Coffee silverskin extract improves glucose-stimulated insulin secretion and protects against streptozotocin-induced damage in pancreatic INS-1E beta cells.

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Abbreviations: CF, caffeine; CGA, chlorogenic acid; CSE, coffee silverskin extract; DCF, dichlorofluorescein; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSIS, glucose-stimulated insulin secretion; KRB, Krebs–Ringer bicarbonate buffer; OPT, *o*-phthaldehyde; ROS, reactive oxygen species; STZ, streptozotocin; t-BOOH, tert-butylhydroperoxide; T2D, Type 2 diabetes.

24 **Abstract**

25 The present research aimed to provide novel information regarding the antidiabetic mechanism
26 of action of coffee silverskin extract (CSE) and its components chlorogenic acid (CGA) and caffeine
27 (CF). Their effect on insulin secretion and biomarkers of oxidative stress in INS-1E cells *in vitro*
28 cultured under physiological and stressed conditions were assessed. Under physiological conditions,
29 CSE and pure CGA and CF did not affect cells' oxidative status and viability. However, concentrations
30 of CSE $\geq 1\mu\text{g/mL}$ and CGA $\geq 5\ \mu\text{M}$ significantly increased ($p < 0.05$) the enzymatic activity of
31 glutathione peroxidase (GPx). Moreover, all concentrations of CSE (1-10 $\mu\text{g/mL}$) and the dose of 10
32 μM of CGA, significantly stimulated ($p < 0.05$) insulin secretion in cells cultured in media containing 4
33 and 10 mM of glucose. CSE (1 $\mu\text{g/mL}$) and CGA (10 μM) reinforced antioxidant defence and increased
34 insulin secretion in response to glucose in beta cells stressed with streptozotocin (STZ). Since CGA
35 concentration in CSE was of $\approx 0.1\ \text{nM}$ it can be assumed that other antioxidants present in this
36 particular extract may also contribute to the observed effect. In conclusion, here we provide evidence
37 that CSE could be a new potential antidiabetic agent through its antioxidant actions and its ability to
38 modulate insulin secretory function.

39

40 **Keywords:** Coffee silverskin, coffee by-products, oxidative stress, insulin secretion, antidiabetic effect

41

42 **Highlights.**

43

44 - Coffee silverskin extract increases GPx activity in INS-1E cells

45 - Coffee silverskin extract increases insulin secretion in response to glucose in INS-1E cells

46 - Coffee silverskin extract protects against STZ induced oxidative stress in INS-1E cells

47 - Coffee silverskin extract may reduce risk of diabetes through pancreas protection

48

1. Introduction

Type 2 diabetes (T2D) is a complex and multifactorial metabolic disorder characterized by persistent hyperglycaemia. Although T2D is caused by a deficiency in insulin secretion associated to decreased responsiveness of peripheral tissues to insulin, it is generally accepted that the inability of beta cells to secrete adequate amounts of insulin is primarily responsible for the development and progression of T2D (Marchetti, Dotta, Lauro, & Purrello, 2008). Hyperglycemia and the subsequent increase in oxidative stress that appears in diabetes mellitus have been largely implicated in the progressive dysfunction of pancreatic beta cells and in the development of diabetic complications (Bensellam, Laybutt, & Jonas, 2012). Micro- and macro-vascular complications of diabetes (blindness, kidney failure, heart disease, stroke and amputations) are the major causes of morbidity and mortality in human populations (IDF Diabetes Atlas Group, 2015). Currently, numerous antidiabetic drugs, including inducers of insulin secretion, are used as treatment of T2D. Nevertheless, all of them exhibit adverse side effects and, actually, even under treatment, beta cell dysfunction worsens driving to loss of glycemic control (Robertson, 2010). Therefore, there is a growing interest in the identification of naturally occurring antioxidant agents that may protect and improve beta cell mass and function since they may offer a natural alternative to reduce risk or treat diabetes and retard the onset of its complications.

