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Green rooibos extract attenuates high glucose induced oxidative stress in a human derived (HepG2) liver cell line

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ABSTRACT

The exposure of hepatocytes to high concentrations of glucose results in a glucotoxic environment and ensuing hepatocellular injury, often due to oxidative stress. The popularly consumed herbal tea, Rooibos (*Aspalathus linearis*), demonstrates pleiotropic pharmacological and antioxidant activities. Our study investigated the molecular events regulating the antioxidant effects of a Rooibos ethanolic extract (REE)in a glucotoxic HepG2 liver cell model. It is hypothesized that REE enhances antioxidant enzyme profiles of HepG2 cells exposed to high glucose to attenuate glucotoxic damage. Glucotoxicity was induced by treating the cells with 25 mM glucose. For this study, colorimetric, luminometric and western blot analyses were used to determine the potential of the REE to reduce biomarkers associated with oxidative damage and its potential to enhance the intracellular antioxidant defense system. The REE was found to be hepatoprotective as evidenced by reduced levels of oxidative damage biomarkers (MDA and protein carbonylation) and enhanced antioxidant capacity under glucotoxic conditions as evidenced by elevated GSH (reduced form). We also observed decreased intracellular hydrogen peroxide levels and caspase activity in the REE treated cells and further showed that the REE enhanced SOD2, CAT, and GPx1 protein expression. These effects were dependent on the transcriptional activity of NRF2. Collectively, our results show that the REE enhances the cellular antioxidant defense capacity and may provide a relevant supportive therapeutic option for treating diabetic complications associated with oxidative stress.

1. Introduction

The increase in macronutrient availability and caloric intake, surplus to physiological requirements, are considered as etiological drivers of diabetes progression (Taylor, 2016; Dendup et al., 2018). Diabetes and its multitude of accompanying complications is associated with hyperglycaemia and consequent glucotoxicity in many tissues. Markers for mitochondrial dysfunction, elevated production of reactive oxygen species (ROS) and lipid peroxidation have been observed in the liver of Zucker rats with type 2 diabetes (T2DM) (Raza et al., 2015) and T2DM patients (Maiese, 2015). Strong evidence has indicated that oxidative stress plays a major role in the pathological processes of T2DM (Henriksen et al., 2011; Tangvarasittichai, 2015) and the development of diabetic complications (Kawahito et al., 2009).

A central mechanism in the cellular defense against the induced-oxidative stress is the activation of the nuclear factor erythroid 2p45-related factor 2 (NRF2) antioxidant response element (ARE) signaling pathway, which regulates the expression of genes whose protein products are responsible for elimination of ROS by enhancing cellular antioxidant capacity (Nguyen et al., 2009). Stimulation of NRF2 antioxidant pathways suppresses oxidative stress and protects tissue against diabetes-mediated oxidative damage (Uruno et al., 2015). There is growing appreciation that the simultaneous targeting of hyperglycaemia and oxidative stress may present a viable strategy to ameliorate cell injury which is commonly observed in the diabetic state (Johansen et al., 2005; Erejuwa, 2012; Mohamed et al., 2016).

Many countries rely on the socio-cultural relevance of ethnopharmacology to meet their primary healthcare needs (Leonti, 2013; Cordell, 2014). There is a growing interest to assess the botanical resources of Southern Africa, and screen indigenous plants for pharmacologically active compounds as novel drug candidates (Douwes et al., 2008; Rybicki et al., 2012). The plant, *Aspalathus linearis* (Burm. f.) R.Dahlgren, Fabaceae, commonly referred to as Rooibos, is endemic to the Cederberg region of South Africa (Malgas et al., 2010). Rooibos is commonly consumed as an herbal tisane and its potential as a phytopharmaceutical has been expanded to intermediate value-added products such as extracts for the nutraceutical and cosmeceutical markets, owing to its unique composition of phytochemicals and bioactive properties (Joubert et al., 2011). Rooibos is well-known for its antioxidant (AO) activity (Villaño et al., 2010; Breiter et al., 2011;

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Marnewick, 2014) which strongly contributes to its therapeutic potential.

Rooibos has been proposed as a nutraceutical intervention for glucose toxicity by modulating antioxidant activities (Ulicna et al., 2006; P.V. Dludla et al., 2017; P.V. Dludla et al., 2020) and metabolic pathways (Kawano et al., 2009; Muller et al., 2012). There is also a growing body of evidence supporting the organo-protective properties of Rooibos under hyperglycaemic conditions (Kawano et al., 2009; Himpe et al., 2016; P.V. Dludla et al., 2020). Thus, Rooibos is uniquely positioned to concurrently target hyperglycemia and oxidative stress and as a result ameliorate damage to organs. The liver is particularly susceptible to glucotoxicity as it plays a prominent role in glucose metabolism and disposal (Klover et al., 2004). Literature has indicated that hepatotoxicity associated with high glucose is strongly linked to oxidative stress (Kawahito et al., 2009; Alnahdi et al., 2019) leading to impaired metabolism and cell signaling (Hensley et al., 2000; Czaja, 2007).

The hepato-protective effect of Rooibos against glucotoxicity has not been documented to date. The present study aimed to verify whether Rooibos protects liver cells *in vitro* against oxidative stress induced by glucotoxicity and whether it improves the cellular antioxidant response. We assessed protein expression profiles of relevant antioxidant enzymes as well as biomarkers associated with oxidative stress to determine the hepato-protective benefits of REE and application in a glucotoxic liver model.

2. Experimental section

2.1. Materials

The HepG2 cell line was purchased from the American Type Culture Collection (ATCC), Glucose (G8270−100 G) was purchased from Sigma-Aldrich (South Africa). Cell culture supplements were procured from ThermoFisher (South Africa). All other reagents and consumables were purchased from Merck (South Africa), unless otherwise stated.

2.2. Plant collection and extraction

The commercially available rooibos plant material used in this study was a generous gift from Rooibos Ltd (Clanwilliam, Western Cape, South Africa) the biggest producers of Rooibos in South Africa (https://rooibosltd.co.za/our-products/). Freshly dried and ground green Rooibos plant material was donated in April 2019 (Batch number: U15/10/JJ). The macerated plant material (20 g) was soaked in a water: ethanol mixture (20:80, 100 mL) for 24 hr at room temperature with constant stirring. The whole plant material was filtered out, and the filtrate evaporated under reduced pressure using a rotary evaporator. The concentrated crude solution was incubated at 37 °C until it was completely dried. The obtained extract (REE) was collected and kept at −20 °C until needed.

2.3. Phenolic content and total antioxidant capacity of REE

The total polyphenol content of the ethanolic green Rooibos extract was determined using the Folin Ciocalteu's phenol reagent according to the method described by Ajuwon (Ajuwon et al., 2014) and results expressed as mg gallic acid equivalents.

The total antioxidant capacity of the Rooibos extract was assessed by trolox equivalent antioxidant capacity (TEAC) and ferric ion reducing antioxidant power (FRAP) according to methods described by Ajuwon (Ajuwon et al., 2013).

2.4. HPLC conditions

Quantification of the major phenolic compounds in the ethanolic Rooibos extract was performed by high performance liquid chromatography (HPLC) on an Agilent 1200 series instrument (Agilent Technologies, Waldbron, Germany) equipped with an in-line degasser, quaternary pump, autosampler and a diode array and multiple wavelength detector, using a 5 μm YMC-Pack Pro-C18 (150 mm × 4.6 mm i.d.) column for separation, with acquisition set at 287 nm for aspalathin and 360 nm for other components. The methods was carried out as per (Ajuwon et al., 2014).

2.5. Cell culture and treatment

Cell culture of the HepG2 cells was done as per Alnahdi et al. (2019) with minor modifications. The HepG2 cells were cultured in DMEM supplemented with 2 mM glutamine, 10% foetal calf serum, and 1% penstrep-fungizone in a humidified incubator (5% CO2 at 37 ° C). Cells were cultured to 80% confluence and then treated with DMEM (Thermo-Fischer, A1443001) supplemented with 5.5 mM glucose (NG, mimics the *in vivo* fasting concentration) or 25 mM glucose (HG, mimics the glucotoxicity found in the *in vivo* diabetic condition) (Alnahdi et al., 2019).

A concentrated stock solution of the REE (1 mg/mL in PBS) was prepared and diluted in the relevant NG or HG supplemented DMEM to the concentrations required for subsequent experiments. Control cells contained respective media with no REE.

