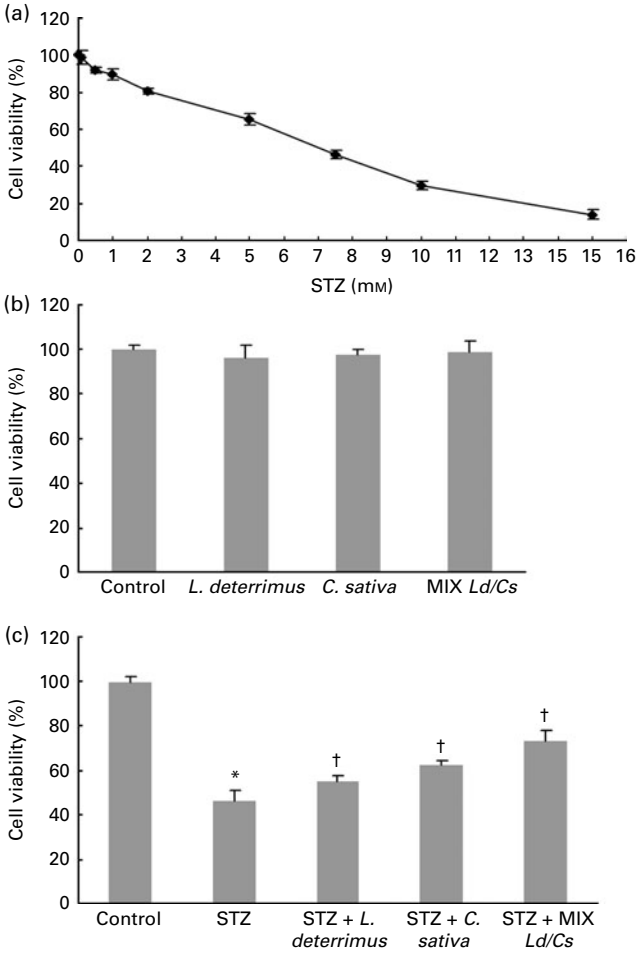

Fig. 2. *Lactarius deterrimus* and *Castanea sativa* extracts and their combination (MIX *Ld/Cs*) increase the survival of pancreatic β-cells *in vitro*. (a) Viability assay performed on Rin-5F cells after treatment with increasing concentrations of streptozotocin (STZ). (b) Application of *L. deterrimus*, *C. sativa* and MIX *Ld/Cs* on Rin-5F cell viability. (c) Viability assay after the co-treatment of Rin-5F cells with STZ and *L. deterrimus*, *C. sativa* or MIX *Ld/Cs*. Values are means of three experiments performed in triplicate, with standard errors represented by vertical bars. * Mean value was significantly different from that of untreated control cells ($P<0·05$). † Mean values were significantly different from those of STZ-treated cells ($P<0·05$).

7·5 mM-STZ induced cell death in more than 50 % of cells and was used in all further experiments.

Initially, cytotoxicities of both extracts and MIX *Ld/Cs* were tested. The two extracts and MIX *Ld/Cs* were applied to Rin-5F cells without previous treatment with STZ. The viability assay revealed that the extracts and MIX *Ld/Cs* did not exhibit cytotoxic activity on Rin-5F cells (Fig. 2(b)). In contrast, treatment of Rin-5F cells simultaneously with 7·5 mM-STZ and either with the mushroom extract, the chestnut extract or MIX *Ld/Cs* led to a statistically significant improvement of Rin-5F cell survival (Fig. 2(c)). Cell viability of 46·3 % obtained after the STZ treatment increased to 55·1 % in the presence of the mushroom extract, to 62·6 % with the chestnut extract and to 75·2 % when the extracts were used in combination. Therefore, the treatment with the extract MIX *Ld/Cs* provided the highest degree of β-cell protection, influencing cell viability more effectively than the sum of effects of the individual extracts.