Coffee consumption, both caffeinated and decaffeinated, has been associated to a wide variety of health beneficial effects, in particular the reduced risk of T2D (Ding, Bhupathiraju, Chen, van Dam, & Hu, 2014; Jiang, Zhang, & Jiang, 2014). Coffee components, caffeine (CF) and chlorogenic acid (CGA), possess potential benefits on glucose homeostasis (Ludwig, Clifford, Lean, Ashiharad, & Crozier, 2014). The effect of CGA and CF on glucose metabolism remains unclear (Akash, Rehman, & Chen, 2014). The use of coffee by-products as natural source of compounds with putative health benefits such

72 as CGA, CF and dietary fiber among others has been proposed (del Castillo et al., 2013; del Castillo,
73 Fernandez-Gomez, Martinez-Saez, Iriondo, & Mesa, 2015; Galanakis, Martinez-Saez, del Castillo,
74 Francisco, & Mitropoulou, 2015). Glucoregulatory properties have been recently ascribed to a coffee
75 silverskin extract (CSE) by-product of coffee roasting (del Castillo, Fernandez-Gomez, Ullate, & Mesa,
76 2014). The antidiabetic effect of CSE has been associated to its capacity to inhibit enzymatic activity of
77 α -glucosidase and lipase. CSE is also able to inhibit *in vitro* the formation of advanced glycation end-
78 products (AGEs) (Mesías et al., 2014; Fernandez-Gomez et al., 2015) which have been involved in the
79 development of diabetes and its complications (Uribarri et al., 2015). However, up to date, the protective
80 effect of CSE on the development and progression of diabetes and, particularly, on beta cell viability
81 and function has not been evaluated.

82 Streptozotocin (STZ) is a potent DNA-methylating agent, which generates ROS that induce
83 oxidative stress in pancreatic beta cells (Szkudelski, 2001). STZ has been used in beta cells to imitate
84 the pathology of T2D and to evaluate the antidiabetic effect of novel compounds (Hong et al., 2013;
85 Kim et al., 2013; Lee, Hang, Ko, Kang, & Jeon, 2013). The aim of the present study was to investigate
86 the mechanism of action of CSE on the pathogenesis of diabetes using an *in vitro* model of beta cells,
87 the INS-1E cells. To achieve this goal, the effect of CSE on redox status and insulin secretion in the
88 pancreatic beta cells was evaluated. Likewise, its capacity to protect pancreatic beta cells against an
89 oxidative damage induced by STZ was also examined. In addition, the main phenolic constituent CGA
90 and the alkaloid CF were individually studied in order to determine their contribution to the beneficial
91 effect of the CSE on the function of pancreatic beta cells.

92

93 **2. Material and Methods**

94 *2.1. Materials and Chemicals*

95 CGA, CF, STZ, glutathione reductase (GR), reduced and oxidized glutathione (GSH and GSSG,
96 respectively), NADH, NADPH, *o*-phthaldialdehyde (OPT), tert-butylhydroperoxide (t-BOOH),
97 gentamicin, penicillin G, streptomycin and bovine serum albumin (fraction V) were purchased from
98 Sigma Chemical (Madrid, Spain). The fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH-DA)
99 was from Molecular Probes (Eugene, OR). Cell culture dishes were from Falcon (Cajal, Madrid, Spain)
100 and cell culture medium and fetal bovine serum from Lonza (Madrid, Spain). Bradford reagent was
101 from BioRad Laboratories S.A.

102

103 *2.2. Preparation of coffee silverskin extract (CSE)*

104 CS from the Arabica (*Coffea arabica*) species was provided by Fortaleza S.A. (Spain).
105 According to the manufacturer, the weight portion of CS represents 0.6 % of the roasted beans. Arabica
106 CSE was prepared by aqueous extraction according to the procedure patented by del Castillo et al.
107 (2013). Briefly, 50 mL of boiling water was added to 2.5 g of CS. The mixture was stirred at 250 rpm
108 for 10 min, filtered by Whatman paper no. 4 and the filtrate was freeze dried. The powdered extracts
109 were stored in dark and dry place until analysis. Concentrations of the bioactive compounds were
110 determined by a capillary electrophoresis and UV-Vis detection, and a detailed description of this CSE
111 is given elsewhere (Mesías et al., 2014). Accordingly, the amounts of CGAs and CF present in the CSE
112 were 11.18 mg/g and 30.26 mg/g, respectively.

