(-)-Epicatechin and the colonic metabolite 3,4-dihydroxyphenylacetic acid protect renal proximal tubular cell against high glucose-induced oxidative stress by modulating NOX-4/SIRT-1 signalling

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Abbreviations: CAT, catalase; DHBA, 2,3-dihydroxybenzoic acid; DHPAA, 3,4-dihydroxyphenylacetic acid; DPI, diphenyleneiodonium; EC, (-)-epicatechin; ERK, extracellular-regulated kinase; FBS, foetal bovine serum; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; HPPA, 3-hydroxyphenilpropionic acid; JNK, c-jun amino-terminal kinase; MAPKs, mitogen-activated protein kinases; NOX, NADPH oxidases; SIRT-1, silent information regulator protein-1; SOD, superoxide dismutase; *t*-BOOH, *tert*-butylhydroperoxide.

Abstract

Oxidative stress plays a main role in the pathogenesis of the diabetic nephropathy. The present study investigated the effect of (-)-epicatechin (EC) and the colonic-derived flavonoid metabolites 2,3-dihydroxybenzoic acid, 3,4-dihydroxyphenylacetic acid (DHPAA), and 3-hydroxyphenylpropionic acid on the redox status in high-glucose-exposed renal proximal tubular NRK-52E cells. EC and DHPAA (10μM) alleviated the redox imbalance induced in high-glucose-challenged cells, as both compounds reverted to control levels reactive oxygen species (ROS) values. EC and DHPAA pre-treatment prevented the decrease of antioxidant defences and silent information regulator protein-1 (SIRT-1), and the increase of phosphorylated mitogen-activated-protein-kinases and NADPH-oxidase-4 (NOX-4) values under high-glucose-conditions. The presence of selective NOX-4 and SIRT-1 inhibitors in EC- and DHPAA-pre-treated cells showed the involvement of both proteins in EC- and DHPAA-mediated protection. These findings suggest that EC and DHPAA protected NRK-52E cells against a high-glucose-challenge by improving the cellular redox status through multiple signalling pathways, playing NOX-4/SIRT-1 a relevant role.

Keywords: Antioxidant defences, colonic-derived flavonoid metabolites, epicatechin, NRK-52E cells, oxidative injury, signalling pathways.

Highlights

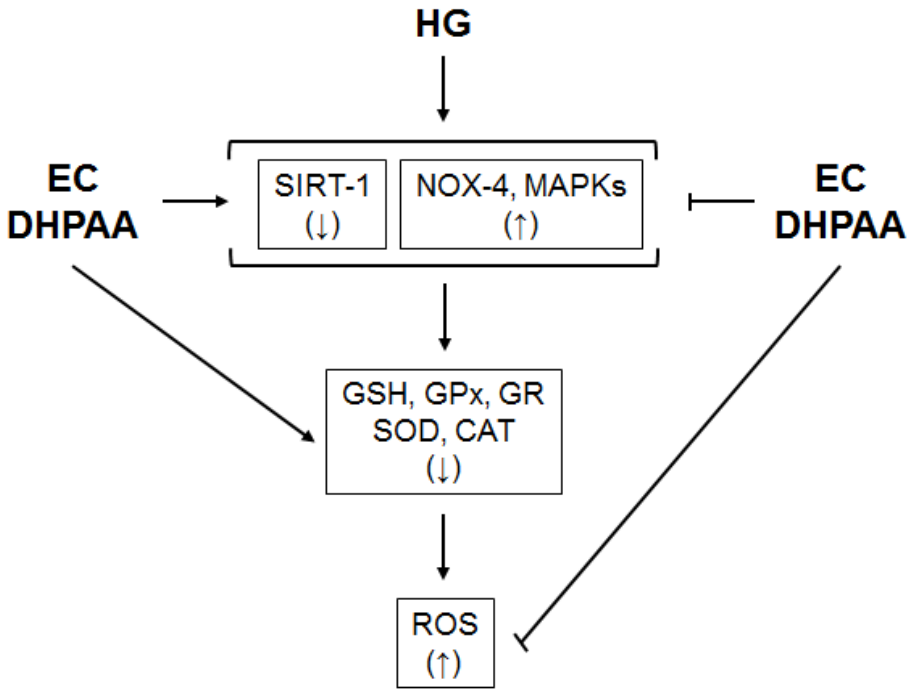
- (-)-Epicatechin (EC) and 3,4-dihydroxyphenilacetic acid (DHPAA) reduce ROS generation.

- EC and DHPAA alleviated impaired antioxidant defenses in high-glucose-treated-cells.

- EC- and DHPAA-induced protective effect involves multiple signalling routes.

- NOX-4/SIRT-1 modulation induced by EC and DHPAA contribute to the redox balance.

Graphical abstract



1. Introduction

Diabetes is a complex metabolic disorder characterized by hyperglycaemia that continues increasing worldwide in association with its complications, such as diabetic nephropathy (Forbes & Cooper, 2013; Stanton, 2011). It is widely accepted that persistent oxidative stress is a key player in the development and progression of diabetes and its complications, which are accompanied by increased production of free radicals and/or impaired antioxidant defences (Stanton, 2011).

In response to an excess of reactive oxygen species (ROS) production mammals have evolved different antioxidant defence mechanisms that include glutathione (GSH), and the enzymes glutathione peroxidase (GPx), glutathione reductase (GR) superoxide dismutase (SOD), and catalase (CAT), among others (Martín & Ramos, 2016). During the generation of ROS superoxide, which is the primary reactive oxygen intermediate, it is rapidly converted to H_2O_2 by spontaneous dismutation and by SOD-catalysed dismutation. Then, H_2O_2 is transformed to water either by CAT or GPx, which utilizes reduced glutathione produced by GR (Martín & Ramos, 2016). In addition, superoxide reacts with the nitrogen-containing oxidant, nitric oxide, to form highly reactive nitrogen species.

In the diabetic nephropathy it has been suggested that the mitochondrial generation of superoxide and NADPH oxidases (NOX)-derived ROS play a significant role in injuring the kidney (Sedeek et al., 2012; Stanton, 2011). Moreover, a number of cellular kinases, including the mitogen-activated protein kinases (MAPKs), are also modulated by oxidative stress (Cordero-Herrera, Martín, Goya, & Ramos, 2015a; Granado-Serrano, Martín, Goya, Bravo, & Ramos, 2009; Granado-Serrano et al., 2007; Martín et al., 2010), and silent information regulator protein-1 (SIRT-1), which is the most studied member of sirtuins family, links the cellular redox state to multiple signalling

and survival pathways (Hao & Haase, 2010). Accordingly, targeting the modulation of the cellular antioxidant defences and close-related key proteins might constitute an effective approach to prevent the development and/or progression of the diabetic nephropathy.

Flavanols, such as (-)-epicatechin (EC), are a group of phenolic compounds and their chemical structure consists of two aromatic rings (A and B) linked through three carbons that forms an oxygenated heterocycle (C ring) (Figure 1). Flavanols, including EC, are abundantly present in cocoa, tea, grapes, and other fruits and vegetables and it has been reported that more than 80% of flavanols ingested in the diet are metabolized by the colonic microbiota generating several phenolic metabolites (Monagas et al., 2010; Ottaviani et al., 2016). In this line, at the first steps in the catabolism of flavanols the C-ring is opened, which is followed by other reactions to generate hydroxyphenylvalerolactones and valeric acids (Monagas et al., 2010). Then, these compounds are oxidised and produce hydroxyphenilpropionic acids, and later dihydroxyphenylacetic acids. The last steps of the catabolism of flavanols involve dihydroxylation of 3,4-dihydroxylated phenolic acids to generate 3- and 4-monohydroxylated phenolic acids (Monagas et al., 2010).

EC has proved antioxidant activity, but also seems to possess other potential health beneficial effects, such as antidiabetic properties (Giacometti, Muhvic, Pavletic, & Đudaric, 2016; Martín, Goya, & Ramos, 2016; Shahidi & Ambigaipalan, 2015), anticarcinogenic activity (Granado-Serrano et al., 2007), and improves parameters related to the endothelium and inflammation in cardiovascular disease and diabetes (Barnett et al., 2015; Loke et al., 2008; Martín et al., 2016; Ottaviani et al., 2016). In addition, the microbial metabolites derived from the colonic fermentation of cocoa flavanols could play an important role in the prevention of diabetic complications

(Alvarez-Cilleros, Martín, & Ramos, 2018; Fernández-Millán et al., 2014). In this line, it has been shown that EC and some colonic phenolic metabolites modulate the oxidative/antioxidative potential and protect their functionality in insulin-sensitive tissues (Cordero-Herrera et al., 2015a; Fernández-Millán et al., 2014; Granado-Serrano et al., 2007; Quine & Raghu, 2005; Ramiro-Puig et al., 2009). Importantly, administration of EC has been reported to cause mild or none adverse effects, although more studies are needed to clearly state the safety of this flavanol (Barnett et al., 2015; Ottaviani et al., 2016). Besides, the precise mechanism for the preventive activities of EC and the microbial phenolic metabolites derived from the flavanol intake related to oxidative stress during diabetes in the renal tubular cells remains largely unknown.

The aim of this study was to evaluate under high glucose conditions the potential protective effects of EC and the colonic-derived flavonoid metabolites 2,3-dihydroxybenzoicacid (DHBA), 3,4-dihydroxyphenylacetic acid (DHPAA), and 3-hydroxyphenylpropionic acid (HPPA) on the redox status in renal proximal tubular NRK-52E cells.