The extracts' effect on genome stability

We used the Comet assay to examine whether the extracts are capable of protecting DNA from damage after the STZ treatment. Rin-5F cells that were treated with 7·5 mM-STZ alone or with STZ in combination with the mushroom extract, the chestnut extract or MIX *Ld/Cs* were subjected to the alkaline Comet assay (Fig. 3). Representative images of the cells are shown in Fig. 3(a; i–v). In the control sample, the great majority of the analysed cells did not exhibit DNA damage (Fig. 3(a), i). The STZ treatment resulted in extensively damaged DNA, since large amounts of DNA were present in the comet tails (Fig. 3(a), ii). Simultaneous treatment of Rin-5F cells with STZ and the mushroom extract (Fig. 3(a), iii), the chestnut extract (Fig. 3(a), iv) and MIX *Ld/Cs* (Fig. 3(a), v) provided increased protection, manifested as lower levels of DNA damage, in comparison with the STZ-treated cells. The extent of DNA damage was expressed as the tail moment, the parameter that considers both the comet tail length and tail intensity (Fig. 3(b)). Analysis of the tail moments revealed that the combination of the extracts MIX *Ld/Cs* provided the best protection against STZ-induced DNA damage, although both the mushroom and chestnut extracts also significantly reduced the tail moment when compared with the STZ-treated cells. Taken together, the results of the Comet assay showed that the mushroom and the chestnut extracts possess a similar and very pronounced potential for genome stabilisation, even though they differ in their respective radical-scavenging activities. Also, in the case of genome

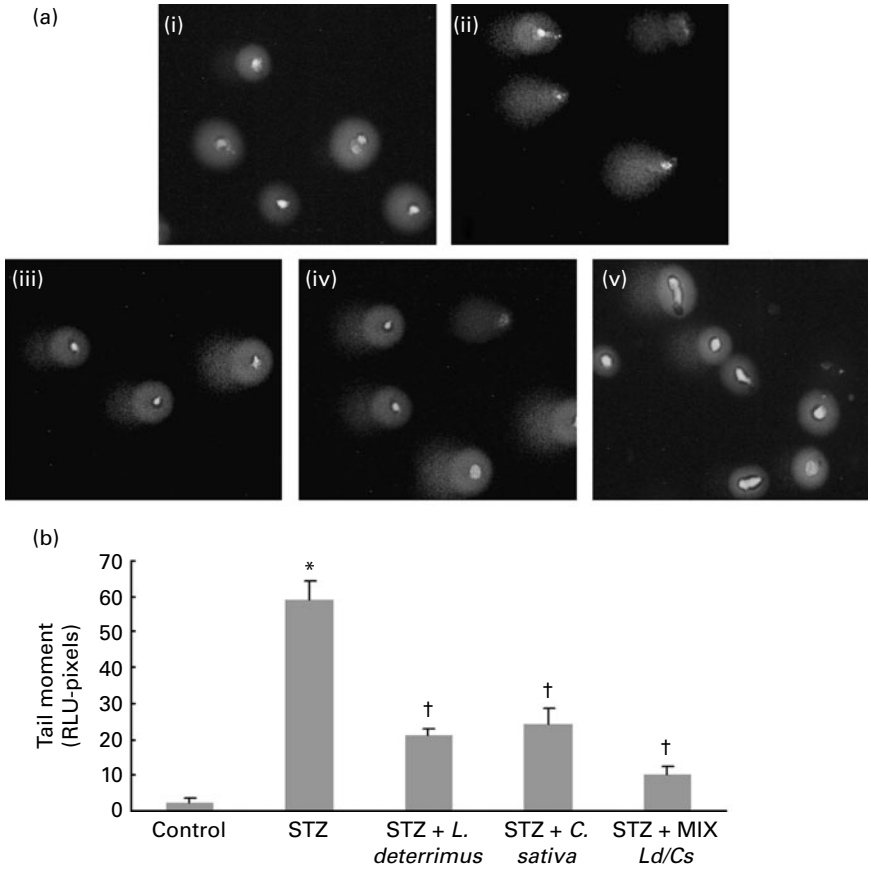

Fig. 3. DNA damage after the streptozotocin (STZ) treatment and co-treatment with *Lactarius deterrimus*, *Castanea sativa* or their combinations (MIX *Ld/Cs*) estimated by the alkaline Comet assay. (a) Representative images of comets: (i) control Rin-5F cells; (ii) cells treated with STZ; (iii) cells co-treated with STZ and *L. deterrimus* extract; (iv) cells co-treated with STZ and *C. sativa* extract; (v) cells co-treated with STZ and MIX *Ld/Cs*. (b) Assessment of DNA damage using tail moment as a parameter of DNA damage. Values are means of three experiments performed in triplicate, with standard errors represented by vertical bars. * Mean value was significantly different from that of untreated control cells ($P<0·05$). † Mean values were significantly different from those of STZ-treated cells ($P<0·05$).

stabilisation, the optimal combination of the extracts provided the most effective DNA protection.