113

114 *2.3. Cell culture and treatments*

115 Rat insulinoma cell line, INS-1E, was kindly provided by Dr. Mario Vallejo of "Alberto Sols"
116 Biomedical Research Institute CSIC, Madrid, Spain. INS-1E cells were maintained in a humidified
117 incubator containing 5 % CO₂ and 95 % air at 37 °C. They were grown in RPMI-1640 medium with 11
118 mM glucose, supplemented with 10 % fetal bovine serum (FBS), 1 % Hepes, 1 mM sodium pyruvate,
119 50 µM betamercaptoethanol and 1 % of the following antibiotics: gentamicin, penicillin and
120 streptomycin.

121 For the treatments with the different compounds, concentrations of CSE (1, 5 and 10 µg/mL),
122 CGA (1, 5 and 10 µM) and CF (1, 5 and 10 µM) diluted in RPMI-1640 culture medium and filtered
123 through a 0.2-µm membrane were added to cell plates during 20 h. For STZ treatment, STZ was
124 dissolved in 0.1 M citrate buffer (pH 4.5) and added to cell plates during different times (3-18 h) and
125 concentrations ranging from 1 to 5 mM.

126

127 *2.4. Evaluation of cell viability and production of reactive oxygen species (ROS)*

128 Cell viability was determined by the crystal violet assay. INS-1E cells were seeded at low
129 density (2 x 10⁵ cells per well) in 24-well plates. After the different treatments, cells were incubated
130 with crystal violet (0.2 % in ethanol) for 20 min. Plates were rinsed with distilled water, allowed to dry,
131 and 1 % sodium dodecyl sulphate (SDS) was added. The absorbance of each well was measured using
132 a microplate reader at 570 nm (Bio-Tek, Winooski, VT, USA).

133 Cellular ROS were quantified by the DCFH assay using a microplate reader (Wang & Joseph,
134 1999).. For the assay, cells were plated in 24-multiwells and incubated with the different treatments.
135 After that, 10 µM DCFH was added to the wells for 30 min at 37 °C. After being oxidized by
136 intracellular oxidants, DCFH will become dichlorofluorescein (DCF) and emit fluorescence. ROS

137 generation was evaluated in a fluorescent microplate reader at an excitation wavelength of 485 nm and
138 an emission wavelength of 530 nm (Bio-Tek, Winooski, VT, USA).

139

140 2.5. *Glucose-stimulated insulin secretion (GSIS) and content*

141 After the different treatments, INS-1E cells were washed and placed in Krebs–Ringer
142 bicarbonate buffer (KRB: 115 mM NaCl, 24 mM NaHCO₃, 5 mM KCl, 1 mM MgCl₂ 6H₂O, 1mM
143 CaCl₂ 2H₂O) supplemented with 5 mg/mL BSA for a quiescent period of two hours. Next, cells were
144 incubated for 90 min in KRB containing 4 or 10 mM glucose and the different concentrations of CSE,
145 CGA and CF. GSIS was evaluated in the medium by an enzyme-linked immunosorbent assay (ELISA)
146 kit (Mercodia, Uppsala, Sweden).

147 For cellular insulin content measurement, treated cells were lysed at 4 °C in a buffer containing
148 25 mM HEPES (pH 7.5), 0.2 mM EDTA, 0.1 % Triton X-100, 200 mM β-glycerolphosphate, 0.1 mM
149 Na₃VO₄, 2 µg/mL leupeptin, and 1 mM phenylmethanesulphonyl fluoride (PMSF). The supernatants
150 were collected and assayed for insulin content by the ELISA kit (Mercodia, Uppsala, Sweden).