2.6. Cell viability – MTT assay

HepG2 cells were seeded into 96 well microtitre plates (15,000 cells/well) and allowed to adhere overnight. Thereafter, cells were treated with varying concentrations of REE (0 μg/mL −1000 μg/mL) under NG and HG conditions at 37 °C for 4 hrs in triplicate. Following treatment, cells were rinsed twice with 0.1 M PBS and incubated (37 °C, 4 hrs) with 20 μl MTT salt solution (5 mg/ml in 0.1 M PBS) and 200 μl DMEM (NG and HG). Subsequently, supernatants were removed and 100 μl of DMSO ($\geq$ 99.9%, MERCK, D8418) per well was added to solubilise the formazan product (15 mins; 37 °C). Optical density was measured at 570 nm and a reference wavelength of 690 nm using a Multiskan Spectrum plate reader (Thermo Fisher Scientific, Waltham, MA, USA). The results are expressed as a percentage of cell viability relative to the control.

The most notable protective effects were seen with 25, 75 and 150 μg/mL REE. Thus, these concentrations were selected for further experiments. Additionally, the cell toxicity of REE was investigated at 1000 μg/mL REE. The time point of 4 h for this study was deemed suitable based on previous studies (Marnewick et al., 2011; Akinfenwa et al., 2021).

2.7. Metabolic output - ATP Assay

The Cell Titer-Glo® assay (Promega, Madison, WI, USA), an ATP bioluminescent assay was used to quantify cellular ATP. Cells were seeded in a white microtitre plate (20,000 cells per well, 50 μL PBS) to which the ATP Cell Titer-Glo® Reagent was added (50 μL) in triplicate. The plate was incubated for 30 min at room temperature (RT) in the dark. Luminescence was measured on a Multiskan Spectrum plate reader (Thermo Fisher Scientific, Waltham, MA, USA). Luminescence is proportional to ATP levels and expressed as relative light units (RLU).

2.8. Oxidative stress biomarkers

Lipid peroxidation was assessed by measurement of malondialdehyde (MDA) using the thiobarbituric acid reactive substances assay.

Supernatant of NG and HG supplemented cells were assessed for TBARS as previously described (Abdul et al., 2016). Absorbance of the samples was read at $\lambda = 532/600$ nm using a Multiskan Spectrum plate reader (Thermo Fisher Scientific, Waltham, MA, USA) using a While the method of (Arumugam et al., 2019) was used to assess extent of protein carbonylation. Absorbance was measured at $\lambda = 370$ nm with a Multiskan Spectrum plate reader (Thermo Fisher Scientific, Waltham, MA, USA)). All experiments were conducted in triplicate.

2.9. Redox status and ROS measurements

The GSH-Glo™ assay (V6912, Promega, Madison, USA) was used to quantify reduced glutathione and carried out as per manufacturer's instructions. A standard curve was constructed using GSH standards and GSH concentrations (μM) in control and REE treated cells were extrapolated. Luminescence was captured using a Multiskan Spectrum plate reader (Thermo Fisher Scientific, Waltham, MA, USA). Experiments were conducted in triplicate.

The ROS-Glo™ H_2O_2 Assay (Promega, Madison, WI, USA) was used to assess the level of hydrogen peroxide, a reactive oxygen species (ROS), directly in cell culture. The assay was carried out according to manufacturer's instructions. Luminescence was captured using a Multiskan Spectrum plate reader (Thermo Fisher Scientific, Waltham, MA, USA). Luminescence was captured using a Multiskan Spectrum plate reader (Thermo Fisher Scientific, Waltham, MA, USA). Experiments were conducted in triplicate.

2.10. Caspase activity (−8, −9, −3/7)

Caspase (−3/7, −8, −9) activity was detected using the Caspase-Glo® assays (Promega, Madison, USA) as per manufacturer's protocol. Luminescence was captured using a Multiskan Spectrum plate reader (Thermo Fisher Scientific, Waltham, MA, USA). Luminescence was captured using a Multiskan Spectrum plate reader (Thermo Fisher Scientific, Waltham, MA, USA). Experiments were conducted in triplicate.

2.11. Protein expression

Protein expression profiles were determined using western blots. We selected the 25 μg/mL concentration of REE to determine the protein expression profile as this concentration was seen as optimum to protect cells against glucotoxic stress.

Cell protein was extracted using the M-PER™ Mammalian Protein Extraction Reagent (78,501, ThermoFisher) and standardised to 1 mg/mL. The samples were then heated (70 °C, 5 min) in NuPAGE™ LDS Sample Buffer (NP0007, ThermoFisher). Prepared protein samples were electrophoresed on sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gels (4% stacking gel, 7.5% resolving gel) at 150 V (Bio-Rad Mini PROTEAN Tetra-Cell System). The separated proteins were then transferred onto nitrocellulose membranes using the Trans-Blot Turbo Transfer system (standard mixed protein program, Bio-Rad). The membranes were blocked with Membrane Blocking Solution for 1 hour (000,105, ThermoFisher). Membranes were incubated with primary antibody (1:1000 dilutions, Table 1) overnight (4 °C). The membranes were then incubated (RT) with HRP-conjugated secondary antibody for 2 h (1:5000 dilution). Protein bands were detected using the Pierce™ ECL Western Blotting Substrate (32,209, ThermoFisher) and images were captured with the ChemiDoc™ XRS + Molecular Imaging System (Bio-Rad). Protein expression was normalised against house-keeping protein, β-actin (CST4970S, Cell Signalling). Protein expression was analysed using Image Lab Software version 5.0 (Bio-Rad) and the results were expressed as relative band density (RBD).

Table 1
Antibodies used for Western Blot Experiments.

Antibody	#Catalogue ID
NRF2	Cell Signalling Technology, CST12721S
NRF2 (phospho S40)	Abcam, ab76026
SOD2	Cell Signalling Technology, CST2770S
Catalase	Cell Signalling Technology, CST12980S
GPX1	Cell Signalling Technology, CST3286S
Anti-mouse IgG, HRP-linked Antibody	Cell Signalling Technology, CST7076S
Anti-rabbit IgG, HRP-linked Antibody	Cell Signalling Technology, CST7074S

2.12. Statistical analysis

Statistical analysis was performed using GraphPad Prism v5.0 software (GraphPad Software Inc., La Jolla, USA). A one-way ANOVA followed by Bonferroni test for multiple group comparison was used where more than two concentrations were being compared. An unpaired *t*-test with Welch's correction was used to assess the differences between means for control *vs.* a selected concentration (25 μg/mL) of REE. Results are expressed as mean with standard deviation (SD). Level of significance was set at $p < 0.05$.

3. Results

3.1. Total phenolics and antioxidant capacity of the REE

Antioxidant compounds generally comprise phenolic group(s). In the present study the total phenolic compounds present in serially diluted REE were measured and expressed as gallic acid equivalents. A dose dependent increase was shown in total polyphenols (Fig. 1A). Since polyphenols contribute significantly to the antioxidant activity, it is hypothesised that the total polyphenol content in the extract would relate to a high antioxidant activity.

To evaluate the antioxidant capacity of REE, two methods were applied in this study. The TEAC and FRAP assays were applied to measure the antioxidant capacity. The use of these two assays with different reaction mechanisms, including Fe^{3+} reducing power (Fig. 1B) and scavenging free radicals (Fig. 1C) validated the AO potential of the extract. Our results indicate a dose dependent increase in antioxidant capacity of the REE.

3.2. HPLC analysis of the REE

The major polyphenolic constituents of the REE were analysed using HPLC (Table 2). Aspalathin, a unique dihydrochalcone of Rooibos was the most abundant polyphenol detected in REE (Table 2). Major and minor peaks corresponding to phytochemical constituents of REE are also shown on the chromatogram (Fig. 2).

3.3. Effect of the REE on metabolic activity under induced glucotoxic conditions

The HepG2 cell viability was determined as a measure of formazan crystal formation in metabolically active cells. The results of the MTT assay post 4 h incubation with increasing concentrations (μg/mL) of REE in HepG2 cells cultured under NG and HG conditions are shown in Fig. 3A. Treatment with the REE in normal glucose- (NG) and high glucose (HG)- supplemented media was shown to increase cell viability at the lower tested concentrations of the green Rooibos extract while toxicity (evidenced by decreased cell viability) was observed at higher concentrations. Based on the cell viability data and available scientific literature, we selected 25, 75, 150 and 1000 μg/mL for 4 h (Akinfenwa et al., 2021) as a wholistic dose range for further experimental evaluation.