The beneficial effects of the extracts on cellular biomarkers of oxidative stress

The role of oxidative stress in β-cell death or dysfunction that ultimately results in the development of diabetes is well established. Lipid peroxidation reflects irreversible oxidative changes of membranes which impair the functioning of cell organelles and thus can be used as a biomarker of oxidative stress[26]. Therefore, our next aim was to evaluate the possible antioxidant effects of the extracts in Rin-5F cells by measuring the level of lipid peroxidation using the thiobarbituric acid-reactive substance assay (Fig. 4(a)). The STZ treatment led to an increase in the lipid peroxidation level in comparison with the control sample. Comparison of MDA levels in the STZ-treated cells with the cells simultaneously treated with STZ and the extracts revealed that both extracts, as well as MIX *Ld/Cs*, exhibited statistically significant inhibition of

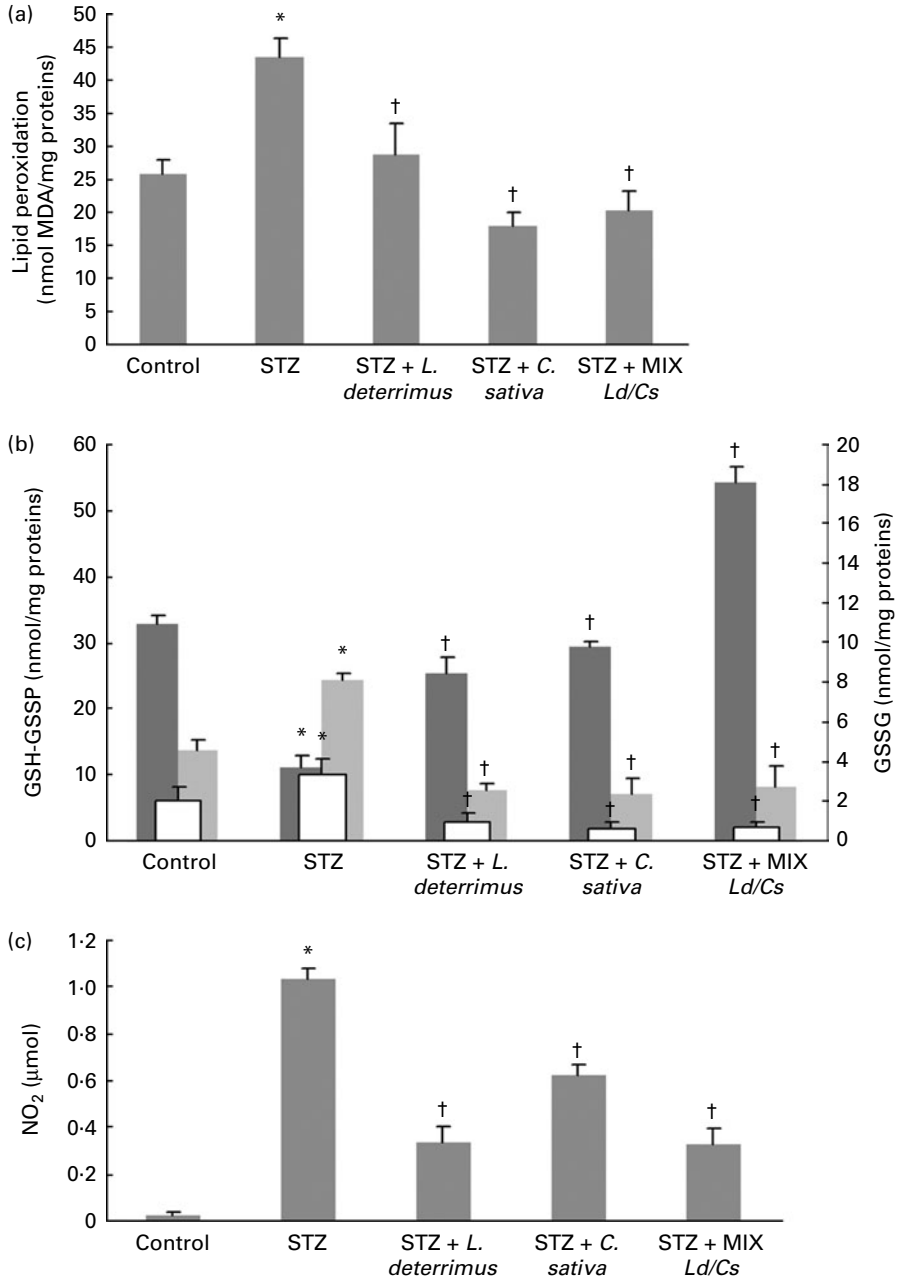

Fig. 4. Evaluation of the redox status of Rin-5F cells after the streptozotocin (STZ) treatment and co-treatment with *Lactarius deterrimus*, *Castanea sativa* or their combination (MIX *Ld/Cs*). (a) Lipid peroxidation level estimated by the thiobarbituric acid-reactive substance assay. MDA, malondialdehyde. (b) Concentration of reduced glutathione (GSH; ■), glutathione disulfide (GSSG; □) and content of glutathionylated proteins (GSSP; ■). (c) Concentration of nitrite determined by the Griess reaction. Values are means of three experiments performed in triplicate, with standard errors represented by vertical bars. * Mean values were significantly different from those of untreated control cells ($P<0·05$). † Mean values were significantly different from those of STZ-treated cells ($P<0·05$).

lipid peroxidation (Fig. 4(a)). The chestnut extract showed the highest potential for the inhibition of lipid peroxidation, based on the MDA level that was 2·4-fold lower than that in the STZ-treated cells. A similar effect was obtained in cells that were treated with MIX *Ld/Cs*, while treatment with the mushroom extract led to a 1·5-fold decrease in the MDA level in comparison with the STZ-treated cells.