151

152 2.6. *Determination of glutathione peroxidase (GPx) and glutathione reductase (GR) activities*

153 Treated INS-1E cells were collected in PBS and centrifuged at low speed (300×g) for 5 min to
154 pellet cells to assay the activities of GPx and GR. Cell pellets were resuspended in 20 mM Tris
155 containing 5 mM EDTA and 0.5 mM beta-mercaptoethanol, sonicated and centrifuged at 3000×g for
156 15 min. Enzyme activities were measured in the supernatants. Determination of GPx activity was based
157 on the oxidation of GSH by GPx, using t-BOOH as a substrate, coupled to the disappearance of
158 NADPH by GR. GR activity was determined by following the decrease of the absorbance due to the
159 oxidation of NADPH utilized in the reduction of GSSG. The methods have been previously described

160 (Rodríguez-Ramiro, Martín, Ramos, Bravo, & Goya, 2011). Protein was measured by the Bradford
161 reagent.

162

163 2.7. *Determination of reduced glutathione (GSH)*

164 The concentration of GSH was evaluated by a fluorometric assay previously described
165 (Rodríguez-Ramiro, Martín, Ramos, Bravo, & Goya, 2011). The method takes advantage of the
166 reaction of GSH with OPT at pH 8.0. After the different treatments, the culture medium was removed
167 and cells were detached and homogenised by ultrasound with 5 % trichloroacetic acid containing 2 mM
168 EDTA. Following centrifugation of INS-1E beta cells homogenates for 30 min at 3.000 rpm, 50 µL of
169 the clear supernatant were transferred to a 96-multiwell plate for the assay. Fluorescence was measured
170 at an emission wavelength of 460 nm and an excitation wavelength of 340 nm. The results were
171 interpolated in a GSH standard curve (5 – 1000 ng) and expressed as nmol GSH/mg protein, which was
172 determined by the Bradford reagent.

173

174 2.8. *Determination of carbonyl groups*

175 Protein oxidation was measured as carbonyl groups content according to a published method
176 (Granado-Serrano, Martín, Bravo, Goya, & Ramos, 2009). The determination was carried out in
177 supernatants of INS-1E cells. Absorbance was measured at 360 nm and carbonyl content was expressed
178 as nmol/mg protein using an extinction coefficient of 22000 nmol/L/cm. Protein concentration was
179 determined by the Bradford reagent.

180

181 2.9. *Statistics*

182 Prior to statistical analysis, the data were tested for homogeneity of variances using Levene test.
183 For multiple comparisons, one-way ANOVA was followed by a Bonferroni test when variances were
184 homogeneous or by the Tamhane test when variances were not homogeneous. The level of significance
185 was $p < 0.05$. A SPSS version 22.0 program was used.

186

187

3. Results and discussion

3.1. Effects of CSE, CGA and CF on the redox status of cultured INS-1E cells

The aim of the present study was to obtain novel information regarding the mechanism of action of CSE and its bioactive components, CGA and CF, on the pathogenesis of diabetes induced by STZ. For this purpose, we used an established cell culture line from rats, INS-1E cells, which show important biological features of the pancreatic islet beta cells and have been widely used as a reliable model of beta cells.

Doses of pure CGA and CF to be tested were selected taking into account the information on physiological concentrations reported by others. After normal dietary intakes, polyphenols and their metabolites appear in the circulatory system at nM- μ M concentrations, so they are the most appropriate doses for *in vitro* studies (Rodríguez-Mateos et al., 2014). On the other hand, plasma concentrations may reach up to 10 μ M of CF after the intake of a normal coffee serving (Martínez-López et al., 2014).

The feasibility of the doses of CSE (1-10 μ g/mL), CGA (1-10 μ M) and CF (1-10 μ M) for treating INS-1E was determined by the analysis of cellular redox status and antioxidant response biomarkers. Treatment of INS-1E with CSE, CGA and CF did not affect intracellular ROS generation or cell viability, indicating no cellular stress or damage (Table 1).