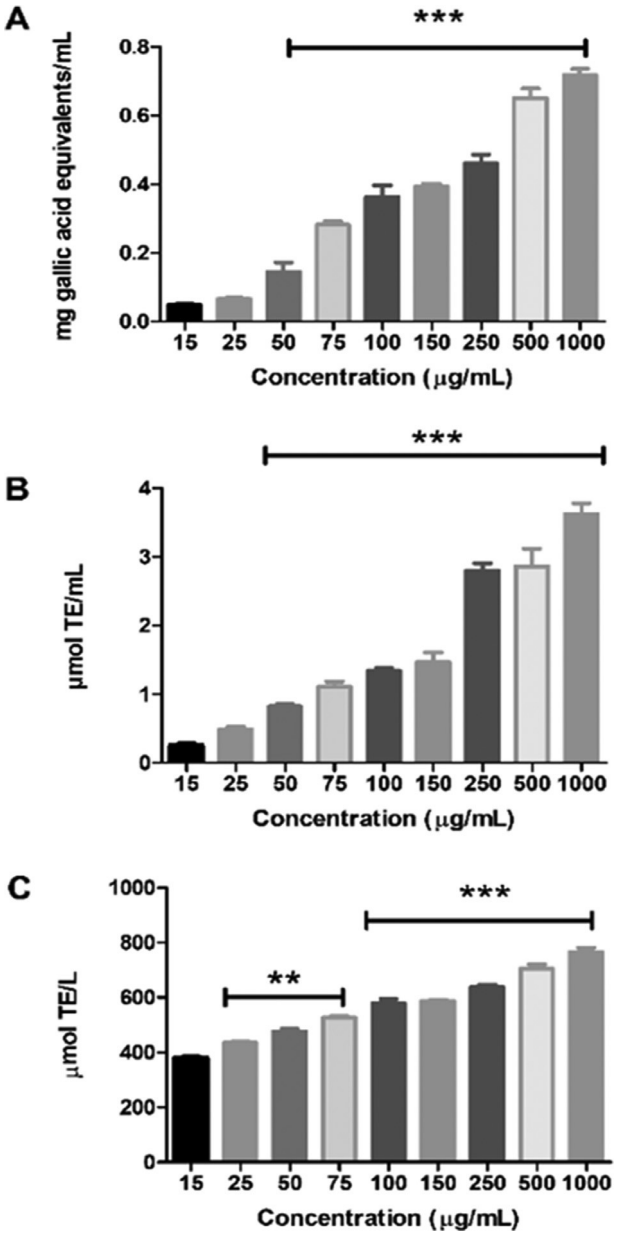

Fig. 1. Evaluation of the Total polyphenol content (A), Fe_3^+ reducing power (B) and free radical scavenging potential (C) of REE. The results are expressed as mean and SD of a triplicate analysis. Significance indicated with $^{**}p \leq 0.005$ and $^{***}p \leq 0.001$.

To further confirm that the selected concentrations (25, 75, 150 and 1000 μg/mL) maintained metabolic activity, we quantified ATP levels in cells treated with the REE. Exposure to REE in NG- and HG-supplemented media significantly ($P<0.05$) elevated ATP output at the lower tested concentrations while a significant ($P<0.05$) decrease in ATP was observed at the highest (1000 μg/mL) tested concentration (Fig. 3B).

Table 2
Quantification of the major polyphenolic constituents present in the ethanolic green Rooibos extract used in this study.

Phytochemical	Quantity (mg/g extract)
Aspalathin	40.79
Orientin	5.07
Isoorientin	3.06
Vitexin	0.71
Hyperoside	6.28
Quercetin	0.04
Luteolin	0.27
Chrysoeriol	0.08

3.4. Effect of the REE on oxidative damage biomarkers in NG and HG supplemented HepG2 cells

Increased ROS production, as an outcome of glucotoxicity, results in the increased oxidative peroxidation of lipids and proteins (Davì et al., 2005; Hecker et al., 2018). The possible protective effect of the REE on HepG2 cells exposed to NG and HG was assessed. Treatment of HepG2 cells with the REE induced a significant ($P<0.05$) decrease in the MDA concentration of both NG and HG supplemented cells, indicating prevention of oxidative damage to cellular lipids (Fig. 4A). A marked reduction ($P<0.05$) in the protein carbonyl levels for both NG and HG supplemented cells after exposure to low concentrations of the REE was observed. However, at the higher REE concentrations, protein damage was not ameliorated (Fig. 4B), but rather enhanced in both the NG (150 ug/ml REE) and HG (150 & 1000 ug/ml REE) – treated cells.

These data suggest a significant and dose dependent inhibition of macromolecule damage in HepG2 cells treated with the REE.

3.5. Effects of the REE on intracellular ROS and reduced glutathione (GSH) in NG and HG cultured HepG2 cells

The ROS-Glo™ H_2O_2 Assay is a rapid and sensitive luminescent assay that measures the level of H_2O_2 in cells with a change in H_2O_2 reflecting a general change in the ROS level. A significant decrease in ROS production was seen with all tested REE concentrations in the presence of NG and HG (Fig. 5A).

Luminometric analysis of GSH, a prominent endogenous antioxidant molecule present in cells, showed a marked ($P<0.05$) elevation in reduced glutathione in both NG and HG supplemented cells treated with the REE (Fig. 5B). These results suggest that the ROS dampening effects of REE and its modulation of the important intracellular antioxidant molecule, GSH are independent of glucotoxicity.

3.6. The effect of the REE on caspase activation

To evaluate whether the antioxidant effect of the REE in NG and HG conditions was associated with apoptosis, caspase activation was assessed. Caspase-8 and −9 are important in the initiation of apoptosis, therefore, the present study examined the effect of the REE on the glucotoxic-induced activation of caspase-8 (Fig. 6A) and −9 (Fig. 6B) and caspase-3/7 (Fig. 6C). The REE significantly ($p \leq 0.05$) reduced the activity of caspase-8 (Fig. 6A) and −9 (Fig. 6B) at the lower tested concentrations but activation was elevated at the highest tested concentration of the REE (1000 μg/mL). This was observed in both NG and HG supplemented media.

The activity of executioner caspase-3/7 was shown to follow a similar trend to the initiator caspases. The REE significantly ($p \leq 0.05$) reduced the activity of caspase-3/7 (Fig. 6C) at the low tested concentrations but activation was elevated at the highest tested concentration (1000 μg/mL).

3.7. Effect of REE on antioxidant enzymes – protein expression profiles

To determine the antioxidant enzyme profile in the REE treated cells, protein expression of ROS detoxifying enzymes was assessed. Fig. 7A shows a marked ($p \leq 0.05$) elevation in the expression of mitochondrial SOD2 only in the 25 μg/mL HG group with no significant effect on the NG supplemented cells. Similarly, expression of CAT, a cytosolic antioxidant enzyme, was also significantly ($p \leq 0.05$) elevated only in the 25 μg/mL HG group (Fig. 7B). The expression of GPx1, an essential enzyme for maintaining glutathione redox