Another valuable marker of oxidative stress is the level of the GSH. During oxidative stress, the concentration of GSH decreases, accompanied by an increase in its oxidised form (GSSG). The STZ treatment of Rin-5F cells induced a powerful oxidative stress, based on the GSH and GSSG levels in this sample (Fig. 4(b)). Treatments with the mushroom extract and, in particular, with the chestnut extract together with STZ preserved glutathione in its reduced state. The strongest antioxidant effect was displayed by MIX *Ld/Cs* which induced an almost 5-fold increase in GSH, together with a 5·1-fold decrease in the level of GSSG in comparison with the STZ-treated cells (Fig. 4(b)).

During prolonged states of oxidative perturbation, GSSG accumulates within the cell and can contribute to protein–glutathione adduct formation[27]. Therefore, in addition to the levels of reduced GSH and GSSG, the content of GSSP can be used as an important marker of oxidative stress[28]. STZ, as an oxidative stress inducer, provoked an increase in the GSSP content in the Rin-5F cells (Fig. 4(b)). However, the presence of the mushroom and chestnut extracts, as well as of the MIX *Ld/Cs*, diminished the amount of glutathionylated proteins. Both extracts and their combination exhibited statistically significant and prominent potential for pancreatic β-cell protection against STZ-induced glutathionylation of proteins.

Although NO participates in numerous physiological processes, it is also the primary source of all RNS in biological systems[29]. We used the Griess reaction to detect nitrite, which is formed by the spontaneous oxidation of NO under physiological conditions and which represents a reliable estimate of the NO output *in vivo*[30]. In the control sample, the presence of nitrites was negligible. The STZ treatment induced a significant increase in the nitrite content (Fig. 4(c)). Treatment with the mushroom extract induced a 3·1-fold decrease in the nitrite content, the same effect as obtained for MIX *Ld/Cs* ($P<0·05$). The chestnut extract was less effective, as it induced a 1·7-fold decrease of nitrites compared with the STZ treatment ($P<0·05$).

