



Rooibos Tea (*Aspalathus Linearis*) Extract Protects against Air Particulate Matter induced Hepatic Oxidative Stress in Nrf2-independent Manner

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ABSTRACT: Diesel exhaust particles (DEP) are a typical example of air particulate matter generated into the environment from industrial, residential and vehicular diesel engines. Studies have implicated the induction of oxidative stress in the adverse health effect of DEP. In this study, the effect of aqueous rooibos extract (RE) on DEP-induced hepatic oxidative stress was examined and the role of Nrf2-regulation in this effect was evaluated. Wistar rats were divided into 4 groups of 5 rats/group and were administered 0.7 mg/kg DEP subcutaneously and 50 mg/kg RE orally. Group one was given vehicle (saline and DMSO), group two was administered with RE for 4 weeks, group three was given RE for 2 weeks followed by concomitant administration of RE and DEP for further 2 weeks and group four was given DEP twice a week at the last 2 weeks of treatment. Malondialdehyde (MDA), Conjugated dienes (CDs) and GSH levels were determined in the liver homogenate. AST and ALT activities were assayed in the serum and RT-qPCR was used to quantify the gene expression of Nrf2 and HO-1 in the liver. Results showed that RE pre-treatment significantly ($p<0.001$) decreased DEP-induced elevated serum AST and ALT levels, decreased DEP-induced MDA ($p<0.001$) and CDs ($p<0.05$) levels. DEP caused significant ($p<0.05$) decrease in GSH levels, which was reversed by rooibos extract pre-treatment. DEP also caused significant induction in Nrf2 and HO-1 mRNA levels. However, these inductions were significantly attenuated by RE. In summary, rooibos extract has the potential to protect against DEP-induced oxidative stress in hepatic tissues probably by a mechanism which may not involve activation of the Nrf2-dependent response.

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Diesel exhaust particles (DEP) are a typical example of air particulate matter released into the atmosphere from incomplete combustion of diesel in diesel engines. Epidemiological and experimental studies have shown that DEP exposure causes damage to different tissues such as the heart, kidney, lung and liver (Yoshizaki *et al.*, 2010; Lawal *et al.*, 2016). These adverse effects have been attributed to its ability to induce oxidative stress (Dybdahl *et al.*, 2003; Yin *et al.*, 2013; Miller *et al.*, 2013). Both *in vitro* and *in vivo* studies have shown that DEP induced reactive oxygen production, lipid peroxidation, production of aldehydes, GSH: GSSG depletion and induction of HO-1 (Lawal *et al.*, 2016; Tobwala *et al.*, 2013; Tseng *et al.*, 2015). We have shown that DEP exposure induced ROS, HO-1 gene expression, lipid peroxidation in both cell line and animal studies (Lawal *et al.*, 2015; Miller *et al.*, 2013). Many studies have shown that antioxidants are capable of protecting the cells against free radical attack and oxidative stress (Pradedova *et al.*, 2011; Lawal *et al.*, 2016). Many phytochemicals derived from plant have been shown to exhibit antioxidant property and thus have been used in many studies to protect or attenuate against free radical attack (Marnewick *et al.*, 2011). Plant with such antioxidant property is Rooibos (*Aspalathus linearis*) tea, a plant native of cape floral kingdom in South Africa (Joubert *et al.*, 2008). This tea is

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commercially available in packs in many local shops and shopping malls in Nigeria and around the world. Rooibos tea has been reported to exhibit antioxidant, antimutagenic, cancer inhibiting properties in many studies (Marnewick *et al.*, 2011; Pansi *et al.*, 2011; Lawal *et al.*, 2019a). This present study examined the antioxidant potential of an aqueous extract from commercially available rooibos tea on DEP-induced oxidative stress in hepatic tissue of exposed rats and the role of Nrf2-dependent antioxidant in this protection.

MATERIALS AND METHODS

Chemicals: Aspartate aminotransaminase (AST) and alanine aminotransaminase assay kits were obtained from Agape Diagnostics (Switzerland GmbH). Primers against rat's cDNA were brought from Inqababiotec (Hatfield, South Africa). RNA isolation TRI Reagent[®] was purchased from Zymo Research (USA). ProtoScript II First Strand cDNA Synthesis Kit, Luna Universal qPCR Master Mix and quick-load purple 100 bp DNA ladder were bought from BioLabs (New England). All other reagents were brought from Sigma-Aldrich (Germany).