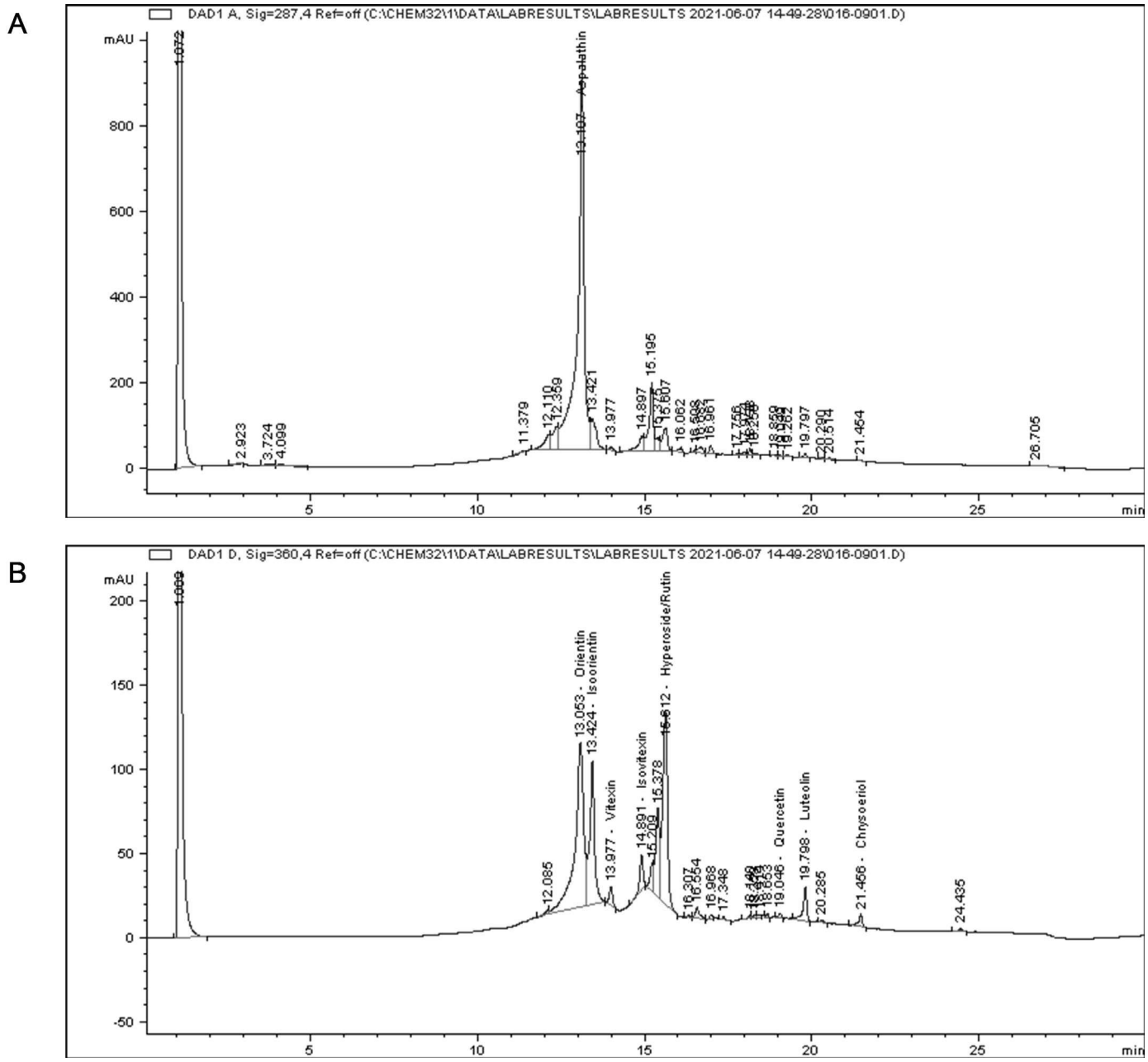

Fig. 2. HPLC chromatogram of REE extract used in the study showing the peak for aspalathin at 287 nm (A) and peaks for other flavonoids (B).

potential, was significantly elevated in the REE treated HG cells (Fig. 7C) and complements our findings on the GSH status of the cells. These results suggest a cellular antioxidant and redox metabolism response to the induced-glucotoxicity in the presence of the REE.

3.8. The effect of REE on NRF2 expression and activation

To gain a better understanding into the regulatory mechanisms used in the liver's antioxidant response to glucotoxic induced-oxidative stress, we determined changes in both total NRF2 and phospho-NRF2 (activated NRF2). The phosphorylation of NRF2 at Ser-40 is a crucial signaling event that results in the ARE-mediated cellular antioxidant response (Huang et al., 2002). We found a significant ($p \leq 0.001$) increase in the phosphorylation status of NRF2 at Serine-40 (Ser-40) by the REE in both the NG and HG groups (Fig. 8).

4. Discussion

Rooibos is a popularly consumed beverage with increasing clinical relevance. Several studies have underscored its bioactive compounds as potential interventions for a plethora of diseases, most of them focussed around non-communicable diseases (Joubert et al., 2011; Magcwebeba et al., 2016; Huang et al., 2019), including diabetes (P.V. Dludla et al., 2017; Ajuwon et al., 2018; Sasaki et al., 2018; P.V. Dludla et al., 2020; P.V. Dludla et al., 2020). Oxidative stress is increased in diabetic patients and is involved in the pathogenesis of the disease and its complications (Niedowicz et al., 2005; Pan et al., 2010; Calderon et al., 2017). Ethnopharmacology may present a novel and underutilized strategy to prevent or delay cell damage caused by oxidative processes under glucotoxic conditions (Sabu et al., 2002). In the present study, we evaluated the antioxidant potential of a green Rooibos ethanolic extract in a glucotoxic liver model. We found that

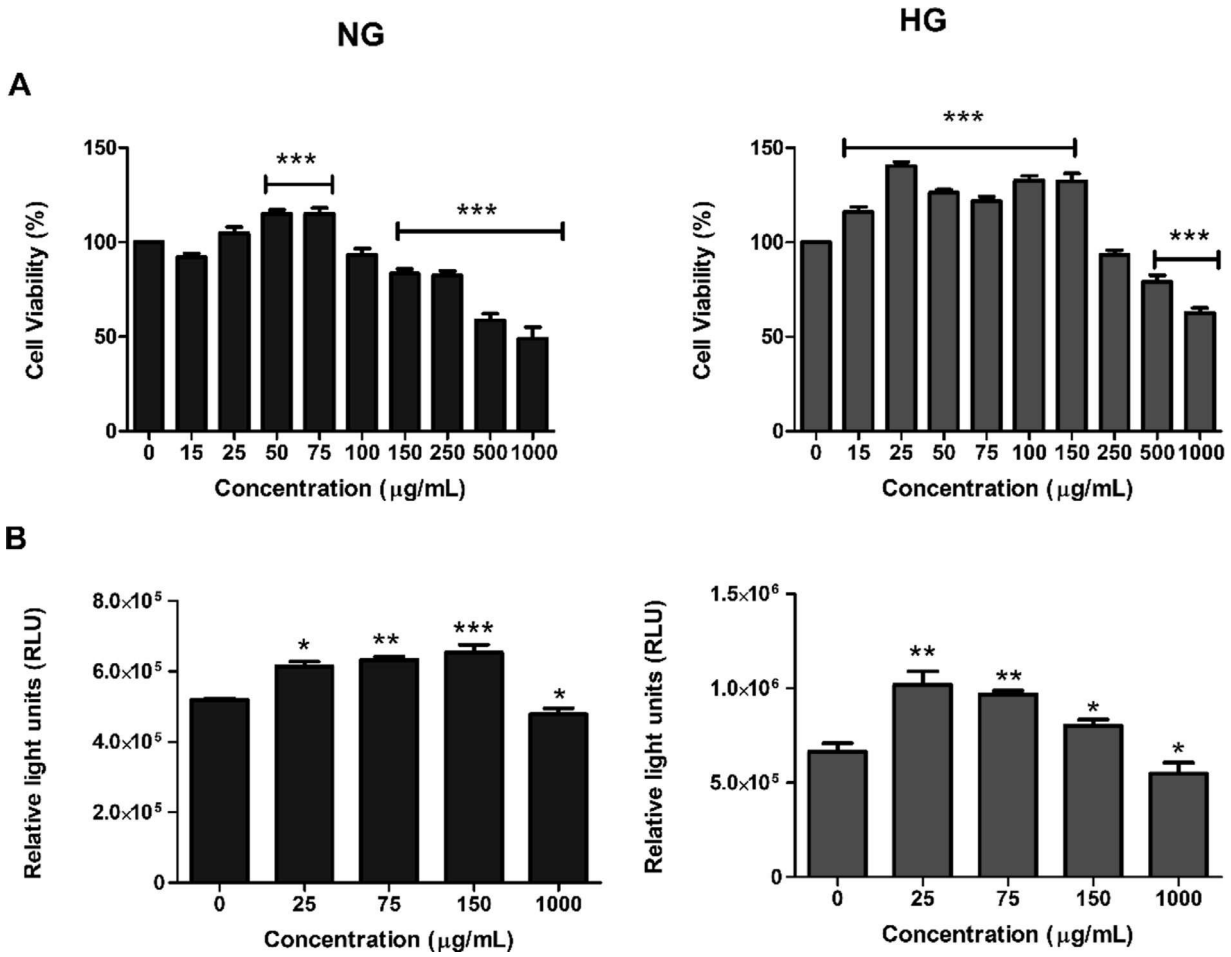

Fig. 3. Effect of the REE on the metabolic output of HepG2 cells cultured under normal glucose (NG) and high glucose (HG) conditions. (A) HepG2 cells exhibited a higher rate of metabolic conversion of MTT salt to the stable formazan product at lower concentrations. (B) Analysis of ATP levels in HepG2 cells for the selected concentrations showed improved metabolic output in NG and HG supplemented cells. Control cells received 0 μg/mL REE. The results are expressed as mean and SD. $^{*}p \leq 0.05$, $^{**}p \leq 0.005$, $^{***}p \leq 0.001$ Control *vs* REE.

the REE treatment ameliorated the induced oxidative damage in liver derived HepG2 cells by improving the antioxidant status. Further molecular analysis revealed that this enhanced antioxidant status was dependent on the transcriptional activity of NRF2. The data support the prospect that REE can protect liver cells from oxidative damage in diabetic patients.