The extracts exhibit a valuable effect on the antioxidant defence system in pancreatic β-cells

Since oxidative stress results from either increased production of reactive species and/or from decreased activity of the antioxidant defence system, our next aim was to determine whether the observed improvement of the redox status after the treatment with the extracts resulted from changes in activities of antioxidant enzymes. Total SOD, MnSOD and CuZnSOD activities were measured in the control Rin-5F cells, STZ-treated cells and cells simultaneously treated with STZ and the extracts (Fig. 5(a)). The STZ treatment led to a significant increase in total SOD activity, which was for the most part the result of an increase in MnSOD activity. Treatment with the mushroom extract did not provoke a considerable effect on overall SOD activity, although some decrease in MnSOD and an increase in CuZnSOD activity could be observed (Fig. 5(a)). However, the chestnut extract almost completely reduced SOD activity to the control level, with only MnSOD remaining 16 % above the activity observed in the control cells. The MIX *Ld/Cs* significantly reduced total SOD and MnSOD activities (by 24 and 15 %, respectively) in comparison with the STZ treatment, although the chestnut extract alone was more effective in reducing SOD activity than the MIX *Ld/Cs* (Fig. 5(a)).

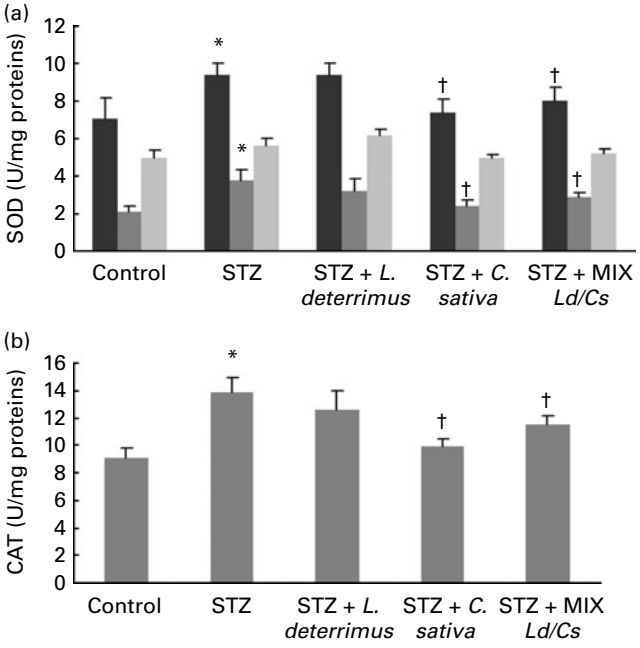

Fig. 5. Activity of antioxidant enzymes in Rin-5F cells after the streptozotocin (STZ) treatment and co-treatment with *Lactarius deterrimus*, *Castanea sativa*) or their combinations (MIX *Ld/Cs*). (a) Total superoxide dismutase (SOD; ■), MnSOD (■) and CuZnSOD (■) activities. (b) Catalase (CAT) activity. Values are means of three experiments performed in triplicate, with standard errors represented by vertical bars. * Mean values were significantly different from those of untreated control cells ($P<0·05$). † Mean values were significantly different from those of STZ-treated cells ($P<0·05$).

In addition to increased SOD activity, the STZ treatment also enhanced CAT activity (Fig. 5(b)). Simultaneous treatments with the mushroom extract, the chestnut extract and with the MIX *Ld/Cs* exerted different effects on the activity of this enzyme. The most effective reduction of CAT activity was observed with the chestnut extract (CAT activity reduction for 28 % relative to the STZ treatment). The mushroom extract reduced CAT activity to a statistically insignificant level, while the MIX *Ld/Cs* led to a statistically relevant diminishment of CAT activity for 17 % (Fig. 5(b)).

Next, we examined the effect of the different treatments on the expression profiles of proteins involved in the maintenance of a physiological redox status in the cell. The Rin-5F cell lysates obtained from the control cells (Fig. 6(a), lane 1), STZ-treated cells (lane 2) and cells treated with the