Collection of DEP and preparation of methanol extract: DEP was collected in Akure, Ondo State,

Nigeria from a MAN tipper truck (1996 model, engine number G0967). The collection of the particles from a running engine was done as previously described (Li *et al.*, 2004) and the collected particles were stored at -80°C away from sunlight. The preparation of the methanol extract of the particles was done as previously described (Lawal *et al.*, 2015). The dried extract was reconstituted in DMSO as 100 µg dried extract/ µl DMSO and stored away from sunlight at -80°C for further use. The redox (oxidative) potential and the polycyclic aromatic hydrocarbons (PAHs) content of the extract had been previously characterised (Lawal *et al.*, 2019b).

Aqueous rooibos extract (RE) preparation: Rooibos tea pack (containing 20 tea bags, batch number 6001156905236), a product of Entryce beverages, South Africa, was purchased from the shoprite shopping mall, Akure, Ondo-State, Nigeria. After pooling the contents of the 20 tea bags together, it was boiled in water at a concentration of 1 g/20 ml distilled water for 15 min. After cooling to room temperature, the solution was filtered using Whatmann paper and then solidified using rotary evaporator. The solidified samples were reconstituted in 0.9% saline solution to 10 mg/ml stock and kept at -20°C for further use.

Exposure protocol and tissue collection: Animals treatments were done according to approved protocols of the Animal Ethics Committee of School of Sciences, Federal University of Technology Akure (FUTA). Six (6)-week-old male wistar rats were assigned into 4 groups (n=6/group) that were exposed to the following: (1) Saline for 4 weeks and DMSO for the last 2 weeks (control), (2) 50 mg/kg RE for 4 weeks, (3) 50 mg/kg RE for 2 weeks followed by concomitant exposure to 50 mg/kg RE and 0.7 mg/kg DEP (twice a week) for 2 additional weeks, and (4) 0.7 mg/kg DEP for 2 weeks (twice a week). RE was administered orally, while DEP was given subcutaneously. The rats were sacrificed by cervical dislocation 24 hr after the last dose and blood were collected from the heart. Liver tissues were excised and stored at -80°C for further analysis.

AST and ALT assays: AST and ALT activities in the serum were assayed using Agape reagent kit (Agape Diagnostics, Switzerland GmbH) as previously described (Reitman and Frankel, 1957).

Oxidative stress measurement: The oxidative damage to membrane lipid was assessed by lipid peroxidation assay using thiobarbituric acid (TBA) method (Yagi, 1976). The conjugated dienes (CDs) levels were assayed as described by Buege and Aust (1978). Reduced glutathione (GSH) content was determined by modified method of Asensi *et al* (1999). Protein determination was done using Bradford method (1976).

Real time-quantitative polymerase reaction (RT-qPCR) for gene expression analysis: TRI Reagent® (Zymo Research, USA) was used to isolate total RNA from the liver tissues as previously described (Ramalho *et al.*, 2004). cDNA was synthesized using 1 µg of the isolated RNA within the purity range of 1.8-2.0. Synthesis was carried out in Multigene™ Mini thermal cycler (Labnet Int'l Inc., USA) using the ProtoScript II First Strand cDNA Synthesis Kit (BioLabs, New England) in a 3-step reaction condition: 65°C for 5 min, 42°C for 1 hr and 80°C for 5 min. RT-qPCR for gene expression analysis was carried out on a StepOnePlus Applied Biosystem qPCR system according to the manufacturer's protocols. Primers to the rat's cDNA were listed in Table 1 and purchased from Inqababiotec (Hartfield, South Africa). The Luna Universal qPCR Master Mix (BioLabs, New England) was used for the real time-quantitative PCR (RT-qPCR) reaction and PCR conditions were as follows: 1 cycle at 95°C for 3 min, 40 cycles at 95°C for 15 sec, 40 cycles at 60°C for 30 sec, and 40 cycles at 72°C for 30 sec. Comparative cycle threshold ($\Delta\Delta CT$) method was used to quantify the relative amount of mRNA in the sample and gene expression was normalised with β -actin gene as housekeeping gene. The PCR product was run in 0.8% agarose gel stained with ethidium bromide.