The Rooibos extract in the present study contained high levels of total polyphenols with a high antioxidant capacity. The phytochemical composition of the extract as revealed by HPLC analysis is presented in Table 2. The phytochemicals identified in this study are typical of those found in *Aspalathus linearis* (Joubert, 1996; Bramati et al., 2003; Schulz et al., 2003). The differences in observed quantities of phytochemicals in REE can be ascribed to the type of solvent used, method of analysis and the plant material used (as the levels of polyphenolics have been shown to be affected by seasonal changes). The use of different solvents is reported to cause clear effects and greater differences in chemical composition of extracts (Lapornik et al., 2005; Pinelo et al., 2005). Antioxidant compounds are the most important constituents of medicinal plants, as they possess redox properties that can neutralize free radicals, quench singlet and triplet oxygen or decompose peroxide in cells (Halliwell, 2001). Among the phytochemicals identified in the REE extract (Table 2), the most prevalent compound was aspalathin, a chalcone unique to Rooibos, which has been appreciated for its strong antioxidant activity and as an intervention for diabetes (Kawano et al., 2009; Sasaki et al., 2018; Moens et al., 2020). Other biological activities associated with the identified compounds include, anticancer, anti-inflammatory and hypocholesterolemic effects (Smith et al., 2016; Orlando et al., 2019; Samodien et al., 2021).

The liver functions as the hub of glucose metabolism (Han et al., 2016). Glucotoxicity induces hepatic oxidative stress that is often associated with metabolic dysfunction (Ruiz et al., 2019; Dewidar et al., 2020). The tetrazolium salt method (MTT) is a reliable indiactor of cell viability as a measure of metabolic activity (Kumar et al., 2018). Our results showed that REE was able to preserve cell viability and stimulate metabolic activity in HG-supplemented HepG2 cells (Fig. 3). Increased MTT reduction implies improved mitochondrial oxidative activities correlating to increased glucose consumption (Takahashi et al., 2002). Thus, the results indicate that the REE potentiated a stimulatory shift to glucose utilization under glucotoxic conditions. The mechanisms of action by which green Rooibos reduces glucose levels in liver cells may be attributed to increased phosphorylation of 5′-adenosine monophosphate-activated protein kinase (AMPK) and serine/threonine kinase (Akt), which promotes translocation of glucose transporter 4 (Glut4) to the plasma membrane (Muller et al., 2012; Kamakura et al., 2015). Studies have further confirmed that the hypoglycemic activity of green Rooibos is strongly linked to the unique phytochemical found only in Rooibos, aspalathin, (Kawano et al., 2009; Mazibuko-Mbeje et al., 2019; Orlando et al., 2019; P.V. Dludla et al., 2020). Furthermore, the beneficial effect of a fermented Rooibos extract (FRE) on mitochondrial bioenergetics was recently observed. Of significant interest was the ability of a FRE

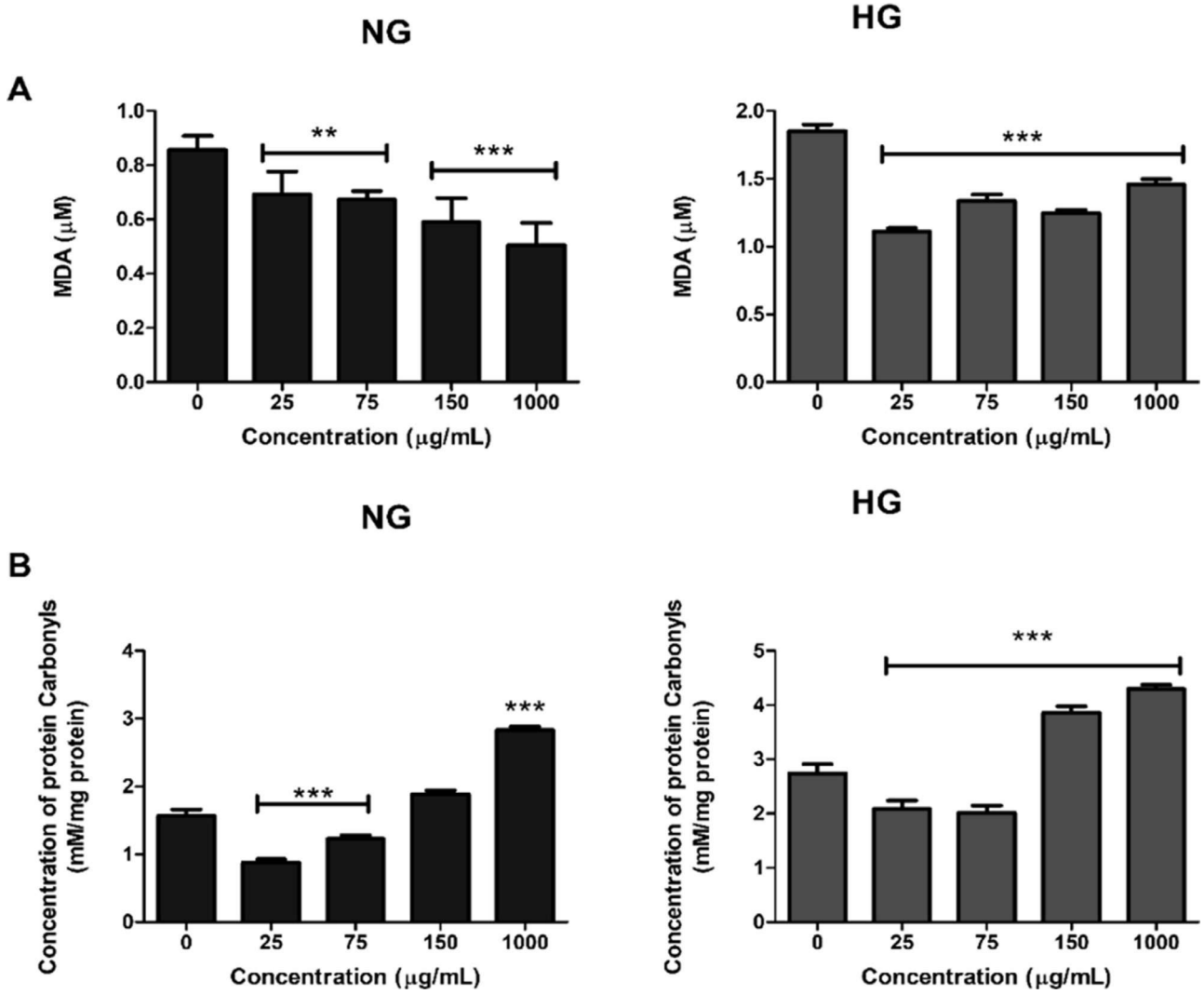

Fig. 4. Inhibition of lipid and protein damage by REE in NG and HG HepG2 cells. (A) The selected doses of REE decreased the presence of MDA and (B) protein carbonyls, which are established biomarkers of lipid and protein oxidation, respectively. Control cells received 0 μg/mL REE. The results are expressed as mean and SD. $^{**}p \leq 0.005$, $^{***}p \leq 0.001$ Control *vs* REE.

to enhance intracellular coenzyme Q_9 levels, a major component of the electron transport chain that plays a crucial role in maintaining efficient aerobic respiration (P.V. Dludla et al., 2020). The ability of the REE to stimulate metabolic output may follow a similar mechanism of action in HepG2 cells and highlights its potential to mop up excess glucose and preserve mitochondrial function.

There is strong evidence linking glucotoxicity to the excessive and harmful generation of ROS (Kawahito et al., 2009) and depletion of the antioxidant defense mechanisms of cells and tissues (Niedowicz et al., 2005). Following this, studies have shown that green Rooibos extracts and its unique component, aspalathin, can protect against ROS damage in diabetic models (Muller et al., 2012; Kamakura et al., 2015; Orlando et al., 2019; P.V. Dludla et al., 2020). In the current study, the REE reduced the negative effect of glucotoxicity on the generation of ROS (Fig. 5A). These results are consistent with our observation of elevated GSH levels in the high glucose-supplemented liver cells after treatment with the REE (Fig. 5B). The exhaustion of cellular GSH levels have previously been confirmed in diabetic rat models and patients (Aaseth et al., 2000; Tachi et al., 2001). Reduced glutathione is a powerful non-enzymatic component of the endogenous antioxidant defense system and its compromised level may leave cells vulnerable to ROS induced damage (Forman et al., 2009). Additionally, GSH is a co-substrate used by glutathione peroxidases to catalyze the detoxification of H_2O_2 to water and oxidised GSH (GSSG) (Forman et al., 2009). Therefore, the induction of GPx1 by the REE reported in this work (Fig. 7C), along with higher levels of its co-factor, GSH, strongly contributes to the protective, antioxidant capability targeted against glucotoxicity. There is a growing body of evidence that supports the improvement of the redox status of glutathione by Rooibos (Marnewick et al., 2003; Marnewick et al., 2011; Pantsi et al., 2011; Ajuwon et al., 2013) and it has recently been shown that Rooibos polyphenols upregulate the expression of γ-glutamylcysteine synthetase (γ-GCS), which is the rate limiting enzyme in the synthesis of GSH (A.O. Lawal et al., 2019). Thus, the REE may reduce oxidative stress under glucotoxic conditions by enhancing the direct scavenging of ROS through increased synthesis or redox cycling of GSH.