Figure 1 shows that the treatment of INS-1E cells with CSE, CGA or CF preserved the GSH store (Figure 1A) and the GR activity (Figure 1C). Interestingly, CSE and the phenolic CGA evoked a significant increase ($p < 0.05$) in the enzymatic activity of GPx (Figure 1B). Glutathione and their related enzymes, GR and GPx, participate in the defence against hydrogen peroxides and superoxides and they are essential to prevent the cytotoxicity of ROS. Several phenolic compounds have shown to enhance the expression and activity of antioxidant enzymes in different tissues such as liver (Martín et al., 2010; Sarriá et al., 2012) or colon (Rodríguez-Ramiro, Martín, Ramos, Bravo, & Goya, 2011) and

also in pancreatic beta cells (Martín, Fernández-Millán, Ramos, Bravo, & Goya, 2014). This outcome should have a significant impact on INS-1E cells because they are particularly susceptible to oxidative stress-induced injury due to the low-level expression of antioxidant enzymes as compared to other types of cells (Robertson, 2010). Furthermore, it has been demonstrated that the overexpression of antioxidant enzymes protects pancreatic beta cells from oxidative stress-induced dysfunction (Harmon et al., 2009). Therefore, the induction of GPx by CSE and CGA sets the cells in favourable conditions to face a potential oxidative challenge and could be an important strategy to improve beta cell survival in diabetes.

219

3.2. CSE and CGA increased GSIS in INS-1E cells

Since the ineffectiveness of beta cells to secrete adequate amounts of insulin is decisive in the development and progression of T2D, agents that may improve beta cell function are considered key to prevent or to treat diabetes (Robertson, 2010). Accordingly, we were next interested in exploring the potential effect of CSE, CGA and CF on insulin secretory function. Indeed, it has been indicated that many phytochemicals present in plant foods, particularly polyphenols, could be able to induce insulin secretion in pancreatic beta cells (Huang et al., 2011; Kappel et al., 2013; Martín et al., 2014; Fernández-Millán et al., 2014).

After the incubation of INS-1E cells in the presence of CSE (1-10 μ g/mL), CGA (1-10 μ M) and CF (1-10 μ M) for 20 hours, the GSIS was assayed for 90 min. As shown in Figure 2A-C, pre-treatment of cells with CSE and CGA, significantly increased ($p < 0.05$) insulin secretion in cells cultured in media containing 4 and 10 mM of glucose. However, CF resulted ineffective stimulating insulin secretion. According to that, a previous work reported a positive effect of CGA on insulin secretion (Tousch et al., 2008), besides the doses of CGA employed in that study were higher (28 and 140 μ M)

234 than those tested in the present study. On the other hand, a very low concentration of CSE (1 $\mu\text{g/mL}$)
235 resulted effective to increase insulin secretion in a glucose-dependent manner in pancreatic beta cells
236 which is a novel and interesting result. This CSE dose contains $9.86 \times 10^{-5} \mu\text{M}$ of CGA which is lower
237 than the effective doses of CGA (10 μM). These data suggest that other compounds are contributing to
238 the observed effect. Synergistic effects of bioactive compounds present in the botanical matrix
239 including those unknown can be responsible of the overall antidiabetic observed effect. Further studies
240 should be conducted to identify such components of this particular extract contributing to the protective
241 effect of pancreatic cells.

242 The observed effect on insulin secretion could be due to an increase of insulin biosynthesis. To
243 confirm this hypothesis, we also measured total insulin content in control and CSE, CGA, and CF 20
244 hour-treated cells. As showed in Figure 2D, there was no difference in insulin levels between the
245 control and treated INS-1E cells. Therefore, we can ensure that the augmented GSIS induced by CSE
246 and CGA was not related to an increase in the biosynthesis of the hormone but rather to the stimulation
247 of insulin secretion. In this regard, from a mechanistic point of view, several insulin secretagogues with
248 recognized therapeutic effect such as sulfonylureas or glinides are able to close K_{ATP} channel and lead
249 to an increase in glucose-induced depolarization. Consequently, voltage-dependent Ca^{2+} channels open,
250 causing the acceleration of Ca^{2+} influx and the increase of the concentration of cytosolic free Ca^{2+} that
251 is necessary and sufficient to trigger insulin secretion (Henquin, 2004). In line with this, it has been
252 recently indicated that a coffee extract (100 $\mu\text{g/mL}$) and its main component CGA (28.2 μM) may
253 block K_{ATP} channel, conferring a regenerative effect on zebrafish pancreatic islet damaged with alloxan
254 (Nam et al., 2015). On the contrary, Tusch et al., (2008) indicated that CGA at stimulating
255 concentrations for insulin secretion (28.2 and 84.6 μM) does not close K_{ATP} channels in rat INS-1E
256 cells, suggesting that a different mechanism of action may be involved.