2 Materials and methods

2.1. Materials and chemicals

(–)-EC (>95% of purity), DHBA (≥99% of purity), DHPAA (>98% of purity), HPPA (>98% of purity), D-glucose, diphenyleneiodonium (DPI), 6-chloro-2,3,4,9-tetrahydro-1H-carbazole-1-carboxamide (EX-527, EX), GR, GSH, glutathione oxidized, NADPH, *tert*-butylhydroperoxide (*t*-BOOH), o-phthaldehyde (OPT) and dichlorofluorescin (DCFH) were purchased from Sigma Chemicals (Madrid, Spain). Anti-ERK1/2 and antiphospho-ERK1/2 recognizing phosphorylated Thr202/Thy204 of ERK1/2, anti-JNK1/2 and antiphospho-JNK1/2 recognizing phosphorylated Thr183/Tyr185 of JNK1/2, anti-phospho-Thr180/Tyr182-p38, and anti-β-actin were obtained from Cell Signalling Technology (Izasa, Madrid, Spain). Anti-p38α (sc-535), anti-NOX-4 (sc-30141), and anti-SIRT-1 (sc-74465) were purchased from Santa Cruz Biotechnology (Qimigen, Madrid, Spain). Bradford reagent, and materials and chemicals for electrophoresis were from BioRad Laboratories S.A. (Madrid, Spain). Cell culture dishes and cell culture medium were from Falcon (Cajal, Madrid, Spain) and Cultek (Madrid, Spain), respectively.

2.2. Cell culture and treatments

Rat NRK-52E cells (kindly provided by Prof. Dr. Patricio Aller, Centro de Investigaciones Biológicas, CSIC, Madrid, Spain) were grown in DMEM medium containing 5.5 mM D-glucose, 2 mM glutamine, supplemented with 10% foetal bovine serum (FBS). Cells were maintained at 37ºC in a humidified atmosphere of 5% CO2.

Cells were treated with different concentrations of EC, DHBA, DHPAA or HPPA (1-20 μM) diluted in serum-free culture medium with 5.5 mM D-glucose and 2 mM glutamine during 24 h.

To evaluate the protective effect of EC, DHBA, DHPAA and HPPA against a high glucose challenge, 1-10 μM of each compound were added to the cells for 2 h. Then, cells were submitted to a challenge with 30 mM glucose for 22 h, as previously reported (Fang et al., 2015; Hou, Zheng, Li, Gao, & Zhang, 2014; Yong et al., 2013; W. Zhang, Miao, Wang, & Zhang, 2013). In the experiments with the inhibitors, cells were pre-incubated with 10 μM DPI (NADH oxidase inhibitor) or 10 μM EX (SIRT-1 specific inhibitor) for 1 h prior to EC or DHPAA incubation for 2 h followed by the glucose challenge.

2.3. Determination of ROS production

Intracellular ROS were quantified by the DCFH assay (Cordero-Herrera et al., 2015a; Granado-Serrano et al., 2007). After being oxidized by intracellular oxidants, DCFH becomes dichlorofluorescein (DCF) and emits fluorescence. Briefly, cells were cultured in 24-well plates ($2x10^5$ cells per well) and incubated with the different treatments. Then, 5 μM DCFH probe was added to for 30 min at 37ºC. The unabsorbed probe was removed, medium with the different treatments added and fluorescence immediately measured in a microplate reader (Bio-Tek, Winooski, VT) at 485 nm/530 nm (excitation/emission wavelengths, respectively).

2.4. Analysis of GSH content

GSH was quantified by Hissin and Hilf fluorimetric assay (Hissin & Hilf, 1976). The method is based on the reaction of GSH with OPT at pH 8.0 and fluorescence was measured at excitation wavelength of 340 nm and emission wavelength of 460 nm. The results of samples were referred to those of a standard curve of GSH.

2.5. Determination of GPx, GR, CAT and SOD activities

Determination of GPx activity is based on the oxidation of GSH by GPx, using *t*-BOOH as a substrate, coupled to the disappearance of NADPH by GR (Cordero-Herrera et al., 2015a; Cordero-Herrera, Martín, Goya, & Ramos, 2015b). GR activity was assayed by following the decrease in absorbance due to the oxidation of NADPH utilized in the reduction of oxidized glutathione (Cordero-Herrera et al., 2015a, 2015b). CAT activity was determined by the decomposition of H_2O_2 as a decrease in absorbance at 240 nm (Cordero-Herrera et al., 2015a, 2015b).

SOD activity was assayed by using a commercial kit following the manufacturer's instructions (Sigma) (Cordero-Herrera et al., 2015b). The assay is based on the capacity of SOD to reduce superoxide anion, couple with the disappearance of Dojindo's highly water-soluble tetrazolium salt (WST-1) to yield a dye. SOD activity was quantified by measuring the decrease in the absorbance at 440 nm. Protein concentration was measured by the Bradford reagent (Bradford, 1976).

2.6. Preparation of total cell lysates and Western blot analysis

To detect ERK, p-ERK, JNK, p-JNK, p38, p-p38, SIRT-1 and NOX-4, cells were lysed at 4ºC in a buffer containing 25mM HEPES (pH 7.5), 0.3M NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 0.5mM dithiothreitol (DTT), 0.1% Triton X-100, 200mM β-glycerolphosphate, 0.1mM Na_3VO_4, 2µg/mL leupeptin and 1mM phenylmethanesulfonyl fluoride (PMSF). Supernatants were collected, assayed for protein concentration by using the Bradford reagent, aliquoted and stored at -80ºC until used for Western blot analyses.

Equal amounts of proteins were separated by SDS polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride filters (Merck, Madrid, Spain). Membranes

were probed with the corresponding primary antibody followed by incubation with peroxide-conjugated anti-rabbit (GE Healthcare) or anti-mouse (Sigma) immunoglobulin. Blots were developed with the ECL system (GE Healthcare). Normalization of Western blot was ensured by β-actin and band quantification was carried out with a scanner and the Scion Image software.

2.7. Statistics

Prior to statistical analysis, data were tested for homogeneity of variances by the test of Levene. For multiple comparisons, one-way ANOVA was followed by the Bonferroni test when variances were homogeneous or by the Tamhane test when variances were not homogeneous. $P<0.05$ was considered significant. An SPSS version 23.0 program has been used.

3. Results

3.1. Effects of EC and colonic phenolic metabolites on ROS generation

To discard that the exposure of renal tubular cells to EC or colonic phenolic metabolites can induce oxidative stress and enhance ROS generation, NRK-52E cells were incubated with realistic doses of EC, DHBA, DHPAA or HPPA (1-20 µM) for 24 h.

As shown in Table 1, treatment of cells with EC alone decreased dose-dependently the production of ROS (10-20 µM), and all microbial metabolites tested maintained levels of ROS in control values. Moreover, the range of concentrations selected for the study did not damage cell integrity during the period of incubation (Alvarez-Cilleros et al., 2018).

Next, to evaluate the long-term protective effect of EC and the colonic phenolic metabolites selected on NRK-52E cultured cells submitted to oxidative stress, cells were pre-treated for 2 h with EC, DHBA, DHPAA or HPPA (1-20 µM) prior to 22 h of 30 mM glucose treatment; then, ROS generation was assayed. Pre-treatment of NRK-52E cells with DHBA or HPPA did not prevent high-glucose-induced ROS generation, whereas 5-20 µM EC or DHPAA reversed the glucose-induced ROS production to pre-stress values (Table 1), showing an IC50 of 3.6 µM and 4.4 µM for EC and DHPAA, respectively. Therefore, the lowest and realistic range of concentrations for both compounds that showed a protective effect against ROS generation, that is 5-10 µM EC and DHPAA, was selected for further studies.

3.2. Effects of EC and DHPAA on antioxidant defences

To continue the study of the protective effect of EC and DHPAA against a high-glucose challenge, cells were pre-treated with both natural substances, incubated with a high glucose concentration (30 mM), and levels of GSH, and activities of GPx, GR, SOD

and CAT were evaluated. As shown in Figure 2, EC and DHPAA alone (5 and 10 µM) did not alter the GSH content or any enzymatic activity tested, just EC (10 µM) alone increased GPx activity when compared to control cells. In addition, incubation of cells with the high dose of glucose (30 mM) diminished all antioxidant defences assayed, as decreased GSH content and activities of GPx, GR, CAT and SOD (Figure 2).

Under high glucose conditions, pre-treatment of NRK-52E cells with 5 and 10 µM EC or 10 µM DHPAA partially prevented GSH depletion, showing cells pre-treated with 10 µM EC higher GSH levels than 5 µM EC and 10 µM DHPAA; in addition, the mentioned concentrations of EC (5 and 10 µM) and DHPAA (10 µM) completely reversed the glucose-induced inhibition of GPx and GR activities to pre-stress values (Figures 2A and 2B). In line with this, pre-treatment with the highest concentration of EC and DHPAA (10 µM) restored CAT and SOD activities to control levels (Figures 2C and 2D). These results indicate that 10 µM EC and DHPAA protect NRK-52E cells against the redox imbalance caused by the high glucose concentration, therefore, this was the dose selected for both compounds in subsequent experiments.

3.3. Effects of EC and DHPAA on MAPKs protein levels

MAPKs are regulated by oxidative stress and induced by high doses of glucose (Cordero-Herrera et al., 2015a). Then, cells were pre-treated for 2 h with 10 µM EC or 10 µM DHPAA prior to 22 h of 30 mM glucose treatment, and levels of MAPKs were evaluated.

As shown in Figure 3, 30 mM glucose increased the phosphorylated levels of all three MAPKs (p-ERK, p-JNK and p-p38). EC and DHPAA alone did not modify p-ERK, p-JNK or p-p38 levels in comparison to control cells. In addition, pre-treatment with EC or DHPAA diminished the enhanced phosphorylated levels of all three MAPKs induced

by 30 mM glucose (Figure 3). Pre-treatment with EC and DHPAA returned the glucose-enhanced p-ERK and p-p38 values to pre-stress levels, whereas the increase in p-JNK values induced by the high concentration of glucose was totally or partly prevented by EC and DHPAA, respectively. Total levels of ERK, JNK and p38 were not modified by any treatment.