Glucotoxicity is a significant driver of liver cell damage due to oxidative stress (Kawahito et al., 2009; Parveen et al., 2010). The excessive production of ROS leads to lipid peroxidation and deterioration of the cell membrane (Sauriasari et al., 2015). Proteins are also susceptible to inactivation or degradation due to oxidative modifications by ROS (Çakatay, 2005). The measurement of MDA and protein carbonylation are considered reliable markers in diabetic patients for

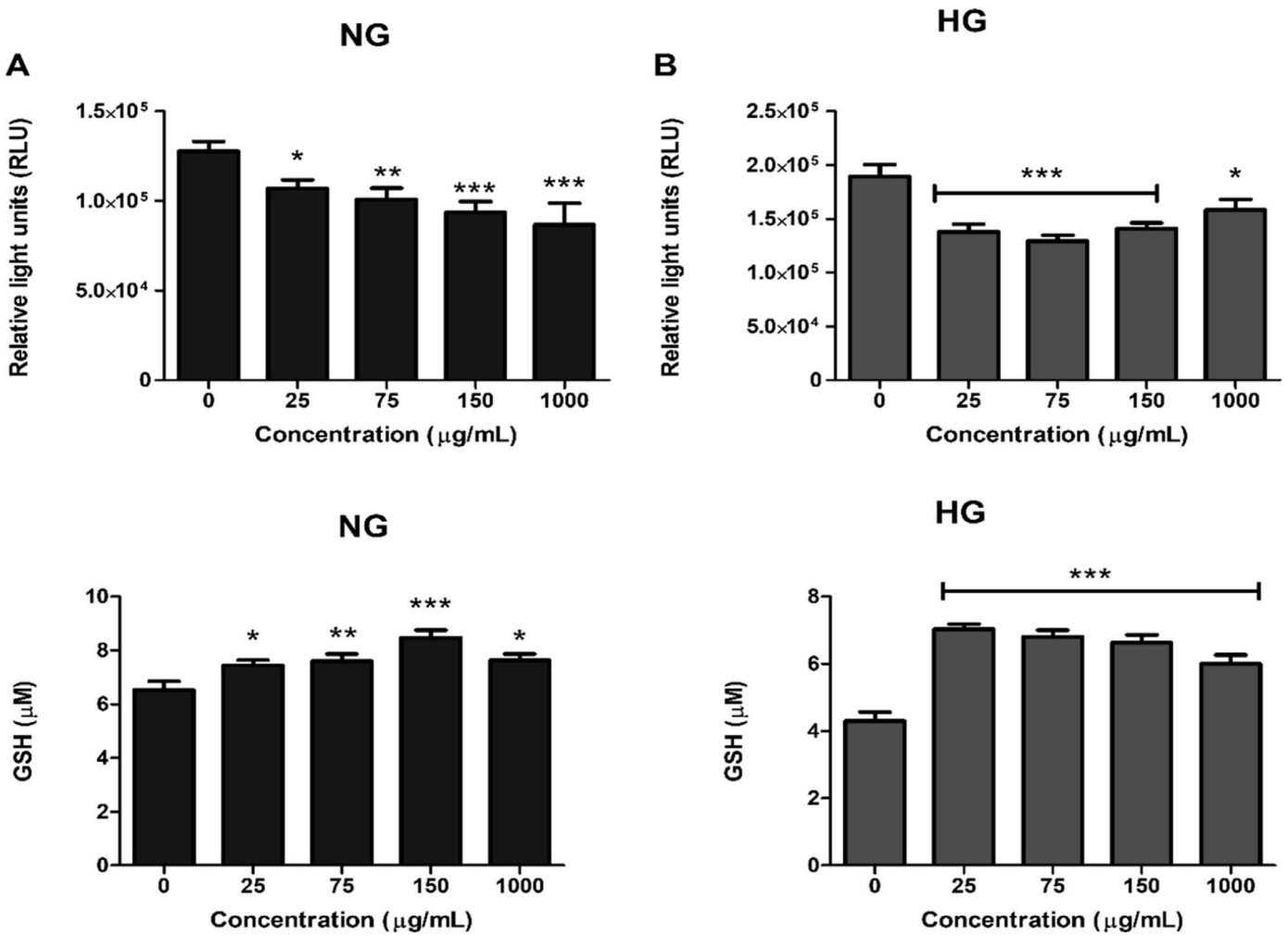

Fig. 5. Effect of REE on ROS production and reduced glutathione (GSH) levels. (A) HepG2 cells treated with different doses of REE under NG and HG conditions showed decreased levels of H_2O_2 suggesting diminished intracellular ROS levels. (B) GSH was significantly elevated by REE in NG and HG supplemented cells. Control cells received 0 μg/mL REE. The results are expressed as mean and SD. $^{*}p \leq 0.05$, $^{**}p \leq 0.005$, $^{***}p \leq 0.001$ Control *vs* REE.

determining the extent of lipid peroxidation and protein damage resulting from excessive ROS production (Çakatay, 2005; Sauriasari et al., 2015). In the current study, treatment with the REE reduced levels of liver MDA and protein carbonylation in HG-supplemented cells, indicating the ROS detoxifying and protective properties of the REE (Fig. 4). Previous reports have indicated that Rooibos reduced MDA formation in rat liver (Ajuwon et al., 2013) and liver microsomal preparations (Bosek et al., 2003). While reports in humans also revealed that Rooibos consumption significantly decreased plasma MDA levels (Marnewick et al., 2011).

The ability of REE to protect against lipid peroxidation and protein carbonylation may be the result of intricate interactions of its different phenolic constituents responsible for its antioxidant activity. This protective effect may be dependent on the ability of Rooibos phenolic constituents to bind lipid peroxides, and inhibit the lipid peroxidation cascade, either by acting as a sacrificial antioxidant or as a chelator of transition metals that promote lipid peroxidation (Nijveldt et al., 2001; Awoniyi et al., 2012). In the current study, the glucotoxicity induced protein damage was assessed by determining the levels of protein carbonylation. Carbonylated proteins are biologically significant, as this process often results in loss of function of the affected proteins (Dalle–Donne et al., 2006; Hecker et al., 2018). Hepatic carbonylation levels were significantly decreased by supplementation with REE. A previous report has indicated that Rooibos reduced protein carbonylation in human umbilical vein endothelial cells exposed to diesel exhaust particles (A.O. Lawal et al., 2019). It is interesting that the protective effects of the REE on liver cells were observed at the low concentration range. The elevation in MDA and protein carbonylation levels in the 1000 μg/mL REE treated group in this study may be due to either overproduction of alkoxyl and peroxyl radicals or their accumulation resulting from a pro-oxidant effect induced by the REE, a phenomenon previously described (Joubert et al., 2005).

High glucose-induced oxidative stress may be further exacerbated by the inactivation or deficiency of supporting antioxidant systems (Sindhu et al., 2004). Thus, investigating glucotoxicity–induced changes in antioxidant enzyme expression was an appropriate way to elucidate REE–related antioxidant recovery *in vitro*. Hepatocytes are particularly susceptible to oxidative damage because they posess a high population of mitochondria (Patche et al., 2017). The mitochondrial specific SOD2 plays a crucial role in dampening ROS toxicity (Kokoszka et al., 2001). Superoxide dismutases (SODs) are a family of enzymes responsible for the detoxification of superoxide into oxygen and H_2O_2, the H_2O_2 is then converted to oxygen and water by catalase and GPx (Maritim et al., 2003). In the current study the REE elicits significant increases in SOD2 and CAT protein expression in cells undergoing glucotoxic stress (Fig. 7A and 7B). From these data it should be appreciated that REE is capable of enhancing mitochondrial