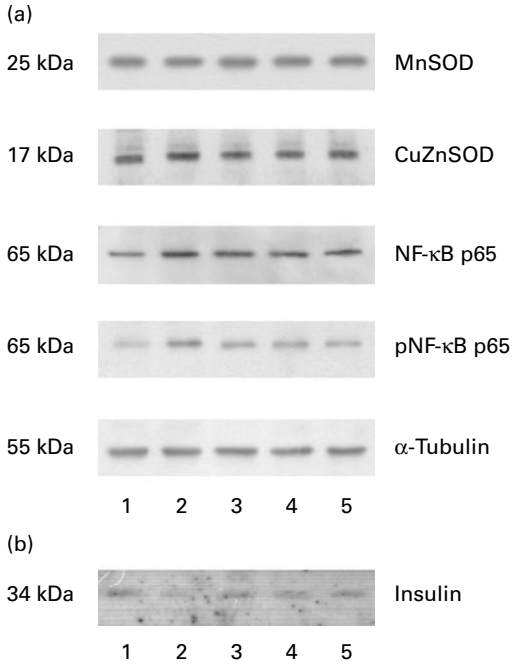

Fig. 6. Immunoblot analysis of Rin-5F lysates (a) and proteins released in cell-culture medium (b) from control cells (lane 1), streptozotocin (STZ)-treated cells (lane 2), STZ + *Lactarius deterrimus*-treated cells (lane 3), STZ + *Castanea sativa*-treated cells (lane 4) and cells treated with STZ and their combinations (lane 5). SOD, superoxide dismutase.

extracts (lane 3, mushroom; lane 4, chestnut; lane 5, MIX *Ld/Cs*) were subjected to immunoblot analysis. Although alterations in MnSOD activity were observed after the treatments, changes at the protein level were not detected. Slight alterations were observed at the protein level of CuZnSOD. Namely, immunoblot analysis of CuZnSOD showed that the STZ treatment of Rin-5F cells induced a small increase in its protein level (Fig. 6(a), lane 2), while the simultaneous treatment with STZ and the mushroom, chestnut and MIX *Ld/Cs* (lanes 3, 4 and 5, respectively) brought about a reduction of CuZnSOD to the level observed in the control sample (lane 1).

Transcription factor NF-κB, sometimes referred to as a redox-sensitive transcription factor, is central to the control of the cellular response, which is triggered by many stimuli including oxidative stress. Once released from the inhibitory molecule IκB, NF-κB is translocated to the nucleus where its phosphorylated form activates transcription of its target genes. In the STZ-treated cell lysates, a higher level of NF-κB p65 and its phosphorylated form pNF-κB p65 compared with the control cell lysate were observed (Fig. 6(a), lane 1), clearly indicating that the STZ treatment induced oxidative stress in the cells (lane 2). A decrease in the level of NF-κB p65 and a decrease in the presence of its phosphorylated form in all samples treated with the extracts (lanes 3, 4 and 5) revealed that the extracts contributed towards an improved redox status of Rin-5F cells.

Bearing in mind that reactive species and oxidative stress often induce β-cell dysfunction, we next examined the protein mix released into the cell-culture medium, with anti-insulin antibody by immunoblot analysis (Fig. 6(b)). The control cells were functional since they released the largest amount of insulin in the medium (lane 1). The STZ treatment led to a considerable reduction in insulin secretion (lane 2), while the augmented presence of insulin in the medium obtained from cells that were treated with STZ and the mushroom extract, the chestnut extract and with MIX *Ld/Cs* points to a preserved functioning of Rin-5F cells (lanes 3, 4 and 5, respectively). This result provides additional support for the conclusion that the examined extracts, and in particular the MIX *Ld/Cs*, possess an important antioxidative potential through which they maintain proper cell functioning and protect pancreatic β-cell from death.

Discussion

In the present paper, the antioxidant properties of extracts obtained from the mushroom *L. deterrimus*, the chestnut *C. sativa* and their combination MIX *Ld/Cs* were assessed by different biochemical assays. The *L. deterrimus* ethanolic extract exhibited relatively low radical-scavenging activity (DPPH assay), reducing power and chelating effect (Fig. 1), which is in agreement with Sarikurkcu *et al.*[31]. In the concentration range we used, the *L. deterrimus* extract exhibited good $H_2O_2^-$ and NO-scavenging activities that, to our knowledge, have not been previously reported. The ethanolic extract from the spiny burrs of *C. sativa* showed remarkably high antioxidant properties based on biochemical assays, with the exception of a chelating effect on the ferrous ion (Fig. 1). It is well known that chestnut extracts from leaves, fruits, skins and flowers possess strong antioxidant properties due to the significant content of total phenolic compounds[7,10]. It has previously been shown that the extract from the spiny burrs of *C. sativa* exhibit a high antioxidative activity against the superoxide anion[32] and can be used as a source of natural antioxidants[33,34]. A specific goal of the present study was to examine the potential beneficial effects of a combination of these two extracts with basically different antioxidant activities. Aside from the observed very high antioxidant capacity of the MIX *Ld/Cs* in those assays in which individual extracts showed good antioxidant properties, MIX *Ld/Cs* was also a very effective chelator of the ferrous ion, despite the very low individual chelating activities of the mushroom and chestnut extracts. It has been acknowledged that no single antioxidant can replace the combination of natural phytochemicals to achieve the health benefits, as a result of their additive and synergistic effects[35]. It is possible that the improved chelation demonstrated by the combination of two extracts was the result of synergism between the phytochemical compounds contained in the chestnut and mushroom extracts.