3.4. Effects of EC and DHPAA on SIRT-1 and NOX-4 protein levels

SIRT-1 has an important cytoprotective role in renal cells against different types of stress, and also during diabetes (Hao & Haase, 2010). NOX-4 is reported to be a major source of ROS in the kidneys during diabetic nephropathy (Sedeek et al., 2012). To evaluate the protective effect of EC and DHPAA against a high glucose challenge, cells were pre-treated for 2 h with 10 μM EC or 10 μM DHPAA prior to 22 h of the 30 mM glucose incubation, and levels of both proteins were evaluated.

EC and DHPAA alone did not alter NOX-4 values and increased SIRT-1 levels, showing EC-incubated cells higher levels of SIRT-1 than those of DHPAA-treated cells (Figure 4). Incubation of NRK-52E cells with the high concentration of glucose diminished the expression levels of SIRT-1 and enhanced the values of NOX-4 (Figure 4). Besides, pre-treatment with EC and DHPAA (10 μM) restored to control values the diminished levels of SIRT-1 induced by 30 mM glucose. EC and DHPAA completely and partly prevented 30 mM glucose-induced NOX-4 expression levels, respectively (Figure 4). All this indicate that DHPAA, and more clearly EC, protect NRK-52E cells against the redox imbalance caused by a high glucose concentration.

3.5. NOX-4 regulates EC- and DHPAA-induced changes of ROS generation, SOD activity and SIRT-1 levels in high glucose-exposed renal proximal tubular cells

Since ROS-mediated injury has been reported in *in vitro* and *in vivo* models of diabetic kidney disease, NOX-4 is consider a main source of ROS in the kidney, and its expression increases in diabetes (Sedeek et al., 2012). Then, the effect of NOX-4 inhibition with DPI on cells pre-treated with EC and DHPAA (10 µM) under high glucose conditions was analysed on ROS production, SOD activity, and NOX-4 and SIRT-1 levels.

As described above, EC and DHPAA pre-treatment prevented the decrease of SOD activity and the increase of ROS generation induced by 30 mM glucose, achieving levels similar to those of untreated cells (Figure 5A). Under high glucose conditions, the incubation with DPI (blockage of NOX-4) suppressed the increase of ROS production induced by the high-glucose concentration, and displayed levels comparable to those of EC-treated cells. Additionally, the inhibition of NOX-4 enhanced the SOD activity to values higher than those of unchallenged cells. Similarly, under high-glucose conditions DPI incubation in cells treated with EC or DHPAA, increased SIRT-1 values and decreased NOX-4 levels in comparison to high glucose-treated cells. Thus, high glucose-treated cells that were incubated with DPI and EC displayed higher levels of SIRT-1 than DHPAA+HG+DPI-treated cells, showing in both cases greater values of SIRT-1 than those of EC- or DHPAA-treated alone (Figures 5B and 5C). Moreover, in the presence of DPI the increased levels of NOX-4 induced by the high dose of glucose were totally or partly averted in EC- and DHPAA-pre-treated cells, respectively. All these suggest that the inhibition of NOX-4 seems to act in a similar manner of that of EC and DHPAA to modulate the cellular redox status and that the NOX-4 inhibitor improved the protection induced by EC and DHPAA.

3.6. SIRT-1 regulates EC- and DHPAA-induced changes of ROS production, SOD activity and levels of NOX-4 in high glucose-challenged renal tubular cells

To further clarify the involvement of SIRT-1 in the modulation of redox status induced by EC and DHPAA (10 μM) on high glucose-challenged cells, namely ROS, SOD activity and NOX-4 levels, the effect of a SIRT-1 selective inhibitor on these parameters was assayed. First, NRK-52E cells were pre-treated for 1 h with the SIRT-1 inhibitor (EX-527), and then treated for 2 h with 10 μM EC or 10 μM DHPAA prior to 22 h high glucose incubation (30 mM).

Figure 6A illustrates that EC and DHPAA pre-treatment prevented the increase of ROS generation, and the decrease of SOD activity induced by 30 mM glucose, showing similar levels to those of EC- and DHPAA-treated, and untreated cells (Figure 6A). In high glucose-challenged cells that were pre-incubated with EC or DHPAA, the inhibition of SIRT-1 maintained ROS production and SOD activity in levels comparable to those of high glucose-treated cells. In addition, EC pre-incubation prevented the alteration in SIRT-1 and NOX-4 levels induced by the high-glucose concentration, similar to DHPAA pre-treatment for SIRT-1 expression values, showing in all cases comparable values to those of control cells, whereas DHPAA pre-incubation partially reverted the enhanced levels of NOX-4 induced with 30 mM glucose (Figure 6B and 6C). Besides, under high-glucose conditions, the inhibition of SIRT-1 in cells pre-treated with EC or DHPAA decreased SIRT-1 levels and increased NOX-4 values in comparison to EC- and DHPAA-high glucose-treated cells, evoking lower and higher values than those of high-glucose-challenged cells for SIRT-1 and NOX-4, respectively. These results suggest the involvement of SIRT-1 in EC- and DHPAA-induced modulation of cellular redox status and indicate that the blockage of SIRT-1 abrogated the beneficial effect of EC and DHPAA on the redox balance.

4. Discussion

Nephropathy is a major complication of diabetes and a leading cause of end-stage renal disease (Forbes & Cooper, 2013; Stanton, 2011). Indeed, tubular dysfunction correlates with the decline of the renal function and early appears in the diabetic nephropathy (Forbes & Cooper, 2013; Stanton, 2011). Therefore, protection of renal tubular proximal cells is important to delay the development and progression of the diabetic nephropathy. Different studies have reported that oxidative stress plays an important role in the aetiology of this disease (Stanton, 2011), then oxidative-stress related pathways might become new targets for protecting tubular cells. In this work, for the first time it has been shown that EC and the colonic metabolite DHPAA prevent the imbalance in the cellular redox status provoked by a high concentration of glucose by reducing ROS overproduction, restoring altered antioxidant defences, and restraining signalling pathways related to stress namely MAPKs, SIRT-1 and NOX-4.

Flavanols, such as (-)-epicatechin (EC), constitute an important group of natural compounds that have been widely reported to possess antioxidant and anti-diabetic properties (Martín et al., 2016; Martín & Ramos, 2016; Shahidi & Ambigaipalan, 2015). However, their potential health effects depend on their bioavailability (Blancas-Benitez, Pérez-Jiménez, Montalvo-González, González-Aguilar, & Sáyago-Ayerdi, 2018; Martín et al., 2016; Monagas et al., 2010; Shahidi & Ambigaipalan, 2015). Moreover, when evaluating the impact of phenolic compounds on health, food polyphenols and their metabolites derived from the dietary intake should be taken into account, as both seem to contribute to the potential health beneficial effects and, after the colonic fermentation, metabolites constitute a large percentage of the amount ingested (Alvarez-Cilleros et al., 2018; Fernández-Millán et al., 2014; Martín et al., 2016; Monagas et al., 2010; Shahidi & Ambigaipalan, 2015). In this regard, it is worth mentioning that the main urinary

microbial phenolic acids derived from the flavanol intake are mono- and di-hydroxylated phenylpropionic and phenylacetic acids, together with hydroxybenzoic acids (Monagas et al., 2010), and that pure compounds are accumulated in tissues as a consequence of a conjugation-deconjugation cycle (Pérez-Vizcaíno et al., 2006). Therefore, in the present study EC and three of the most abundant phenols found in urine after the intake of flavanol-rich foods have been selected to evaluate their protective effect against a high-glucose challenge in terms of redox status in renal proximal tubular cells. In addition, it should be mentioned that a realistic range of phenolic compound concentrations (1-20 μM) has been selected to perform the work, since these levels could be found in biological fluids after the intake of flavanols and they are considered to be within the range recommended for *in vitro* studies (Monagas et al., 2010).

In diabetes oxidative stress has been proved as a result of long-term high glucose levels exposure, and mediates diabetic complications (Stanton, 2011). Consistent with this, our results showed that a high concentration of glucose led to an overproduction of ROS, and diminished antioxidant defences such as GSH content, and activities of GPx, GR, SOD and CAT in renal proximal tubular NRK-52E cells. Indeed, both the augmented production of oxidants and the decreased actions of antioxidants play roles in the increased oxidative stress in diabetic nephropathy and have been reported to occur in renal cells under high glucose conditions *in vitro* and *in vivo* (Hou et al., 2014; Quine & Raghu, 2005; W. Zhang et al., 2013).

Under high glucose conditions, EC and DHPAA prevented the imbalance of the redox status caused by the high glucose challenge in renal tubular cells, as they restrained the increase in ROS generation and the decrease of GSHcontent, as well as in GPx, GR, SOD and CAT activities. In agreement with our results, other natural compounds such

as peppermint infusions, glycyrrhizic acid and beta-casomorphin-7 alleviated the high-glucose-induced alteration in the redox status of the kidney in diabetic animals and in renal cultured cells (Figueroa-Pérez et al., 2018; Hou et al., 2014; W. Zhang et al., 2013). Similarly, EC and the NADPH oxidase inhibitor, aponycin, have been reported to prevent the renal imbalance of the redox status in animal models of diabetes (Quine & Raghu, 2005; Winiarska et al., 2014). EC and DHPAA also prevented protein oxidation under oxidative stress conditions in insulin-sensitive tissues (Cordero-Herrera et al., 2015a; Fernández-Millán et al., 2014), and EC has demonstrated a dose-dependent superoxide anion and hydroxyl radical scavenging activity *in vitro* (P. S. M. Prince, 2013). Therefore, a relevant role of EC and DHPAA in the redox balance by quenching superoxide anions and hydroxyl radicals should not be ruled out. Indeed, all these preventive effects have been related to a more efficient ROS quenching in flavanol pre-treated cells or animals, and to a reinforcement in the activity of antioxidant enzymes, which prepares cells to deal with ROS generation in the presence of stressors and thus to suppress or minimize the oxidative-stress induced damage (Cordero-Herrera et al., 2015a; Quine & Raghu, 2005; Vranješ et al., 2016; Winiarska et al., 2014). These subjects deserve further studies.