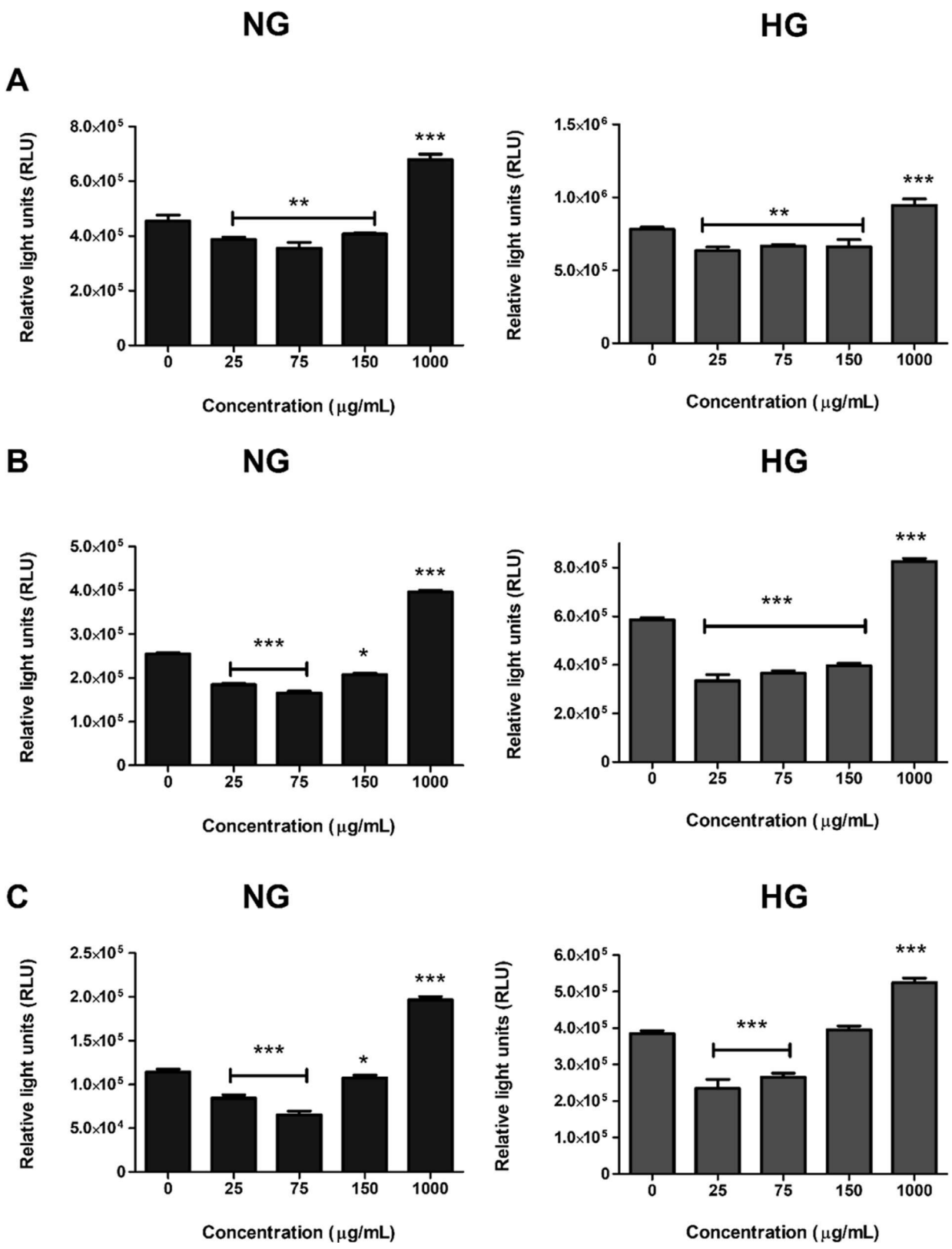

Fig. 6. REE inhibited caspase activation. Both the extrinsic (caspase 8, A) and intrinsic (caspase 9, B) initiator caspases were significantly inhibited at low REE concentrations. The executioner caspases (caspases 3/7, C) also showed decreased activation at low REE concentrations. Control cells received 0 μg/mL REE. The results are expressed as mean and SD. $^{*}p \leq 0.05$, $^{**}p \leq 0.005$, $^{***}p \leq 0.001$ Control *vs* REE.

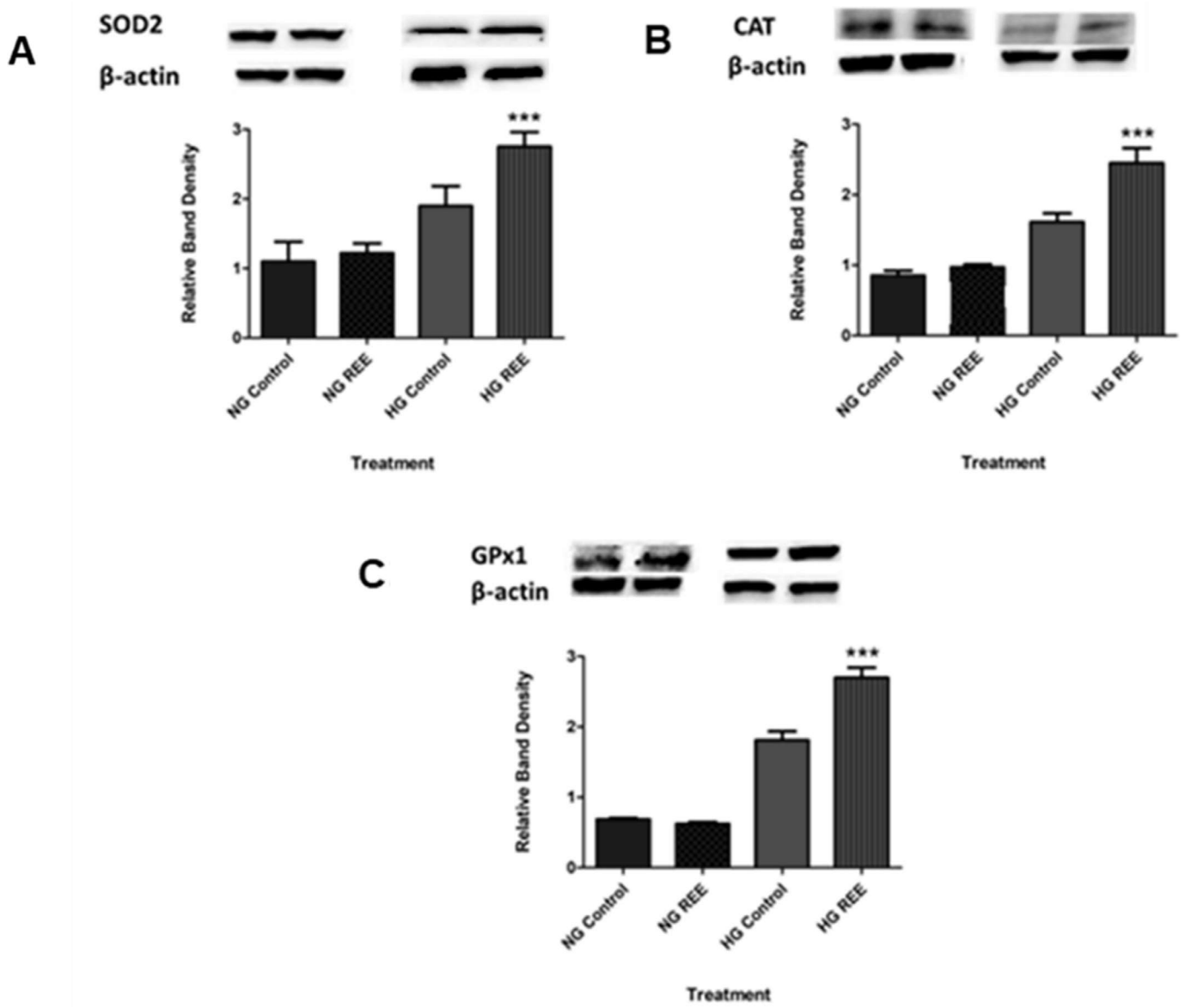

Fig. 7. REE enhanced antioxidant and GSH redox modulating enzyme content in HG conditions. REE at 25 μg/mL significantly enhanced protein expression of (A) the mitochondrial dismutase (SOD2) and (B) catalase in HG supplemented HepG2 cells. (C) REE at 25 μg/mL treatment significantly enhanced expression of GPx1 as evidenced by higher protein levels post REE treatment. Control cells received 0 μg/mL REE. The results are expressed as mean and SD. $^{**}p \leq 0.005$, $^{***}p \leq 0.001$ Control *vs* REE.

and cytosolic antioxidant enzyme capacity. Previously, Dludla and co-workers (P.V. Dludla et al., 2017) reported that aspalathin modulated the changes observed in the expression of antioxidant enzymes (SOD2, CAT, GPx) in a cardiomyocyte glucotoxic model. Another study reported that changes in the transcriptional profile of antioxidant enzymes are observed in the heart cells exposed to lipotoxic conditions (Johnson et al., 2017).