Table 1: Primer sequence for real time quantitative PCR

Gene	Sequence
β -actin	Forward: CTCCTGGAGAAGAGCTATGA Reverse: AGGAAGGAAGGCTGGAAGA
Nrf2	Forward: ACGTGATGAGGATGGGAAAC Reverse: TATCTGGCTCTTGCTCTTGG
HO-1	Forward: GATGGCCTCCTGTACCATATC Reverse: AGCTCCTCAGGAAGTAGAG

Nrf2, nuclear factor erythroid 2 [NF-E2]-related factor 2; *HO-1*, heme oxygenase-1

Statistical analysis: GraphPad Prism5 Software was used for statistical analysis. Analysis was done using one-way analysis of variance (ANOVA) and comparison between group pairs was carried out with the Bonferroni post-hoc test. Data were expressed as mean \pm SEM and differences were considered statistically significant at the p-value of <0.05.

RESULTS AND DISCUSSION

The present study investigates the protective effects of aqueous rooibos extract on diesel exhaust particles induced hepatic oxidative stress in exposed wistar rats. The data indicate that while DEP-induced significant hepatotoxic effect and oxidative stress, the presence of aqueous rooibos extract attenuated these effects.

To assess the protective effects of rooibos extract on diesel exhaust particles-induced hepatotoxicity, the serum levels of AST and ALT in rats exposed to 0.7 mg/kg DEP in the presence and absence of 50 mg/kg aqueous rooibos extract were determined (Fig. 1). The data showed that DEP caused a 1.52- and 2.31-fold significant ($p < 0.001$) increase in serum AST (Fig 1A)

and ALT (Fig. 1B) activities respectively, when compared to control. The data also showed that pre-treatment with aqueous rooibos extract significantly

($p < 0.001$) caused a 1.55- and 1.42-fold decrease in serum AST and ALT activities when compared to DEP exposure only (Fig. 1A & B),

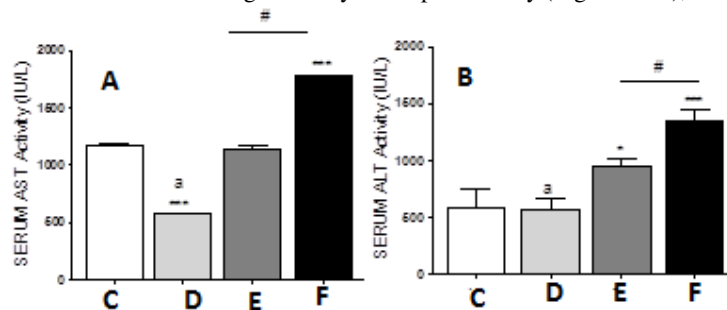


Fig 1. Effects of aqueous rooibos tea extract on DEP-induced serum AST and ALT activities: C = 0; D = RE (50 mg/kg); E = RE (50 mg/kg) + DEP 0.7 mg/kg; F = DEP 0.7 mg/kg

Wistar rats were exposed to 0.7 mg/kg DEP in the presence and absence of 50 mg/kg rooibos tea extract. (A) AST and (B) ALT activities were determined in the serum as described in the materials and methods. Values are mean \pm SEM of three different experiments done in triplicate ($n=3$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as significant difference as compared to the controls. # $p < 0.001$ as significant difference between rooibos extract pre-treatment vs DEP, ° $p < 0.01$ as significant difference between rooibos extract pre-treatment vs rooibos extract

Exposure of rats to nanoparticle rich-diesel exhaust has been reported to increase serum ALT and AST activities (Ito *et al.*, 2016). The present study was in agreement with this earlier study. In the present study, DEP caused significant increase in ALT and AST activities, which is a measured of liver damage. Though some studies have shown the use of endogenous and exogenous antioxidants to protect against diesel exhaust particles induced damage (Tseng *et al.*, 2015; Lawal *et al.*, 2015), no study has yet reported on the effects of rooibos extract on the activity of the serum enzymes biomarkers of the hepatotoxic effect of diesel exhaust particles. This study showed that rooibos extract attenuates the effects of DEP on serum ALT and AST activities, indicating the hepato-protective role of rooibos extract on diesel exhaust particles induced hepatic damage. These findings were in agreement with the reported health promoting effects of rooibos in mice and humans (Watanabe *et al.*, 2014; Marnewick *et al.*, 2011).