Oxidative stress induced by hyperglycaemia activates key stress mediators, such as MAPKs, which play an important role in the pathogenesis of the diabetic nephropathy (Forbes & Cooper, 2013). Accordingly, increased phosphorylated levels of all three MAPKs were found in high-glucose challenged NRK-52E cells. Additionally, pre-treatment with EC or DHPAA prevented the activation of the three MAPKs in renal tubular cells treated with the high concentration of glucose. Consistent with this, in renal proximal tubular cells exposed to 60 mM glucose for 48 h the dimer of EC, procyanidin B2, decreased the phosphorylation of p38, showing a protective anti-

fibrotic effect (D. Li et al., 2015). Similarly, it has been shown that in renal cells other phenolic compounds such as apigenin, a novel chalcone, a curcumin derivative, and the main flavanol in green tea, epigallocatechin-3-gallate, exert anti-diabetic effects through the down-regulation of MAPKs *in vitro* and *in vivo* (Fang et al., 2015; Malik et al., 2017; Pan et al., 2013; Yang et al., 2016).

NOX-4 has been recently suggested to contribute to the initiation and development of renal injury in diabetic nephropathy, whereas the inhibition of NOX-4 alleviates the tubular-induced injury (Qi, Niu, Qin, Qiao, & Gu, 2015; Sedeek et al., 2012; Stanton, 2011). In agreement, the present results demonstrated for the first time that EC and DHPAA prevented the high-glucose-induced NOX-4 expression and contribute to the cellular redox balance. This is supported by our findings that inhibiting NOX-4 in EC- and DHPAA-pre-treated cells prevented the high-glucose-induced ROS generation, as well as the decreased SOD activity and SIRT-1 levels, since the NOX-4 inhibitor enhanced the protection induced by EC and DHPAA. Previous studies performed with a cocoa-enriched in flavanols, naringin and antioxidants such as N-acetylcysteine (NAC) or NOX-4 inhibitors (DPI and plumbagin) have demonstrated that those compounds exerted renoprotective effects by improving the functionality of renal tubular cells *in vitro* and *in vivo* (Papadimitriou, Peixoto, Silva, Lopes de Faria, & Lopes de Faria, 2014; Qi et al., 2015; Shimizu, Saito, Higashiyama, Nishijima, & Niwa, 2013; Yong et al., 2013; J. Zhang, Yang, Li, Chen, & J, 2017). Moreover, in renal proximal tubular cells it has been shown that NOX-4 levels were upregulated and SOD reduced in diabetic nephropathy, and in concordance, the blockage of NOX-4 increased SOD activity and reduced oxidative stress markers (Jeong et al., 2018; Yong et al., 2013). All these suggest for the first time that EC and DHPAA might have a key role to inhibit NOX-4 and to prevent the damage in high-glucose-treated renal tubular cells.

The activation of SIRT-1 contributes to increase the resistance to metabolic and oxidative stress (Hao & Haase, 2010). In agreement with our results, it has been reported that SIRT-1 levels are decreased in diabetic patients, as well as in high-glucose-treated renal tubular cells (Hao & Haase, 2010; Hou et al., 2014); indeed the therapeutic use of sirtuins is currently in clinical trials, as they are likely to improve the diabetic condition in humans (Hao & Haase, 2010). In the present work, EC and DHPAA alone increased SIRT-1 levels in NRK-52E cells, in agreement with other studies performed with other flavonoids such as puerarin, and with the stilbene resveratrol in renal cultured cells, and cocoa and EC in adipose tissue of rats (Huang et al., 2016; X. Li et al., 2017; Rabadan-Chávez, Quevedo-Corona, Miliar-Garcia, Reyes-Maldonado, & Jaramillo-Flores, 2016). Additionally, pre-treatment with EC and DHPAA prevented the decrease of SIRT-1 levels induced by the high glucose challenge. Consistent with these findings, grape seed procyanidin B2, glycyrrhizic acid, and puerarin avoided SIRT-1 diminution under high-glucose conditions, which was associated with a protective effect and improved redox balance in renal cells (Bao, Cai, Zhang, & Li, 2015; Hou et al., 2014; X. Li et al., 2017).

The inhibition of SIRT-1 enables to understand better the cross-talk between SIRT-1 and the modulation of the cellular redox status. In our study, the blockage of SIRT-1 abolished the protective effects exerted by EC and DHPAA under high glucose conditions, as enhanced ROS generation and NOX-4 levels, and decreased SOD activity were found. These features suggested that SIRT-1 directly or indirectly regulates key proteins to modulate the cellular redox status. As far as we know, the role of SIRT-1 induction evoked by flavanols or their metabolites on NOX-4 and SOD activity in renal tubular cells has not been previously studied. However, it has been demonstrated that the inhibition of SIRT-1 caused by high concentrations of glucose was associated with a

deterioration in the redox status of renal cells, i.e: enhanced ROS generation and NOX-4 levels, and decreased SOD activity (Hou et al., 2014; X. Li et al., 2017). Additionally, it could be mentioned that in podocytes puerarin conferred a cytoprotective effect by decreasing NOX-4 expression through SIRT-1 activation (X. Li et al., 2017).

Interestingly, antioxidant treatments, such as N-acetylcysteine administration, have demonstrated to play a role against the oxidative stress in renal cells during diabetes, as restored both decreased levels of GSH and diminished activities of GPx and SOD, and inhibited NOX enzymes, resulting in the attenuation of the oxidative damage in renal cells (Lei et al., 2012; Ribeiro et al., 2011; Shimizu et al., 2013). In addition, it should be considered that enhanced levels of ROS activate inflammatory mediators and different signalling pathways apart from the mechanisms studied in the present work (Forbes & Cooper, 2013; Martín & Ramos, 2016). In this line, it has been reported that EC is able to prevent the inflammatory response in the kidney through the downregulation of redox-sensitive pro-inflammatory pathways, such as NF-κB, NOX, TNFα, iNOS, and IL-6 (P. D. Prince, Fischerman, Toblli, Fraga, & Galleano, 2017; P. D. Prince et al., 2016). Likewise, an extract from *Albizia harveyi* containing EC showed an anti-diabetic effect in rats, which could be related to a modulatory effect on relevant enzymes implicated in the renal dysfunction in diabetes, such as aldose reductase (Sobeh et al., 2017); indeed docking studies showed that EC interacted with the active site of α-amilase, maltase-glucoamylase, and aldose reductase (Sobeh et al., 2017). Further studies are needed to elucidate how different molecular mechanisms related to the oxidative stress are modulated by EC and DHPAA in renal tubular cells.

In summary, we show for the first time that EC and the flavanol colonic metabolite DHPAA exert renoprotective effects by contributing to the balance of the cellular redox status, as under high glucose conditions both compounds prevented ROS generation,

activation of stress related key proteins (MAPKs and NOX-4), as well as the diminution of antioxidant defences and SIRT-1 levels. We have also revealed a new mechanism by which EC and DHPAA modulate the redox balance in NRK-52E cells, in which the NOX-4/SIRT-1 signalling plays a main role. All together, this study provides new evidences on the beneficial effects of the cocoa flavanol EC and the colonic metabolite DHPAA on the protection of renal function under a high glucose condition.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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5. References.

Alvarez-Cilleros, D., Martín, M. A., & Ramos, S. (2018). (-)-Epicatechin and the colonic 2,3-dihydroxybenzoic acid metabolite regulate glucose uptake, glucose production, and improve insulin signaling in renal NRK-52E cells. *Mol Nutr Food Res, DOI: 10.1002/mnfr.201700470*.

Bao, L., Cai, X., Zhang, Z., & Li, Y. (2015). Grape seed procyanidin B2 ameliorates mitochondrial dysfunction and inhibits apoptosis via the AMP-activated protein kinase–silent mating type information regulation 2 homologue 1–PPARg co-activator-1a axis in rat mesangial cells under high-dose glucosamine. *Brit J Nutr, 113*, 35-44.

Barnett, C. F., Moreno-Ulloa, A., Shiva, S., Ramirez-Sanchez, I., Taub, P. R., Su, Y., . . . Villarreal, F. (2015). Pharmacokinetic, partial pharmacodynamic and initial safety analysis of (-)-Epicatechin in healthy volunteers. *Food & Function, 6*, 824-833.

Blancas-Benitez, F. J., Pérez-Jiménez, J., Montalvo-González, E., González-Aguilar, G. A., & Sáyago-Ayerdi, S. G. (2018). In vitro evaluation of the kinetics of the release of phenolic compounds from guava (*Psidium guajava* L.) fruit. *J Funct Food, 43*, 139–145.

Bradford, M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein, utilizing the principle of protein-dye binding. *Anal Biochem., 72*, 248.

Cordero-Herrera, I., Martín, M. A., Goya, L., & Ramos, S. (2015a). Cocoa flavonoids protect hepatic cells against high glucose-induced oxidative stress. Relevance of MAPKs. *Mol Nutr Food Res, 59*, 597-609.

Cordero-Herrera, I., Martín, M. A., Goya, L., & Ramos, S. (2015b). Cocoa intake ameliorates hepatic oxidative stress in young Zucker diabetic fatty rats. *Food Res Int, 69*, 194-201.

Fang, Q., Zhao, L., Wang, Y., Zhang, Y., Li, Z., Pan, Y., . . . Liang, G. (2015). A novel chalcone derivative attenuates the diabetes-induced renal injury via inhibition of high glucose-mediated inflammatory response and macrophage infiltration. *Toxicol Appl Pharmacol, 282*, 129-138.