Other insulin secretagogues amplify Ca^{2+} -induced insulin secretion through the activation of diverse protein kinases, including protein kinase A (PKA), protein kinase C (PKC) or extra cellular regulated kinases (ERKs), that are involved in the mechanism of insulin exocytosis itself (Bratanova-Tochkova et al., 2002). Accordingly, several phenolic compounds such as quercetin (Youl et al., 2010) and microbial-derived flavonoid metabolites (Fernández-Millán et al., 2014) have demonstrated to increase glucose-stimulated insulin secretion via ERKs activation. Therefore, a potential role of the phenolic CGA on signalling pathways cannot be ruled out. Further studies about the molecular mechanisms involved in the action of CGA and CSE on beta cell insulin secretion are necessary.

3.3. CSE and CGA protect INS-1E cells against STZ-induced oxidative stress

It is generally accepted that oxidative stress is involved in the loss of beta cell function and viability, thus, the protection of beta cells from oxidative stress is one of the mechanisms potentially involved in the prevention of diabetes (Bonora, 2008). Consequently, we finally investigated the protective effect of CSE, CGA and CF against oxidative stress. To this end, we used STZ, a chemical compound commonly used to induce diabetes through its toxic effects on pancreatic beta cells (Kim et al., 2003). The cytotoxic action of STZ is associated with the generation of ROS and the consequent beta cell destruction and suppression of insulin secretion (Szkudelski, 2001).

Our first goal for this outcome was to determine the conditions leading to oxidative stress and cell death in INS-1E cells. Figure 3 reveals that increasing concentrations of STZ induced a dose-dependent increase in ROS generation and cell toxicity as shown by the decrease in cell viability. Since the STZ concentration of 5 mM at 6 hours caused a significantly increase ($p < 0.05$) in ROS generation and nearly 40 % cell death (Figure 3), we decide to choose this concentration and that time for the following experiments.

280 To investigate the potential of CSE, CGA and CF to protect beta cells against the impairment of
281 viability or insulin secretion induced by STZ, we used effective doses of each compounds on antioxidant
282 defence and insulin secretion. INS-1E cells were treated with 1 µg/mL CSE, 10 µM CGA or 10 µM CF
283 during 20 hours followed by treatment with 5 mM of STZ for 6 hours (for oxidative damage assays and
284 cell viability) or for 90 minutes (for insulin secretion assays). Figure 4 illustrates the effect of the
285 different treatments on the appearance of INS-1E cells. STZ treatment led to morphological changes
286 such as cell shrinkage related to cell death. However, pre-treatment with CSE or CGA, but not CF,
287 prevented these morphological alterations. In agreement with those data, treatment of cells with STZ
288 enhanced ROS generation (Figure 5A) and protein cell oxidative damage (measured as carbonyl groups)
289 (Figure 5B) resulting in a remarkable decrease of INS-1E cell viability (Figure 5C). Pre-treatment with
290 CSE or CGA significantly reduced ($p < 0.05$) the ROS over production induced by STZ and prevented
291 beta cell death. Since the increase in ROS generation induced by STZ has been directly implicated in
292 pancreatic beta cell apoptosis (Zheng, et al., 2015; Bhakkiyalakshmi et al., 2014), it is reasonable to
293 suggest that the effect of CGA and CSE reducing ROS over production possibly will contribute to
294 reduce apoptosis and enhance cell survival of INS-1 beta cells. Likewise, STZ treatment also induced a
295 significant decrease ($p < 0.05$) in insulin secretion in INS-1E cells (Figure 5d) whereas the pre-
296 incubation with CSE and CGA completely restored GSIS to control levels. CF did not protect against
297 oxidative stress, cell death or impaired insulin secretion induced by STZ. In parallel, the oxidative stress
298 induced by STZ caused a significant decrease ($p < 0.05$) in GSH (Figure 6A) and a remarkable increase
299 of GPx (Figure 6B) and GR (Figure 6C) activities in order to enhance the antioxidant cell defence
300 against ROS. Under these extreme oxidative conditions pre-treatment of INS-1E cells with CSE and
301 CGA greatly prevented GSH depletion and completely recovered GPx and GR activities. Altogether,
302 our results indicate that the concentrations of CSE and CGA hereby assayed efficiently protect the