The current study further investigated the possible mechanisms underlying the increased antioxidant capacity by examining transcriptional regulation as major contributors to the antioxidant effects of the REE in the context of hyperglycemia. The NRF2 transcription factor is considered the master regulator of the antioxidant defense system by modulating the collective expression of antioxidant genes by binding to the ARE (Harvey et al., 2009; Tan et al., 2014; Lee, 2017). Previous studies have demonstrated that upregulation of NRF2 enhances SOD2, GPx1 and CAT expression in cells under oxidative stress induced by hyperglycemia (Wang et al., 2015; Dinić et al., 2016; Ding et al., 2019; Jerotic et al., 2019). There is also evidence suggesting that hyperglycemia-induced oxidative stress leads to the stabilization of NRF2 by releasing it from its repressor, Keap1, allowing NRF2 to translocate to the nucleus (Tan et al., 2014). Several studies using diabetic models have focused on the potential of phytochemicals to activate NRF2 and increase the antioxidant response against hyperglycemic damage (Jiménez-Osorio et al., 2015). In line with this, our study shows that the REE was able to induce phoshphorylation and activation of NRF2 in HG- and NG-supplemented cells (Fig. 8A). Phosphorylation of NRF2 at serine 40 promotes its dissociation from Kelch-Like-ECH Associated Protein 1 (KEAP1) (Huang et al., 2002). These results are supported by previous studies that report on the protective effect of Rooibos and aspalathin by attenuating oxidative stress through the NRF2 signaling pathway (P.V. Dludla et al., 2017; P.V. Dludla et al., 2017; A.O. Lawal et al., 2019). The flavonoids present in the green Rooibos may enhance the transcription of antioxidant enzyme genes, resulting in increased protein expression. The increased co-expression of these antioxidant enzymes suggest that the REE is successful at establishing ROS

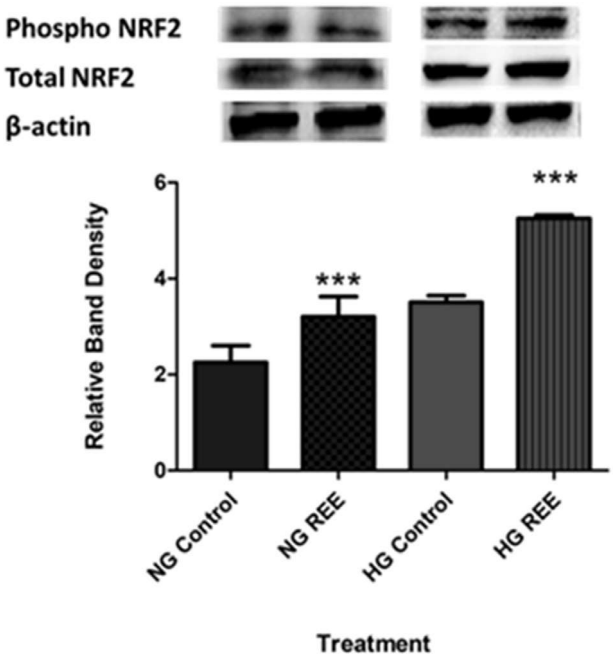

Fig. 8. The REE (25 ug/mL) promotes NRF2 activation (elevated phosphorylation status) under glucotoxic conditions suggesting a role for REE in maintaining antioxidant defences under glucotoxic stress. Control cells received 0 μg/mL REE. The results are expressed as mean and SD. $^{***}p \leq 0.001$ Control *vs* REE.

detoxifying pathways that complement each other in a diabetes simulated model. Fig. 9

Glucotoxicity causes death of various cell types in several organs (Maedler et al., 2001; Odetti et al., 2003) including the liver (Behrends et al., 2010; Francés et al., 2010). High glucose enhances the production of mitochondrial ROS and stimulates mitochondrial release of cytochrome-c, cleavage of procaspase-9, and caspase-9 enzyme activity, suggesting an intrinsic pathway of proapoptotic signaling (Mishra et al., 2005; Francés et al., 2010). In the current study, the REE significantly reduced the activation of caspase-9 that may be closely related to its protective effects on mitochondria. The foremost pathway of caspase-8 activation, known as the extrinsic apoptotic pathway, is based on death receptor signaling, which in turn promotes dimerization and subsequent activation of this caspase (Tummers et al., 2017). However, a growing body of evidence has now suggested that caspase-8 activation can be triggered independent of the death receptor pathway (Inman et al., 2000; von Haefen et al., 2003). A study by Dumont *et al*., revealed the ability of H_2O_2, to act as an intrinsic trigger, that can induce apoptosis which is Fas-independent (Dumont et al., 1999). Moreover, caspase-8 activation has also been observed in various cell types during apoptosis in response to oxidative stress (Schrantz et al., 2001; Marques et al., 2003). This is consistent with and may explain the decreased activity of caspase-8 and elevated oxidative stress by REE shown in current study. The present study further demonstrated significant decreases in caspase-3/7 activity in HG-supplemented cells treated with the REE. Caspase-3/7 are activated by initiator caspases and induces cell death in response to stimuli. The decrease in caspase-3/7 activity indicates reduction of apoptotic cell death in the HG-supplemented group which correlates with the loss of initiator caspase activity. Taken together, the data suggest that hyperglycemia-induced liver cell death *in vitro* is most likely ameliorated by the antioxidant effects of the REE. The organ protective, and in particular, hepatoprotective effects of Rooibos are well established *in vitro* and *in vivo* models (Marnewick et al., 2009; Ajuwon et al., 2013; Ajuwon et al., 2014).

5. Conclusion

Targeting hyperglycemia and oxidative stress may ameliorate liver damage and hepatic oxidative stress which are common in diabetes mellitus (Czaja, 2007; Kawahito et al., 2009; Francés et al., 2010; Mohamed et al., 2016). Considering the roles of the liver in glucose homeostasis, antioxidant phytochemicals may improve liver function and contribute to improved glycaemic control. The targeting of antioxidant pathways by phytochemicals to alleviate glucotoxic-associated oxidative stress has shown considerable success *in vitro* and *in vivo* models. Rooibos extracts are positioned to take full advantage of the critical NRF2 antioxidant pathways for improvement of

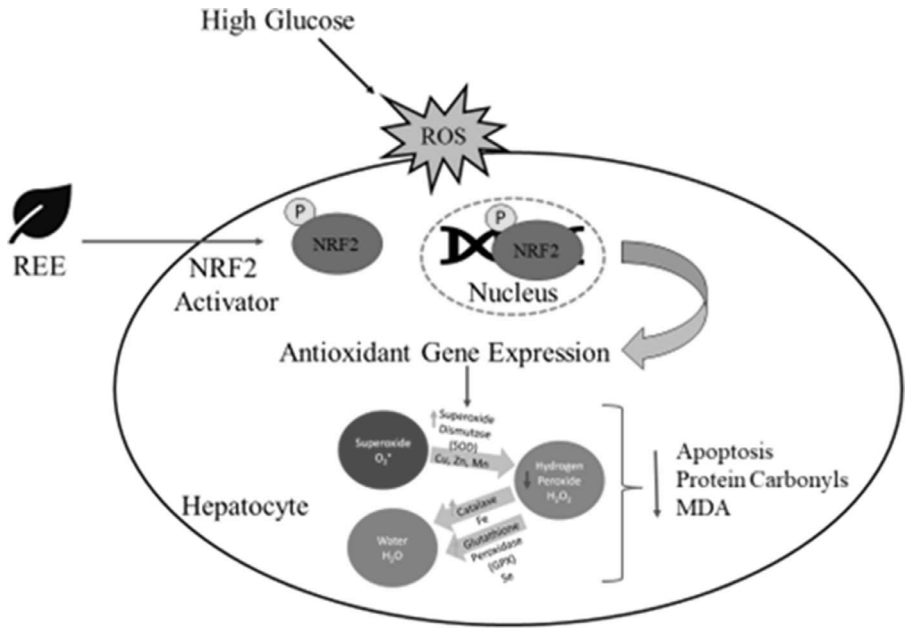

Fig. 9. Mechanism of action summary. By upregulating the expression of antioxidant enzymes through NRF2 activation REE can detoxify reactive oxygen species present in hepatocytes. This has a protective effect on macromolecules such as lipids and proteins in cells under glucotoxic conditions. Our data further shows that REE is hepatoprotective and inhibits apoptosis and maintains cell viability under glucotoxic conditions.

ROS-induced damage in various metabolic diseases including diabetes. It is clear from the current study that the ethanolic green Rooibos extract promoted significant hepatic cell recovery under glucotoxic conditions for all the tested oxidative stress parameters. This study adds to the growing body of evidence that Rooibos has tangible therapeutic benefits for diabetes treatment and management and should be further explored.

6. CRediT author statement

Conceptualization, N.S.A. and J.L.M; investigation, N.S.A.; writing—original draft preparation, N.S.A.; writing—review and editing, N.S.A. and J.L.M; supervision, J.L.M. All authors have read and agreed to the presented version of the manuscript.

Declaration of Competing Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Ethical statement

The experiments did not make use of human or animal subjects. Only commercially sourced HepG2 liver cells (ATCC) were used for experiments.

Data availability

All data pertaining to the study has been included.

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