Fernández-Millán, E., Ramos, S., Alvarez, C., Bravo, L., Goya, L., & Martín, M. A. (2014). Microbial phenolic metabolites improve glucose-stimulated insulin secretion and protect pancreatic beta cells against tert-butylhydroperoxide-induced toxicity via ERKs and PKC pathways. *Food Chem Toxicol, 66*, 245-253.

Figueroa-Pérez, M. G., Pérez-Ramírez, I. F., Enciso-Moreno, J. A., Gallegos-Corona, M. A., Salgado, L. M., & Reynoso-Camacho, R. (2018). Diabetic nephropathy is ameliorated with peppermint (*Mentha piperita*) infusions prepared from salicylic acid-elicited plants. *J Funct Food, 43*, 55-61.

Forbes, J. M., & Cooper, M. E. (2013). Mechanisms of diabetic complications. *Physiol Rev, 93*, 137-188.

Giacometti, J., Muhvic, D., Pavletic, A., & Đudaric, D. (2016). Cocoa polyphenols exhibit antioxidant, antiinflammatory, anticancerogenic, and antinecrotic activity in carbon tetrachloride intoxicated mice. *J Funct Food, 23*, 177-187.

Granado-Serrano, A. B., Martín, M. A., Goya, L., Bravo, L., & Ramos, S. (2009). Time-course regulation of survival pathways by epicatechin on HepG2 cells. *J Nutr Biochem, 20*, 115-124.

Granado-Serrano, A. B., Martín, M. A., Izquierdo-Pulido, M., Goya, L., Bravo, L., & Ramos, S. (2007). Molecular mechanisms of (-)-epicatechin and chlorogenic acid on the regulation of the apoptotic and survival/proliferation pathways in a human hepatoma cell line. *J Agric Food Chem., 55*, 2020-2027.

Hao, C.-M., & Haase, V. H. (2010). Sirtuins and their relevance to the kidney. *J Am Soc Nephrol, 21*, 1620-1627.

Hissin, P., & Hilf, R. (1976). A fluorimetric method for determination of oxidized and reduced glutathione in tissues. *Anal Biochem., 74*, 214-226.

Hou, S., Zheng, F., Li, Y., Gao, L., & Zhang, J. (2014). The protective effect of glycyrrhizic acid on renal tubular epithelial cell injury induced by high glucose. *Int J Mol Sci, 15*, 15026-15043.

Huang, Y. T., Chen, Y. Y., Lai, Y. H., Cheng, C. C., Lin, T. C., Su, Y. S., . . . Lai, P. C. (2016). Resveratrol alleviates the cytotoxicity induced by the radiocontrast agent, ioxitalamate, by reducing the production of reactive oxygen species in HK-2 human renal proximal tubule epithelial cells in vitro. *Int J Mol Med, 37*, 83-91.

Jeong, B. Y., Lee, H. Y., Park, C. G., Kang, J., Yu, S. L., Choi, D. R., . . . Yoon, S. H. (2018). Oxidative stress caused by activation of NADPH oxidase 4 promotes contrast-induced acute kidney injury. *PLoS One, 13*(1), e0191034.

Lei, S., Liu, Y., Liu, H., Yu, H., Wang, H., & Xia, Z. (2012). Effects of N-acetylcysteine on nicotinamide dinucleotide phosphate oxidase activation and antioxidant status in heart, lung, liver and kidney in streptozotocin-induced diabetic rats. *Yonsei Med J, 53*, 294-303.

Li, D., Zhao, T., Meng, J., Jing, Y., Jia, F., & He, P. (2015). Procyanidin B2 inhibits high glucose‐induced epithelial‐mesenchymal transition in HK‐2 human renal proximal tubular epithelial cells. *Mol Med Rep*, 8148-8154.

Li, X., Cai, W., Lee, K., Liu, B., Deng, Y., Chen, Y., . . . Zhong, Y. (2017). Puerarin attenuates diabetic kidney injury through the suppression of NOX4 expression in podocytes. *Sci Rep, 7*, 14603.

Loke, W. M., Hodgson, J. M., Proudfoot, J. M., McKinley, A. J., Puddey, I. B., & Croft, K. D. (2008). Pure dietary flavonoids quercetin and ()-epicatechin augment nitric oxide products and reduce endothelin-1 acutely in healthy men. *Am J Clin Nutr 88*, 1018-1025.

Malik, S., Suchal, K., Khan, S. I., Bhatia, J., Kishore, K., Dinda, A. K., & Arya, D. S. (2017). Apigenin ameliorates streptozotocin-induced diabetic nephropathy in rats via MAPK-NF-κB-TNF-α and TGF-β1-MAPK-fibronectin pathways. *Am J Physiol Renal Physiol, 313*(2), F414-F422.

Martín, M. A., Goya, L., & Ramos, S. (2016). Antidiabetic actions of cocoa flavanols. *Mol Nutr Food Res, 60*, 1756-1769.

Martín, M. A., Granado-Serrano, A. B., Ramos, S., Izquierdo Pulido, M., Bravo, L., & Goya, L. (2010). Cocoa flavonoids up-regulate antioxidant enzyme activity via the ERK1/2 pathway to protect against oxidative stress-induced apoptosis in HepG2 cells. *J Nutr Biochem, 21*, 196-205.

Martín, M. A., & Ramos, S. (2016). Cocoa polyphenols in oxidative stress: Potential health implications. *J Funct Food, 27*, 570-588.

Monagas, M., Urpi-Sarda, M., Sanchez-Patan, F., Llorach, R., Garrido, I., Gomez-Cordoves, C., . . . Bartolome, B. (2010). Insights into the metabolism and microbial biotransformation of dietary flavan-3-ols and the bioactivity of their metabolites. *Food & Function, 1*, 233-253.

Ottaviani, J. I., Borges, G., Momma, T. Y., Spencer, J. P. E., Keen, C. L., Crozier, A., & Schroeter, H. (2016). The metabolome of [2-14C](−)-epicatechin in humans: implications for the assessment of efficacy, safety, and mechanisms of action of polyphenolic bioactives. *Sci Rep, 6*, 29034.

Pan, Y., Zhu, G., Wang, Y., Cai, L., Cai, Y., Hu, J., . . . Liang, G. (2013). Attenuation of high-glucose-induced inflammatory response by a novel curcumin derivative B06 contributes to its protection from diabetic pathogenic changes in rat kidney and heart. *J Nutr Biochem, 24*, 146-155.

Papadimitriou, A., Peixoto, E. B. M. I., Silva, K. C., Lopes de Faria, J. M., & Lopes de Faria, J. B. (2014). Increase in AMPK brought about by cocoa is renoprotective in experimental diabetes mellitus by reducing NOX4/TGFβ-1 signaling. *J Nutr Biochem, 25*, 773-784.

Pérez-Vizcaíno, F., Bishop-Bailley, D., Lodi, F., Duarte, J., Cogolludo, A., Moreno, L., . . . Warner, T. (2006). The flavonoid quercetin induces apoptosis and inhibits JNK activation in intimal vascular smooth muscle cells. *Biochem Biophys Res Comm, 346*, 919-925.

Prince, P. D., Fischerman, L., Toblli, J. E., Fraga, C. G., & Galleano, M. (2017). LPS-induced renal inflammation is prevented by (−)-epicatechin in rats. *Redox Biol, 11*, 342-349.

Prince, P. D., Rodríguez-Lanzi, C., Toblli, J. E., Elesgaray, R., Oteiza, P. I., Fraga, C. G., & Galleano, M. (2016). Dietary (-)-epicatechin mitigates oxidative stress, NO metabolism alterations,and inflammation in renal cortex from fructose-fed rats. *Free Radic Biol Med, 90*, 35-46.

Prince, P. S. M. (2013). (-) Epicatechin attenuates mitochondrial damage by enhancing mitochondrial multi-marker enzymes, adenosine triphosphate and lowering calcium in isoproterenol induced myocardial infarcted rats *Food Chem Toxicol, 53*, 409-416.

Qi, W., Niu, J., Qin, Q., Qiao, Z., & Gu, Y. (2015). Glycated albumin triggers fibrosis and apoptosis via an NADPH oxidase/Nox4-MAPK pathway-dependent mechanism in renal proximal tubular cells. *Mol Cell Endocrinol, 405*, 74-83.

Quine, S. D., & Raghu, P. S. (2005). Effects of (–)-epicatechin, a flavonoid on lipid peroxidation and antioxidants in streptozotocin-induced diabetic liver, kidney and heart. *Pharmacol Rep, 57*, 610-615.

Rabadan-Chávez, G., Quevedo-Corona, L., Miliar-Garcia, A., Reyes-Maldonado, E., & Jaramillo-Flores, M. E. (2016). Cocoa powder, cocoa extract and epicatechin attenuate hypercaloric diet-induced obesity through enhanced β-oxidation and energy expenditure in white adipose tissue. *J Funct Food, 20*, 54-67.

Ramiro-Puig, E., Casadesus, G., Lee, H. G., Zhu, X., McShea, A., Perry, G., . . . Castell, M. (2009). Neuroprotective effect of cocoa flavonoids on in vitro oxidative stress. *Eur J Nutr, 48*, 54-61.

Ribeiro, G., Roehrs, M., Bairros, A., Moro, A., Charão, M., Araújo, F., . . . Garcia, S. C. (2011). N-acetylcysteine on oxidative damage in diabetic rats. *Drug Chem Toxicol*, 1-8.

Sedeek, M., Montezano, A. C., Hebert, R. L., Gray, S. P., Di Marco, E., Jha, J. C., . . . Touyz, R. M. (2012). Oxidative stress, Nox isoforms and complications of diabetes-potential targets for novel therapies. *J Cardiovasc Trans Res, 5*, 509-518.

Shahidi, F., & Ambigaipalan, P. (2015). Phenolics and polyphenolics in foods, beverages and spices: Antioxidant activity and health effects–A review. *J Funct Food, 18*, 820-897.