(Dybdahl *et al.*, 2003; Lawal *et al.*, 2016; Montiel-Davalos *et al.*, 2010). We and others have shown that diesel exhaust particles caused elevated lipid peroxidation (MDA and CDs) levels in the liver of exposed rats (Yin *et al.*, 2013; Dybdahl *et al.*, 2003; Miller *et al.*, 2013). The results obtained from this present study were in agreement with these earlier reports. This study showed that diesel exhaust particles exposure caused a 3.15- and 1.77-fold significant ($p < 0.001$) increase in hepatic MDA and conjugated dienes (CDs) levels respectively, when compared to control (Fig. 2). However, rooibos extract pre-treatment significantly ($p < 0.001$) decreased the DEP-induced MDA and CDs levels by 1.92- and 1.29- fold respectively (Fig. 2A & B). These findings agreed with the earlier work reported by Marnewick *et al* (2011), where it was shown that consumption of rooibos in adults at the risk of cardiovascular disease caused a significant reduction in MDA and CDs levels. This emphasized the importance of polyphenol-rich beverages in dietary intervention in human.

Several studies have implicated oxidative stress in the adverse health effects of diesel exhaust particles

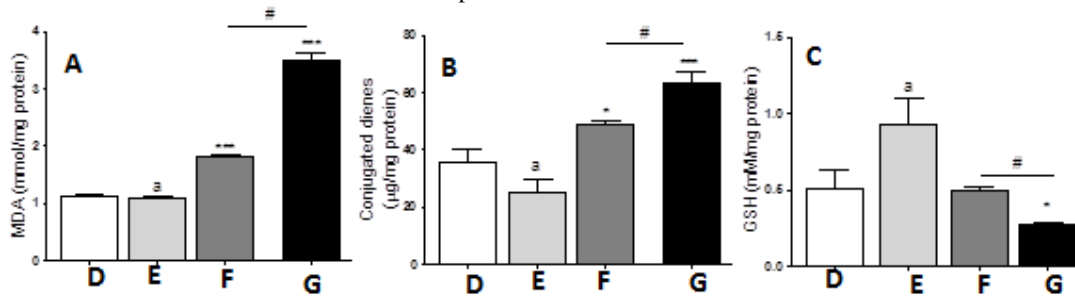


Fig 2. Effects of aqueous rooibos tea extract on DEP-induced hepatic oxidative stress D = 0; E = RE (50 mg/kg); F = RE (50 mg/kg) + DEP 0.7 mg/kg; G = DEP 0.7 mg/kg

Wistar rats were exposed to 0.7 mg/kg DEP in the presence and absence of 50 mg/kg rooibos tea extract. (A) MDA, (B) CDs and (C) GSH levels were determined in the homogenised liver tissue as described in the materials and methods. Values are mean \pm SEM of three different experiments done in triplicate ($n=3$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as significant difference as compared to the controls. # $p < 0.001$ as significant difference between rooibos extract pre-treatment vs DEP, ° $p < 0.01$ as significant difference between rooibos extract pre-treatment vs rooibos extract.

The present study also showed that diesel exhaust particles caused significant ($p < 0.05$) decrease (1.62-fold) in the reduced glutathione (GSH) level in the liver (Fig. 2C). Pre-treatment with 0.7 mg/kg rooibos extract, however, caused a significant ($p < 0.01$) elevation in the intracellular GSH level compared to DEP exposure only (Fig. 2C). GSH is an important endogenous antioxidant that has been implicated in the maintenance of redox homeostasis (Lawal *et al.*, 2017). Depletion of GSH level with accumulation of oxidised glutathione (GSSG) is a hallmark of oxidative stress. In the present study, diesel exhaust particles caused significant depletion in hepatic GSH levels and this may account for the increased oxidative damage in the liver. This result was in consistent with other studies that have shown depletion in GSH and accumulation of GSSG in DEP exposure (Lawal *et al.*, 2016; Tobwala *et al.*, 2013; Tseng *et al.*, 2015). The pre-treatment with aqueous rooibos extract, however, significantly reversed this DEP-induced GSH

reduction, confirming the antioxidant potential of the extract. These data was consistent with the clinical data that showed the GSH boosting activity in the serum of subjects given oral dose of rooibos herbal tea (Marnewick *et al.*, 2011).