The antioxidant compounds could be toxic at higher concentrations, especially if they are rich in phenolics[36]. Therefore, it is important to evaluate the cytotoxicity of a compound or plant extract *in vitro* and *in vivo* before its consideration as a natural antioxidant for human use. We showed that *C. sativa, L. deterrimus* and their combination MIX *Ld/Cs* did not exhibit any cytotoxic effect on the Rin-5F pancreatic

β-cell line (Fig. 2(b)). Also, both extracts and especially their combination exhibited a protective effect against STZ-induced β-cell death (Fig. 2(c)). The improved viability was, in part, the result of a significant reduction of DNA damage, as established by the Comet assay (Fig. 3). Using the Comet assay, a positive effect on genome stability and antigenotoxic potential was shown for another species from the genus *Lactarius*, *L. vellereus*[37], and for the extract from the chestnut inner shell[38]. The observed reduction of DNA damage after the application of STZ with the chestnut extract could result from the high content of ellagic acid and its derivatives that were present in the chestnut extract. It was shown earlier that treatment with ellagic acid leads to a noticeable reduction of $H_2O_2^-$ and bleomycin-induced DNA damage[39]. Based on the mechanism of STZ-induced DNA damage and β-cell death, which include generation of both ROS and RNS[25], the decreased amount of DNA breaks implies potent antioxidant activities of both extracts, and in particular their combination.

Oxidative stress can be alleviated either by increasing activities of antioxidant enzymes or by free radical scavenging by different antioxidants. Experiments with transgenic mice with β-cell-specific overexpression of the antioxidant proteins MnSOD and CAT revealed that elevated expression of these enzymes enhanced β-cell ROS scavenging, rendering β-cells resistant to STZ-mediated damage[40]. It was shown that some antioxidants realise their antioxidant properties by inducing antioxidant enzymes[41–44]. Herein, the chestnut and mushroom extracts decreased the level of MnSOD activity in comparison with STZ-induced increase, without changing MnSOD protein levels. The activity of CuZnSOD did not alter significantly throughout the treatments, although a slight increase in the CuZnSOD protein level was observed in the STZ-treated cells. The observed increase in the CuZnSOD protein level could be the result of NF-κB activation, as it was shown that NF-κB is involved in the transcriptional regulation of the *CuZnSOD* gene[45]. Since the treatment with the extracts did not increase the protein expression level neither for CuZnSOD nor for MnSOD, we assumed that the extracts lowered oxidative stress via a mechanism other than the induction of antioxidant enzyme gene expression.

Besides an overall improvement of the oxidative status (Fig. 4), some differences in the individual effects of the mushroom and chestnut extracts on β-cell survival were noted. They were probably mediated by different mechanisms of antioxidative action. In contrast to the mushroom extract, the chestnut extract had a greater influence on the activity of SOD and CAT. The diminishment of the STZ-induced elevation of both MnSOD and CAT activities after *C. sativa* extract administration is most probably due to the lowering of ROS concentrations as a result of the pronounced scavenging activity of the extract against ROS. Antioxidant properties of the *C. sativa* extract directly correlates with the extremely high content of phenolic compounds, especially hydrolysable tannins (ellagic and galic acids and their derivatives). The beneficial effects of these compounds have been extensively studied and their *in vivo* biological effects are related to the high free radical-scavenging activity they exhibit *in vitro*[46]. The proposed antioxidant mechanism of ellagic acid, the most pronounced compound in the *C. sativa* extract, is scavenging of superoxide, hydroxyl and peroxyl radicals[47]. This is in the strict correlation with our conclusion that the *C. sativa* extract accomplishes its effects by ROS scavenging.