Shimizu, H., Saito, S., Higashiyama, Y., Nishijima, F., & Niwa, T. (2013). CREB, NF-κB, and NADPH oxidase coordinately upregulate indoxyl sulfate-induced angiotensinogen expression in proximal tubular cells. *Am J Physiol Cell Physiol, 304*(7), C685-C692.

Sobeh, M., Mahmoud, M. F., Abdelfattah, M. A. O., El-Beshbishy, H. A., El-Shazly, A. M., & Wink, M. (2017). *Albizia harveyi:* phytochemical profiling, antioxidant, antidiabetic and hepatoprotective activities of the bark extract. *Med Chem Res, 26*, 3091-3105.

Stanton, R. C. (2011). Oxidative stress and diabetic kidney disease. *Curr Diab Rep, 11*, 330-336.

Vranješ, M., Popovic´, B. M., Štajner, D., Ivetic´, V., Mandic´, A., & Vranješ, D. (2016). Effects of bearberry, parsley and corn silk extracts on diuresis, electrolytes composition, antioxidant capacity and histopathological features in mice kidneys. *J Funct Food, 21*, 272-282.

Winiarska, K., Focht, D., Sierakowski, B., Lewandowski, K., Orlowska, M., & Usarek, M. (2014). NADPH oxidase inhibitor, apocynin, improves renal glutathione status in Zucker diabetic fatty rats: a comparison with melatonin. *Chem Biol Interact, 218*, 12-19.

Yang, X. H., Pan, Y., Zhan, X. L., Zhang, B. L., Guo, L. L., & Jin, H. M. (2016). Epigallocatechin-3-gallate attenuates renal damage by suppressing oxidative stress in diabetic db/db mice. *Oxid Med Cell Longev, 2016*, 2968462.

Yong, R., Chen, X. M., Shen, S., Vijayaraj, S., Ma, Q., Pollock, C. A., & Saad, S. (2013). Plumbagin ameliorates diabetic nephropathy via interruption of pathways that include NOX4 signalling. *PLoS One, 8*(8), e73428.

Zhang, J., Yang, S., Li, H., Chen, F., & J, S. (2017). Naringin ameliorates diabetic nephropathy by inhibiting NADPH oxidase 4. *Eur J Pharmacol, 804*, 1-6.

Zhang, W., Miao, J., Wang, S., & Zhang, Y. (2013). The protective effects of beta-casomorphin-7 against glucose -induced renal oxidative stress in vivo and vitro. *PLoS One, 8*(5), e63472.

Legends to figures

Figure 1. Chemical structure of (-)-epicatechin (EC) and 3,4-dihydroxyphenilacetic acid (DHPAA).

Figure 2. Protective effect of (A and C) EC and (B and D) DHPAA on GSH levels and the activity of antioxidant enzymes GPx, GR, CAT and SOD. NRK-52E cells treated with 10 µM EC or 10 µM DHPAA for 2 h were later incubated with 30 mM glucose (HG) for 22 h. Data are expressed as percentage of controls. Values are means±SD of 6-9 different samples per condition. Different letters over a bar of the same parameter indicate statistically significant differences ($P<0.05$). Different styles of letters (plain, bold and italics) have been used for each parameter depicted within the same graph.

Figure 3. Effect of EC and DHPAA on phosphorylated and total levels of ERK, JNK, p-38 in NRK-52E. Cells treated with 10 µM EC or 10 µM DHPAA for 2 h were later incubated with 30 mM glucose (HG) for 22 h. (A) Bands of representative experiments. Percentage data of (B) p-ERK/ERK, (C) p-JNK/JNK and (D) p-p38/p38 ratios relative to control condition (means±SD, n=5-8). Equal loading of Western blots was ensured by β-actin. Means without a common letter differ significantly ($P<0.05$).

Figure 4. Effect of EC and DHPAA on SIRT-1 and NOX-4 in NRK-52E. Cells were incubated with 10 µM EC or 10 µM DHPAA for 2 h prior to a 22 h-glucose (HG, 30 mM) challenge. (A) Bands of representative experiments. Densitometric quantification of total levels (B) SIRT-1 and (C) NOX-4. Values are expressed as percentage relative to the control condition (means±SD, n=6-9). Equal loading of Western blots was ensured by β-actin. Means without a common letter differ significantly ($P<0.05$).

Figure 5. Effect of EC and DHPAA and an inhibitor of NOX (DPI) on ROS generation, SOD activity, and total levels of SIRT-1 and NOX-4. NRK-52E cells incubated for 1 h with 10 μM DPI were later treated with 10 μM EC or DHPAA for 2 h prior to 22 h-glucose (HG, 30 mM) challenge. (A) Intracellular ROS production and activity of SOD are expressed as percentage of control (means±SD, n=6-8). (B) Bands of representative experiments. (C) Percentage data of total levels of SIRT-1 and NOX-4 relative to the control condition (means±SD, n=6-9). Equal loading of Western blots was ensured by β-actin. Means without a common letter differ significantly ($P<0.05$). Different styles of letters (plain, bold and italics) have been used for each parameter depicted within the same graph.

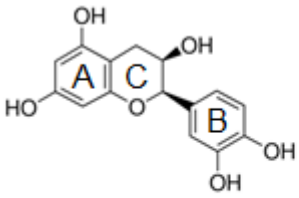
Figure 6. Effect of EC and DHPAA and a selective inhibitor of SIRT-1 (EX, EX-527) on intracellular ROS production, SOD activity, and SIRT-1 and NOX-4 total levels. NRK-52E cells incubated with 10 μM EX for 1 h were later incubated with 10 μM EC or DHPAA for 2 h prior to 22 h-glucose (HG, 30 mM) challenge. (A) Intracellular ROS production and activity of SOD are expressed as percentage of control (means±SD, n=6-9). Different letters over bars indicate statistically significant differences ($P<0.05$). Different styles of letters (plain, bold and italics) have been used for each parameter depicted within the same graph. (B) Bands of representative experiments. (C) Percentage data of total levels of SIRT-1 and NOX-4 relative to the control condition (means±SD, n=7-9). Equal loading of Western blots was ensured by β-actin. Different letters over bars indicate statistically significant differences ($P<0.05$). Different styles of letters (plain, bold and italics) have been used for each parameter depicted within the same graph.

Table 1. Direct and protective effect of EC, DHPAA, DHBA and HPPA on ROS generation. NRK-52E cells were treated with the noted concentrations of mentioned compounds during 24 hours (direct effect, without glucose, left column) or for 2 hours, and then 30 mM glucose (HG) was added for 22 hours to all cultures except controls (protective effect, high glucose, right column). Results are expressed as a percent relative to the control condition. Values are means ± SD of 6-10 data. Means in a column without a common letter differ, $P< 0.05$.

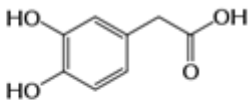
		Direct effect			**Protective effect**
	Concentration	**ROS** (% of control)		**Concentration**	**ROS** (% of control)
Control		100.01 ± 2.95^{a}	**Control**		99.99 ± 2.13^{a}
		-	**Glucose (30 mM)**		124.13 ± 3.04^{b}
EC	**1 µM**	93.91 ± 7.25^{a}	**EC+HG**	**1 µM**	126.75 ± 6.46^{b}
	5 µM	91.96 ± 5.54^{ab}		**5 µM**	100.53 ± 6.21^{a}
	10 µM	89.39 ± 4.90^{bc}		**10 µM**	103.32 ± 3.60^{a}
	20 µM	80.17 ± 4.39^{c}		**20 µM**	102.11 ± 4.69^{a}
DHPAA	**1 µM**	98.04 ± 3.63^{a}	**DHPAA+HG**	**1 µM**	129.82 ± 8.19^{b}
	5 µM	96.44 ± 8.58^{a}		**5 µM**	110.73 ± 6.74^{ab}
	10 µM	93.73 ± 6.66^{a}		**10 µM**	98.14 ± 6.09^{a}

	20 µM	100.65 ± 9.11[a]		**20 µM**	98.23 ± 5.87[a]
DHBA	**1 µM**	94.70 ± 5.48[a]	**DHBA+HG**	**1 µM**	126.69 ± 8.21[b]
	5 µM	100.18 ± 4.16[a]		**5 µM**	121.05 ± 8.97[b]
	10 µM	98.86 ± 1.50[a]		**10 µM**	126.78 ± 5.98[b]
	20 µM	102.18 ± 3.52[a]		**20 µM**	132.38 ± 9.61[b]
HPPA	**1 µM**	99.31 ± 1.97[a]	**HPPA**	**1 µM**	121.97 ± 5.69[b]
	5 µM	97.25 ± 4.32[a]		**5 µM**	120.40 ± 6.13[b]
	10 µM	94.81 ± 5.34[a]		**10 µM**	128.64 ± 8.52[b]
	20 µM	92.99 ± 3.35[a]		**20 µM**	126.66 ± 1.12[b]