Nrf2 is an important transcription factor that regulates the gene expression of antioxidant enzymes. We have shown that exposure of *Apoc^{-/-}* (null) mice to diesel exhaust caused induction in Nrf2, HO-1, NQO1, GCLC and SOD1 genes in the liver (Yin *et al.*, 2013; Miller *et al.*, 2013). Consistent with these findings, the present study shows that diesel exhaust particles induced hepatic expression of Nrf2 and HO-1 genes probably in response to oxidative stress. The results showed that diesel exhaust particles induced hepatic expression of Nrf2 and HO-1 genes significantly ($p < 0.001$) by 1.74- and 2.07- fold respectively, when compared to control (Fig. 3A & 4A).

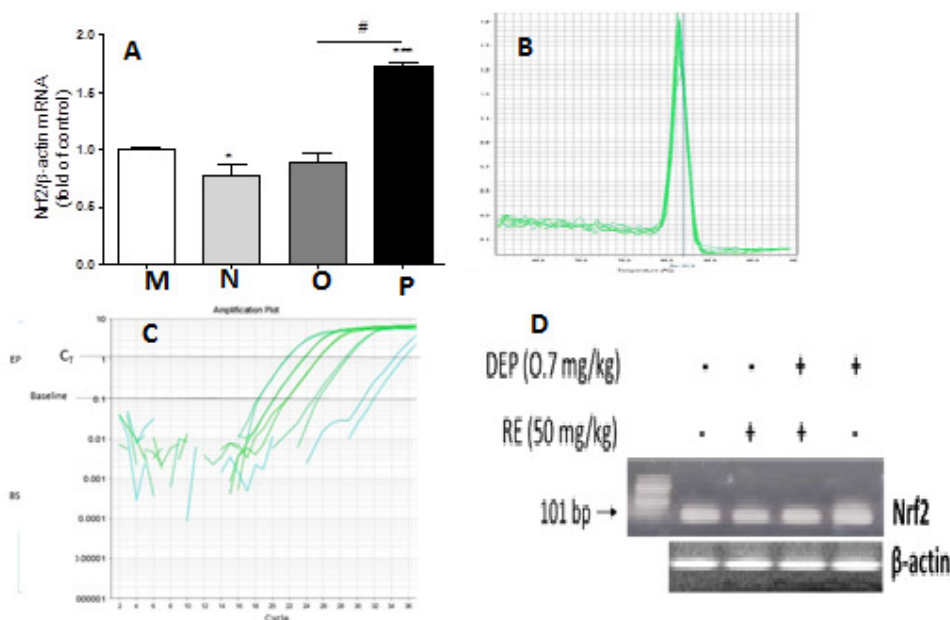


Fig 3. Effects of rooibos extract on Nrf2 gene expression in the presence of DEP (M = 0; N = RE 50 mg/kg; O = RE 50 mg/kg + DEP 0.7 mg/kg; P = DEP 0.7 mg/kg)

(A) Relative quantification of Nrf2 gene expression from the C_T values of cDNA obtained from the liver of rats exposed to 0.7 mg/kg DEP in the presence and absence of 50 mg/kg rooibos tea extract using Nrf2 primers and β-actin as endogenous reference. Values are mean ± SEM of three different experiments done in triplicate ($n = 3$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as significant difference as compared to the controls. # $p < 0.001$ as significant difference between rooibos extract pre-treatment vs DEP, ^a $p < 0.01$ as significant difference between rooibos extract pre-treatment vs rooibos extract. (B) Instrument-derived melt curve showing a single amplification product (single peak) (C) Instrument derived amplification curve. (D) agarose gel analysis of the qPCR product (101 bp). BS, background signal; EP, exponential phase; Rn, emission intensity of the reporter/emission intensity of passive reference (normalised reporter); ΔRn, the difference between the Rn at the end point and at the starting point and it is directly proportional to the DNA amount during the exponential phase.

The presence of rooibos extract, however, caused a significant ($p < 0.001$) 2.23- and 1.32- fold decrease in hepatic Nrf2 and HO-1 gene expression induced by diesel exhaust particles respectively (Fig. 3A & 4A). The melting curves obtained from the qPCR instrument showed that only the desired amplicons-Nrf2 (Fig. 3B) and HO-1 (Fig. 4B)- were amplified

during the qPCR runs. Also, the instrument derived amplification curves showed that there were amplifications of the Nrf2 (Fig. 3C) and the HO-1 (Fig. 4C) genes during the qPCR runs. The results of the RT-qPCR were confirmed by the agarose gel analysis of the Nrf2 (Fig. 3D) and HO-1 (Fig. 4D), which gave the amplicon sizes of 101 bp (Nrf2) and 99 bp (HO-1).