303 viability and function of pancreatic beta cells against STZ while CF was ineffective on that since did not
304 significantly inhibit ROS production.

305 The cyto-protective effect of coffee and its phenolic components against an oxidative injury has
306 previously been described in different cultured cells (Deng et al., 2013; Ahn et al., 2014; Baeza et al.,
307 2014). Results obtained for pure CGA are in line with those reporting a protective effect of different
308 antioxidant compounds on pancreatic beta cells (Karunakaran & Park, 2013; Fernández-Millán et al.,
309 2014; Martín et al., 2014). However, the present study is the first to demonstrate a specific chemo-
310 protective effect of CSE on pancreatic beta cells. This effect produced by CSE cannot be ascribed to
311 isolated CF or CGA, but to a synergic effect of different components present in the extract. Further
312 studies are necessary to identify the compounds responsible for that property. On the other hand, our
313 findings highlight the potential of CSE in the protection against diabetes supporting those described in
314 the patented with number P201431848.

315

316

317 **4. Conclusion**

318 In conclusion, for the first time we provide scientific evidences regarding the protective effects
319 of CSE in pancreatic beta cells through its antioxidant actions and its ability to modulate insulin
320 secretory function. In addition, our results suggest that physiological concentrations of pure CGA (10
321 μM) are able to protect pancreatic cells against oxidative stress while CF in the same concentration is
322 ineffective. On the other hand, since the concentrations of CGA present in CSE seems to be ineffective
323 to protect pancreatic cells against diabetogenic agents causing cell oxidative stress (STZ), further
324 research should be conducted to identify which compound/s are responsible for those benefits. Our
325 findings support the potential of CSE as anti-diabetic phytodrug and functional ingredient for the
326 prevention of diseases related to oxidative stress such as diabetes. Results also suggest that coffee by-
327 products present added value confirming that coffee is not only a drink.

328

329

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335

336 **Conflict of interest**

337 The authors declare that there are no conflicts of interest.

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471 **Figure captions**

472 **Figure 1.-** Effect of coffee silverskin extract (CSE), chlorogenic acid (CGA) and caffeine (CF) on GSH
473 concentration and GPx and GR activities. C represents untreated control cells. INS-1E cells were
474 treated with 1–10 µg/mL CSE and 1-10 µM of CGA and CF for 20 h and GSH concentration (A) and
475 GPx (B) and GR (C) activities were evaluated. Data represent means ± SD of 6-8 samples per
476 condition. Different letters denote statistically significant differences, $p < 0.05$.

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481 **Figure 2.-** Effect of coffee silverskin extract (CSE), chlorogenic acid (CGA) and caffeine (CF) on
 482 insulin secretion and insulin content in INS-1E cells. C represents untreated control cells. Cells were
 483 treated with 1–10 $\mu\text{g/mL}$ CSE (A) and 1-10 μM CGA (B) and CF (C) for 20 h and then incubated in
 484 KRB medium containing 4 or 10 mM glucose for 90 min and insulin release was evaluated in the KRB
 485 medium. Insulin content was determined in non-treated control cells and in cells treated with CSE,
 486 CGA and CF for 20 h (D). Data represent means $\pm$ SD of 6-8 samples per condition. Different letters
 487 denote statistically significant differences, $p < 0.05$.