Figure 1



EC



DHPAA

Figure 2

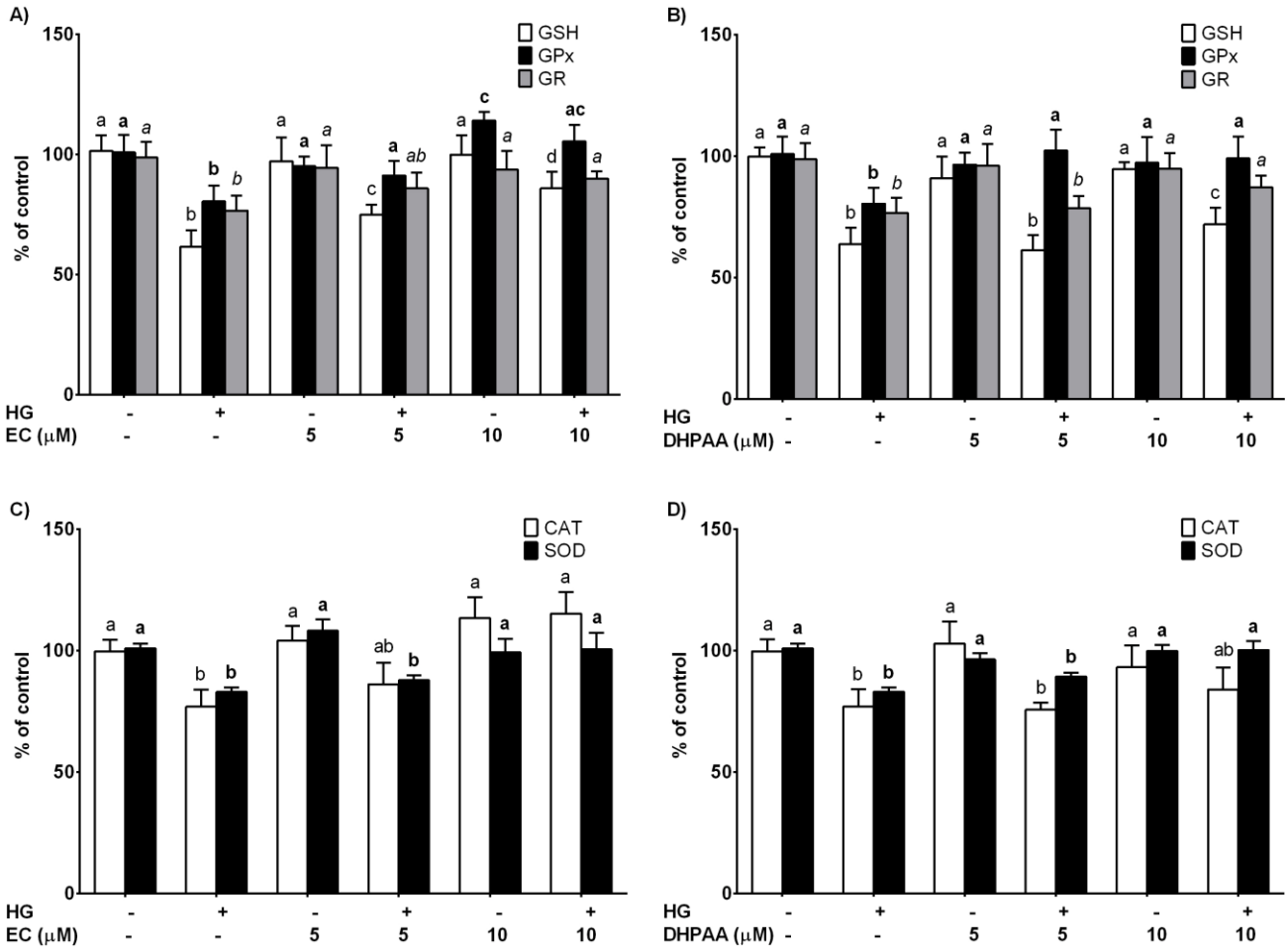

A)
GSH
GPx
GR
% of control
150
100
50
0
HG - + - + - +
EC (μM) - - 5 5 10 10
B)
GSH
GPx
GR
% of control
DHPAA (μM) - - 5 5 10 10
C)
CAT
SOD
% of control
HG - + - + - +
EC (μM) - - 5 5 10 10
D)
CAT
SOD
% of control
DHPAA (μM) - - 5 5 10 10

Figure 3

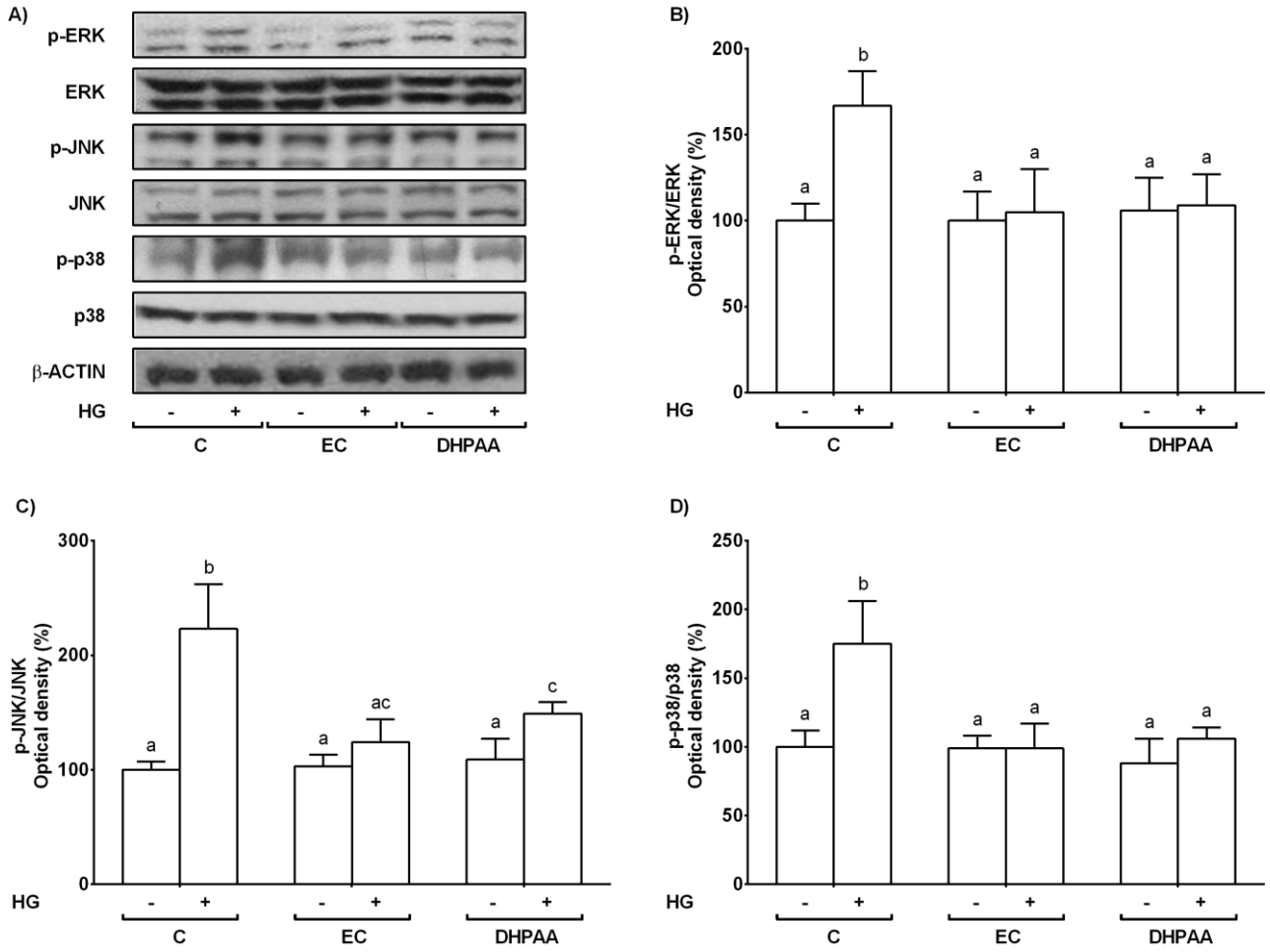

A)
p-ERK
ERK
p-JNK
JNK
p-p38
p38
β-ACTIN
HG
- + - + - +
C
EC
DHPAA
B)
p-ERK/ERK
Optical density (%)
200
150
100
50
0
a
b
a
a
a
a
HG
- + - + - +
C
EC
DHPAA
C)
p-JNK/JNK
Optical density (%)
300
200
100
0
a
b
a
ac
a
c
HG
- + - + - +
C
EC
DHPAA
D)
p-p38/p38
Optical density (%)
250
200
150
100
50
0
a
b
a
a
a
a
HG
- + - + - +
C
EC
DHPAA