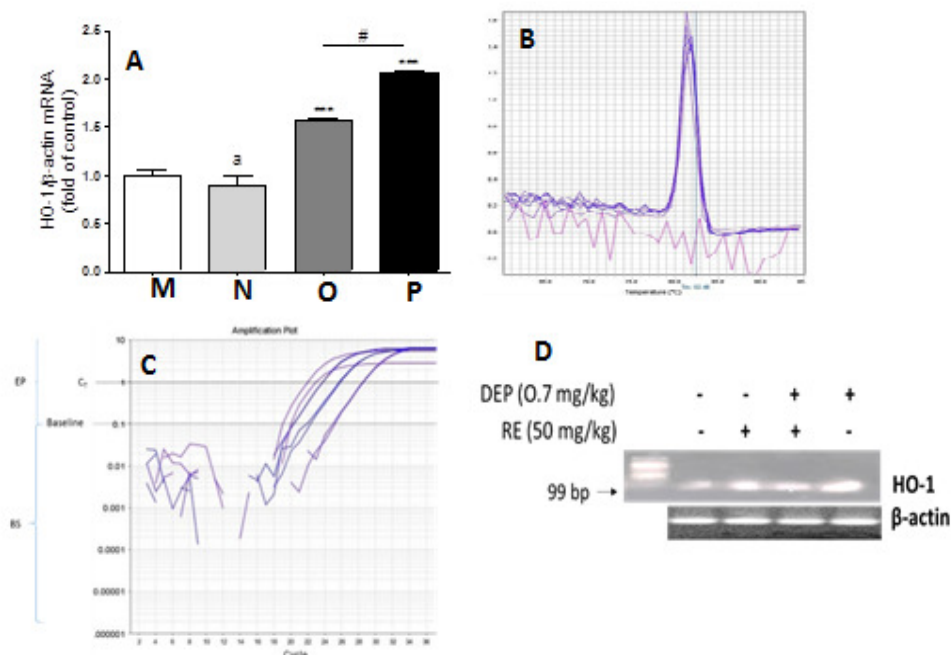


Fig 4. Effects of rooibos extract on HO-1 gene expression in the presence of DEP.(M = 0; N = RE 50 mg/kg; O = RE 50 mg/kg + DEP 0.7 mg/kg; P = DEP 0.7 mg/kg)
 (A) Relative quantification of Nrf2 gene expression from the C_T values of cDNA obtained from the liver of rats exposed to 0.7 mg/kg DEP in the presence and absence of 50 mg/kg rooibos tea extract using HO-1 primers and β -actin as endogenous reference. Values are mean \pm SEM of three different experiments done in triplicate (n=3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as significant difference as compared to the controls. # $p < 0.001$ as significant difference between rooibos extract pre-treatment vs DEP, * $p < 0.01$ as significant difference between rooibos extract pre-treatment vs rooibos extract. (B) Instrument-derived melt curve showing a single amplification product (single peak). (C) Instrument derived amplification curve. (D) agarose gel analysis of the qPCR product (99 bp). BS, background signal; EP, exponential phase; Rn, emission intensity of the reporter/emission intensity of passive reference (normalised reporter); ΔRn , the difference between the Rn at the end point and at the starting point and it is directly proportional to the DNA amount during the exponential phase.

We have also shown that the induction of HO-1 protects against diesel exhaust particles induced oxidative damage in human microvascular endothelial cells (Lawal *et al.*, 2015). In addition, studies have shown that administration of exogenous antioxidants are effective in protecting against diesel exhaust particles induced oxidative stress in different endothelial cells (Frikke-Schmidt *et al.*, 2011; Li *et al.*, 2009; Tseng *et al.*, 2015).

Consistent with these studies, the present study showed that the use of rooibos extract protects against the oxidative stress induced by diesel exhaust particles in hepatic cells. However, this protection does not involve the up regulation of Nrf2 and HO-1 from the levels seen in diesel exhaust particles exposure. Thus, the protective effects of the extract may be due to the activity of its antioxidant phytochemicals, which has earlier been reported by Marnewick (2009)

Conclusion: In conclusion, these data seem to suggest that rooibos extract exerts protection against DEP-induced hepatic oxidative stress without triggering the up-regulation of Nrf2 mediated pathways. Therefore, rooibos tea may provide an alternate therapeutic approach to combating the adverse health effects of diesel exhaust particulate matter on hepatic tissue.

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