In contrast, the effect of the *L. deterrimus* extract on the activity of antioxidant enzymes was negligible, pointing to its considerably lower influence on ROS scavenging. In spite of that, an overall improvement of the oxidative status after the co-treatment of β-cells with the *L. deterrimus* extract and STZ was detected. The antioxidant properties of the *L. deterrimus* extract can be explained by a strong NO-scavenging activity that was observed in the biochemical assay (Fig. 1) and also on β-cells *in vitro* (Fig. 4(c)). Since STZ is a potent NO donor[48,49], the improved viability of the STZ-treated β-cells observed after administration of the *L. deterrimus* extract could be the result of its NO-scavenging activity. However, based on the phenolic content of the *L. deterrimus* extract, we cannot identify a compound responsible for its antioxidant effects. It would appear that some other non-phenolic compounds or secondary metabolites[50] were responsible for the beneficial effect of the mushroom extract. One possibility is that the antioxidant properties of the *L. deterrimus* extract are derived from essential trace elements, Se and Zn. Se functions as a cofactor of some antioxidant enzymes and thus is involved in the elimination of free radicals[51], while Zn protects certain enzyme sulfhydryls from oxidation and reduces the formation of hydroxyl radical from H_2O_2 through the antagonism of redox-active transition metals[52]. The *L. deterrimus* extract is enriched in Se (0·946 (SD 0·002) μg/g of extract) and Zn (57·03 (SD 0·001) μg/g of extract) (S Vidorić, unpublished results), the feature that may underlie the antioxidant and beneficial effect of the *L. deterrimus* extract.

Although both extracts possess an antioxidant potential and exert a positive effect on cell viability after the STZ treatment, their combination provided the highest viability and the lowest level of DNA damage in β-cells after the STZ treatment. We assume that MIX *Ld/Cs* exhibited the most beneficial result on cell survival due to the additive and synergistic effects[53] of different antioxidant mechanisms of the mushroom and chestnut extracts. Namely, based on the present results and HPLC analysis, we assumed that the chestnut extract exerts an antioxidative and protective effect by ROS scavenging. The mushroom extract primarily appears to exert its effect as a NO scavenger. It is known that NO rapidly reacts with other reactive species, in particular with the superoxide ion to form peroxynitrite ($ONOO^-$). Peroxynitrite is chemically unstable under physiological conditions and reacts with all major classes of biomolecules, mediating cytotoxicity[29]. Thus, the simultaneous presence of both *C. sativa* and *L. deterrimus* extracts could prevent not only the cytotoxicity mediated by NO and ROS, but also the cytotoxic effect mediated by highly reactive species formed through their interaction. This particular effect would be specific for a given combination of extracts but not for a single extract. Also, we observed a synergistic effect of MIX *Ld/Cs* in the Fe^{2+}-chelating assay. Fe^{2+} chelation is an essential feature of antioxidants since

'free' Fe can catalyse the formation of very injurious compounds, such as the hydroxyl radical, from H_2O_2 through the Fenton reaction. The mushroom and chestnut extracts individually possess moderate chelating capability, while their combination demonstrates a high potential for preventing the Fenton reaction, which could be particularly beneficial *in vivo*. Therefore, mixtures of extracts acquire new qualities with respect to individual extracts, which explains the improved antioxidant and beneficial effects on β-cells. We also believe that combined actions are especially manifest in mixtures that contain phytochemicals from evolutionarily distant groups, as in our case. Identification of the phenolic compounds confirmed the diversity of the secondary metabolites present in the *L. deterrimus* and *C. sativa* extracts. Underlining that antioxidant properties of the mushroom and chestnut extracts need to be confirmed *in vivo* on a rat model of STZ-induced diabetes (work in progress) and with complete awareness of the limitations of the *in vitro* model system, we suggest that the combination of the *C. sativa* and *L. deterrimus* extracts may have beneficial effects on the reduction of oxidative stress in β-cells and could potentially attenuate the process that underlies the development and progression of diabetes.

Acknowledgements

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