Figure 4

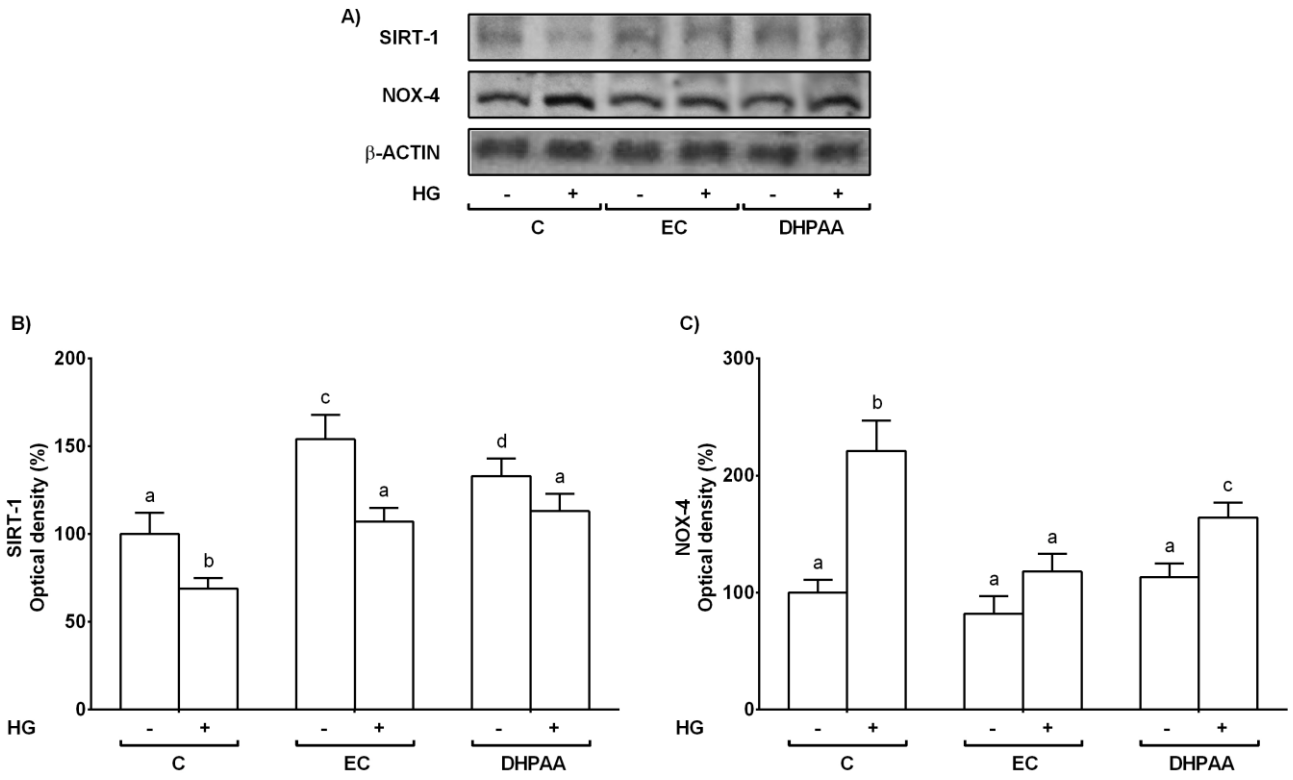

A)
SIRT-1
NOX-4
β-ACTIN
HG
- + - + - +
C
EC
DHPAA
B)
SIRT-1 Optical density (%)
200
150
100
50
0
a
b
c
a
d
a
HG
- + - + - +
C
EC
DHPAA
C)
NOX-4 Optical density (%)
300
200
100
0
a
b
a
a
a
c
HG
- + - + - +
C
EC
DHPAA

Figure 5

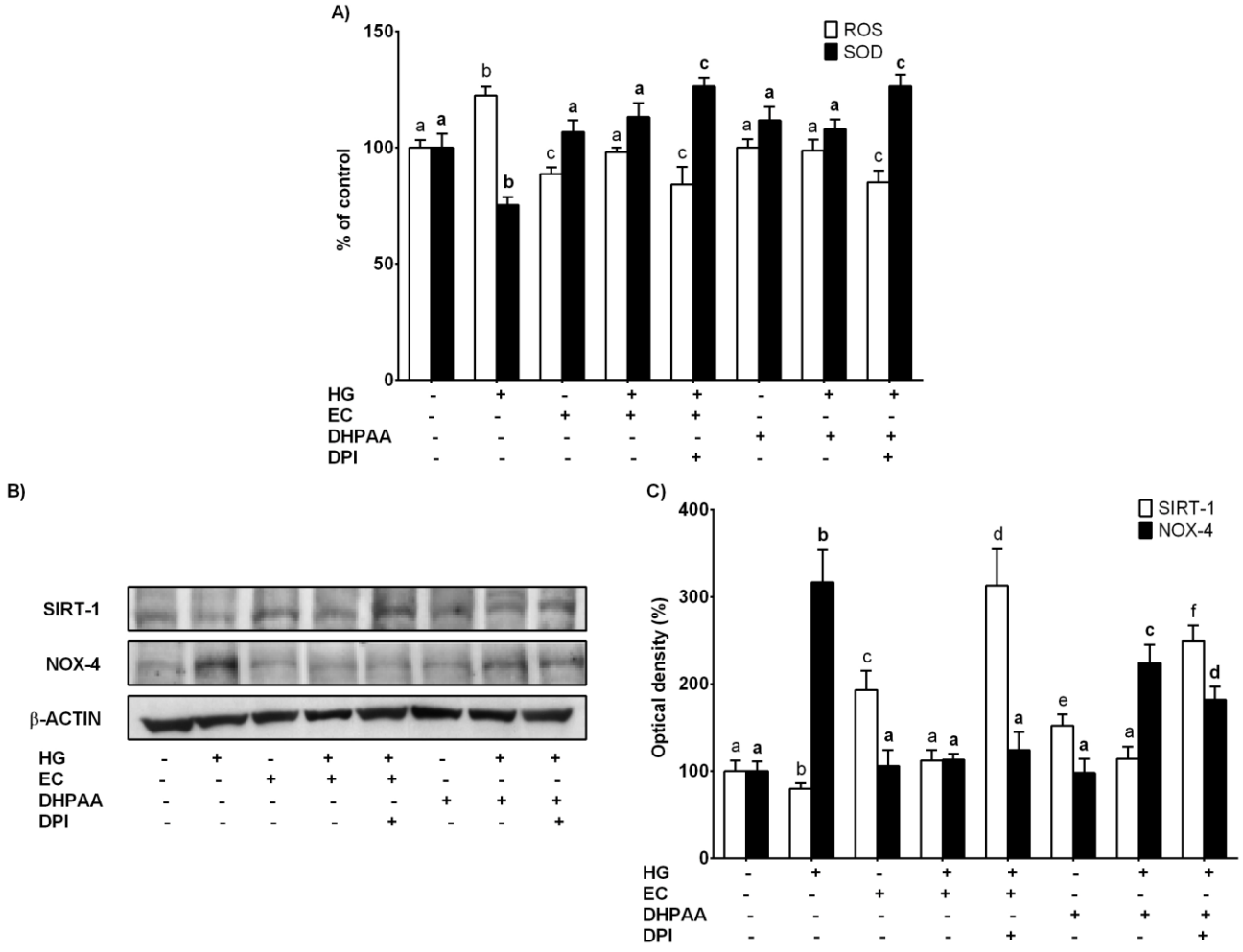
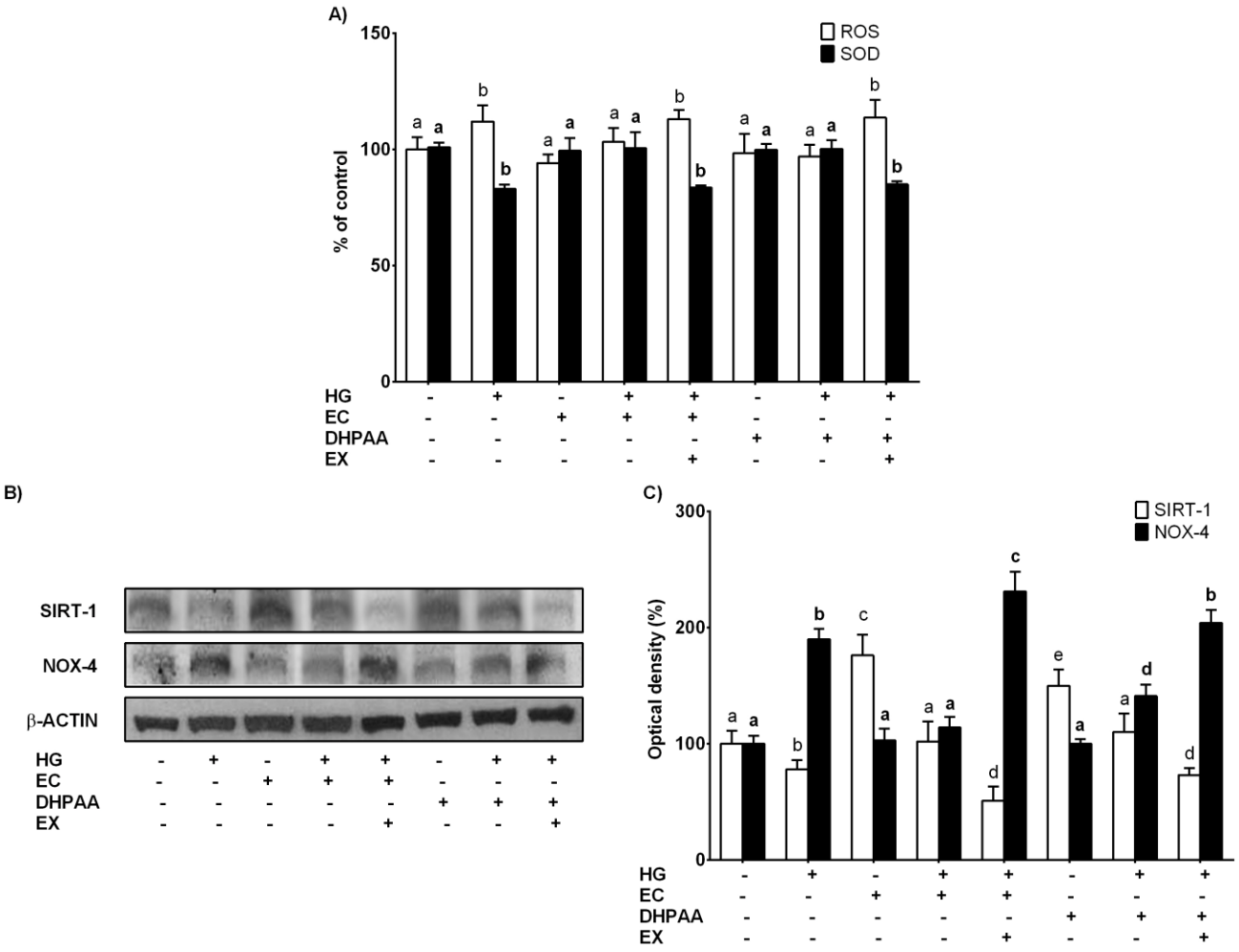

A)
ROS
SOD
% of control
150
100
50
0
HG
EC
DHPAA
DPI
B)
SIRT-1
NOX-4
β-ACTIN
HG
EC
DHPAA
DPI
C)
SIRT-1
NOX-4
Optical density (%)
400
300
200
100
0
HG
EC
DHPAA
DPI

Figure 6


A)
ROS
SOD
% of control
150
100
50
0
HG
EC
DHPAA
EX
B)
SIRT-1
NOX-4
β-ACTIN
HG
EC
DHPAA
EX
C)
SIRT-1
NOX-4
Optical density (%)
300
200
100
0
HG
EC
DHPAA
EX