Figure 2

490 **Figure 3.-** Effect of streptozotocin (STZ) on ROS production and cell viability in INS-1E cells. Cells
 491 were treated with 1-5 mM STZ for 3, 6, 9 and 18 hours and ROS levels (A) and cell viability (B) were
 492 evaluated. Data represent means $\pm$ SD of 8-10 samples per condition. Different letters denote
 493 statistically significant differences, $p < 0.05$.

497 **Figure 4.-** Representative microscopy images of INS-1 cells after different treatment. Untreated cells
498 (C). Cells treated with 5 mM STZ for 6 h (STZ). Cells treated with 1 µg/mL CSE and further exposed
499 to 5 mM STZ for 6 h (CSE + STZ). Cells treated with 10 µM of CGA and further exposed to 5 mM
500 STZ for 6 h (CGA + STZ). Cells treated with 10 µM of CF for 20 h and further exposed to 5 mM STZ
501 for 6 h (CF + STZ)

509 **Figure 5.-** Effect of coffee silverskin extract (CSE), chlorogenic acid (CGA) and caffeine (CF) against
 510 oxidative damage induced by STZ. C represents untreated control cells. Cells were treated with 1
 511 $\mu\text{g/mL}$ CSE (A) and 10 μM of CGA (B) and CF (C) for 20 h and further exposed to 5 mM STZ for 6 h.
 512 Then, intracellular ROS generation (A), carbonyl group production (B) and cell viability (C) were
 513 measured. To evaluate cell functionality, after 20 h of CSE, CGA and CF treatment, control and treated
 514 cells were placed in KRB containing 10 mM glucose and 5 mM STZ and insulin secreted during 90
 515 min was evaluated (D). Data represent means $\pm$ SD of 8-10 samples per condition. Different letters
 516 denote statistically significant differences, $p < 0.05$.

520 **Figure 6.-** Effect of coffee silverskin extract (CSE), chlorogenic acid (CGA) and caffeine (CF) on GSH
 521 concentration and GPx and GR activity in STZ-treated cells. C represents untreated control cells. INS-
 522 1E cells were treated with 1 μ g/mL CSE (A) and 10 μ M of CGA (B) and CF (C) for 20 h and further
 523 exposed to 5 mM STZ for 6 h. Then, GSH concentration (A) and GPx (B) and GR (C) activities were
 524 evaluated. Data represent means $\pm$ SD of 6-8 samples per condition. Different letters denote statistically
 525 significant differences, $p < 0.05$.

526

527 **Table 1.-** Effect of 20 hours treatment with noted concentrations of coffee silverskin extract (CSE),
 528 chlorogenic acid (CGA) and caffeine (CF) on cell viability and intracellular ROS generation in
 529 pancreatic INS-1E cells. C represents untreated control cells. Data represent means $\pm$ SD of 8-10
 530 samples per condition. Same letter **a** as superscript indicates that no significant differences were found,

531 $P < 0.05$.

		% Cell Viability	ROS (% Fluorescence Units) ⁵³²
C		100.3 $\pm$ 2.5 ^a	100.2 $\pm$ 6.3 ^a 533
CSE	1 μg/mL	103.4 $\pm$ 9.4 ^a	101.2 $\pm$ 6.4 ^a
	5 μg/mL	104.2 $\pm$ 4.4 ^a	99.8 $\pm$ 7.2 ^a
	10 μg/mL	102.1 $\pm$ 4.6 ^a	100.2 $\pm$ 8.7 ^a
CGA	1 μM	99.8 $\pm$ 5.4 ^a	97.4 $\pm$ 8.2 ^a
	5 μM	98.9 $\pm$ 8.7 ^a	100.3 $\pm$ 9.2 ^a
	10 μM	98.7 $\pm$ 7.7 ^a	101.2 $\pm$ 5.8 ^a
CF	1 μM	100.3 $\pm$ 5.8 ^a	99.7 $\pm$ 5.8 ^a
	5 μM	102.4 $\pm$ 4.0 ^a	96.8 $\pm$ 8.8 ^a
	10 μM	100.4 $\pm$ 2.0 ^a	97.3 $\pm$ 9.3 ^a